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# Alkaline pH and Internal Calcium Increase $Na^+$ and $K^+$ Effluxes in LK Sheep Red Blood Cells in $Cl^-$-free Solutions

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**Abstract.** We examined the effects of pH, internal ionized Ca ($Ca_i^{2+}$), cellular ATP, external divalent cations and quinine on Cl-independent ouabain-resistant $K^+$ efflux in volume-clamped sheep red blood cells (SRBCs) of normal high (HK) and low (LK) intracellular $K^+$ phenotypes. In LK SRBCs the $K^+$ efflux was higher at pH 9.0 (350%) than at pHs 7.4 and 6.5, and was inhibited by external divalent cations, quinine, and cellular ATP depletion. The above findings suggest that the increased $K^+$ efflux at alkaline pH is due to the opening of ion channels or specific transporters in the cell membrane. In addition, $K^+$ efflux was activated (100%) when $Ca_i^{2+}$ was increased (+A23187, +$Ca_o^{2+}$) into the μM range. However, in comparison to human red blood cells, the $Ca_i^{2+}$-induced increase in $K^+$ efflux in LK SRBCs was fourfold smaller and insensitive to quinine and charybdotoxin. The $Na^+$ efflux was also higher at pH 9.0 than at pH 7.4, and activated (about 40%) by increasing $Ca_i^{2+}$. In contrast, in HK SRBCs the $K^+$ efflux at pH 9.0 was neither inhibited by quinine nor activated by $Ca_i^{2+}$. These studies suggest the presence in LK SRBCs, of at least two pathways for $Cl^-$-independent $K^+$ and $Na^+$ transport, of which one is unmasked by alkalinization, and the other by a rise in $Ca_i^{2+}$.

**Key words:** pH — Erythrocytes — Calcium — Sodium — Potassium — Efflux

## Introduction

Sheep is one of the species that shows a genetically controlled dimorphism with respect to the membrane transport systems controlling the cation content of their red blood cells. This dimorphism results in two phenotypes: cells with normal high (HK) or low (LK) intracellular $K^+$ concentration [29]. In both LK and HK sheep red blood cells (SRBCs) there is a basal $K^+$ efflux that operates in $Cl^-$-free solutions [18]. K-Cl cotransport (a mechanism specific for $K^+$ and dependent on $Cl^-$), however, is the major pathway of passive $K^+$ transport in the mature erythrocytes of the LK, but not the HK, phenotype [*for review see 19*]. LK SRBCs are used as a model for studies of volume regulation (through K–Cl cotransport), but little is known about the nature of the ouabain-resistant $Cl^-$-*independent* $K^+$ efflux in these cells. The latter is elevated at alkaline pH (pH > 7.4) in control, Mg-depleted, and swollen LK SRBCs [20, 22, 25], and is inhibited by the nonspecific $K^+$ channel blocker quinidine [1].

Several types of cation channels have been identified in the erythrocyte membrane of different species. In human red blood cells, as in most cell types [7], a rise in cytosolic free (ionized) Ca ($Ca_i^{2+}$) induces a rapid increase in the $K^+$ permeability (''Gárdos effect'' [14]), resulting in membrane hyperpolarization [*for review see* 16, 23, 27]. Quinine, quinidine, [16, 23, 27], and the peptide toxin charybdotoxin [4, 30] are some of the inhibitors of the $Ca_i^{2+}$-activated $K^+$ channels ($K_{Ca}$). In human erythrocytes, the permeability of the $K_{Ca}$ channels to $K^+$ and $Rb^+$ is very similar, with a considerable selectivity for $K^+$ over $Na^+$ (27, review). However, in the nucleated red cells of chicken and certain species of fish $K_{Ca}$ channels do not discriminate between $K^+$ and $Na^+$, and furthermore, in chicken erythrocyte $K_{Ca}$ channels are insensitive to quinidine [24]. It has been reported that the red blood cells of cow, mature sheep, and goat lack $K_{Ca}$ channels [17], but that they are present in the fetal erythrocytes of LK and HK sheep [3].

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Other types of channels, which do not discriminate for $Na^+$ and $K^+$ and are activated by membrane depolarization, are present in human red cells, and in HK but not in LK SRBCs [6, 15].

The purpose of the present study was: (i) to ascertain whether the effect of alkaline pH on $K^+$ efflux in SRBCs is mediated by a generalized effect on the membrane, or on specific sites or ion channels, and (ii) to investigate the presence of $K_{Ca}$ channels in SRBCs with K-Cl cotransport inhibited by either methane sulfonate ($MSF^-$) or nitrate replacement of $Cl^-$. The rise in cytosolic $Ca_i^{2+}$ was induced by the ionophore A23187 in the presence of external $Ca_o^{2+}$.

The results indicate that at pH 9.0 a fraction of the $Cl^-$-independent $K^+$ efflux in LK SRBCs occurred through specific membrane sites or channels and was inhibited by external divalent cations, quinine and cellular ATP depletion. In HK SRBCs, however, the $K^+$ efflux at pH 9.0 was not inhibited by quinine. Furthermore, a rise in $Ca_i^{2+}$ activated $K^+$ efflux (100%) in LK but not in HK SRBCs. In LK SRBCs the $Na^+$ efflux was also higher at pH 9.0 than at pH 7.4, and activated (about 40%) by increasing $Ca_i^{2+}$. These studies suggest the presence, in LK SRBCs, of two $Cl^-$-independent $K^+$ and $Na^+$ pathways, one that is activated by alkalinization, and another by a rise in $Ca_i^{2+}$. The $Ca_i^{2+}$-induced $K^+$ permeability in LK SRBCs differed from that reported in human red cells [16, 23, 27] not only in its magnitude (2-fold *vs.* 8-fold) but also in its lack of cation specificity and its insensitivity to quinine and charybdotoxin. Cell heterogeneity in the samples might explain the discrepancy with previous reports [3, 17]. Part of this work has been presented in abstract form [26].

## Materials and Methods

### Chemicals

All reagents were of analytic grade. The following chemicals were obtained through Sigma Chemical (St. Louis, MO): methane sulfonic acid (HMSF), ethylene diamine tetra-acetic acid (EDTA), ethylene glycol tetra-acetic acid (EGTA), N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) (HEPES), Piperazine-N,N-bis-[2-ethanesulfonic acid] (PIPES), N,N-bis[2-Hydroxyethyl]-glycine (BICINE), A23187, 2-deoxy-D-glucose, dibutyl phthalate, N-methyl-D-glucamine, quinine, and ouabain. KCl, $Na_2HPO_4$, $NaH_2PO_4$, $Mg(NO_3)_2$, $Ca(NO_3)_2$, glucose and sucrose were obtained through Fisher Scientific (Fair Lawn, NY) and $RbNO_3$ from Johnson Matthey Materials Technology U.K. (Royston, England).

### Solutions

All solutions were prepared using double-distilled water. Methane sulfonate ($MSF^-$) was the major anion in the solutions used for cell washing, pH equilibration, and flux measurement. The composition of the solutions used for cell washing and pH equilibration before the flux incubation was as follows (with solute concentration given in mM and pH at 4°C): Solution ''*A*'' (pH 6.5): 160 NaMSF, 20 Na-PIPES, 3 EDTA, 330 mOsm; Solution ''*B*'' (pH 7.4): 140 NaMSF, 20 Na-HEPES, 3 EDTA, 290 mOsm; and Solution ''*C*'' (pH 9.0): 120 NaMSF, 20 Na-BICINE, 3 EDTA, 250 mOsm. The flux solutions contained (pH at 37°C; concentration in mM): solution ''*AI*'' for pH 6.5: 100 NaMSF, 20 Na-PIPES, 0.05 EGTA, 0.1 ouabain and sucrose for a final osmolality of 330 mOsm; (*b*) solution ''*BI*'' for pH 7.4, same composition as ''*AI*'' but Na-PIPES was replaced by Na-HEPES and sucrose was added to obtain a final osmolality of 290 mOsm; and solution ''*CI*'' for pH 9.0, same composition as solution ''*AI*'' but Na-HEPES was replaced by Na-BICINE, and sucrose was added for a final osmolality of 250 mOsm. Incubation medium for divalent cations extraction (in mM, pH at 37°C) ''*M*'': 137 NaCl, 3 EDTA, 20 Na-BICINE, pH 9.0, 290 mOsm. Na-free medium for washing and incubation: ''*G*'' (in mM, pH 9.0 at 37°C): 100 N-methyl-D-glucamine, 100 HMSF, 0.05 EGTA, 20 TRIS-BICINE, and with sucrose added for a final osmolality of 250 mOsm; for the flux incubation (medium ''*GI*'') 0.1 mM ouabain was added to medium ''*G*''. Medium (''*D*'') for ATP-depletion: (in mM, pH 7.4 at 37°C, 290 mOsm): 137 NaCl, 5 KCl, 1 $NaH_2PO_4$, 0.05 EGTA, 20 Na-HEPES, and 10 2-deoxy-D-glucose. Osmolalities were measured with an Advanced DigiMatic Osmometer, Model 3DII (Advanced Instruments, Needham Heights, MA).

### Preparation of Red Blood Cells

Blood from mixed breed LK and HK sheep was drawn by personnel from the Laboratory of Animal Resources by jugular venipuncture into a heparinized syringe (10 i.u. heparin $ml^{-1}$ blood) and used within 1 hr. The hematocrit (Hct) and hemoglobin content of the fresh blood were measured, and the blood was centrifuged in a Sorval RC-5B or RC2-B centrifuge at 10,000 rpm for 1 min at 4°C to separate cells from plasma and buffy coat. The cells were washed three times (4°C) in isosmotic NaCl (290 mOsm) prior to washing in the medium appropriate for each experimental protocol.

### ATP Depletion

The cells were depleted of ATP [21] by incubation at 5% Hct for 4 hr at 37°C, in medium ''*D*'' (*see* Solutions). The control cells (Fed) were incubated in the same solution with glucose replacing 2-deoxy-D-glucose. The ATP content of the cells was measured by the luciferin-luciferase assay (Sigma Assay Kit FL-AA, Sigma Chemical, St. Louis, MO) in neutralized supernatants of perchloric acid-precipitated cells [11].

### Divalent Cation ($Me^{2+}$) Extraction

When the experimental protocol required extraction of cellular $Me^{2+}$, this procedure was carried out after depletion of cellular ATP. Divalent cation extraction was as follows: The washed sheep erythrocytes were incubated (10% Hct, 20 min, 37°C) in solution ''*M*'' at pH 9.0 (*see* Solutions, above) with 10 μM A23187; this pH was chosen since we have recently shown [25] that the extraction of cellular $Me^{2+}$ (in LK SRBCs) by the combined use of A23187 and EDTA is more effective at pH 9.0. This solution was 290 mOsm, although it should have been 250 mOsm to prevent shrinkage at alkaline pH (*see below*, and [22]), because we found that the higher osmolality (290 mOsm) prevented cell lysis during pH equilibration and incubation with A23187. At the end of the 20 min incubation, the cells were washed once in solution ''*M*'' and 2–3 times in the solution of the appropriated pH and osmo-

lality according to the experimental protocol as described below for $K^+$ efflux determination.

### Cell Volume

The volume of cells in samples was referred to the volume (liter) of original cells (Loc) and was determined as described elsewhere [18]. Briefly, the hematocrit and the optical density at 527 nm ($OD^{527}$; using a Gilford 300-N spectrophotometer) of a lysed aliquot of the fresh blood were both measured to obtain the ratio of $OD^{527}$ to hematocrit ($OD^{527}_{pc}$). The $OD^{527}$ of an aliquot of the cell suspension was then compared to the ratio $OD^{527}_{pc}$.

### $K^+$ Efflux

All fluxes were measured at constant cell volume in the presence of $10^{-4}$ M ouabain and in $K^+$-free solutions. To avoid changes in cell volume during cell acidification (swelling [22]) or alkalinization (shrinkage [22]) and to maintain a constant ionic strength, the concentration of the principal anion ($MSF^-$) was kept constant (100 mM) and the tonicity of the solution was changed accordingly with pH (250 mOsm at pH 9.0, and 330 mOsm at pH 6.5) by addition of sucrose [22]. The cells were washed (three times), suspended (5–10% Hct) in either solution A, B, or C, and then the flasks containing the cell suspensions were placed into an ice-water bath for 15 min to allow equilibration of internal and external pHs. At the end of 15 min, the cell suspensions were centrifuged (1 min at 10,000 rpm, 4°C) and resuspended at 5% Hct in the flux solutions (AI, BI, CI) at 4°C; the ionophore A23187 (10 μM, final concentration) was added and one aliquot was removed for determination of cell $K^+$ and hemoglobin. To start the flux, plastic flasks containing the cell suspensions were placed into a water bath at 37°C (time = 0) and incubated for 30–60 min. Aliquots were removed every 10–20 min, placed into conical microtubes containing 100 μL of dibutyl phthalate and immediately centrifuged for 30 sec at 13,000 rpm in an Eppendorf microcentrifuge. The supernatants were analyzed for $K^+$ and hemoglobin content. The hemoglobin content was determined from the optical density at 527 nm and the $K^+$ content was measured using an atomic absorption spectrophotometer (Perkin Elmer 5000). The rate constant ($k$) for ouabain-resistant $K^+$ loss ($h^{-1}$) was calculated from the $K^+$ concentrations in supernatants (corrected for cell lysis) using a linear regression program as reported elsewhere [18].

### $Na^+$ Efflux

$Na^+$ efflux was measured as described above for $K^+$ efflux, but the cells were washed in medium ''*G*'' and the flux incubation carried out in medium ''*GI*'', both $Na^+$-free. The $Na^+$ content of cells and supernatants was measured by atomic absorption spectroscopy as described for $K^+$.

### Effect of Charybdotoxin on $Ca^{2+}$-activated $K^+$ Transport

The tubes to be used in the incubations were preincubated overnight in a low ionic strength ''binding medium'' (in mM: 18 $NaNO_3$, 20 BICINE, 230 Sucrose, pH 9.0) containing 0.25% bovine serum albumin (BSA) to avoid binding of charybdotoxin to polystyrene [4]. Two experimental approaches were used to test the effect of charybdotoxin, as follows: (i) washed cells were suspended (10% Hct) in ''binding medium'' and divided into three aliquots, A, B, and C. Charybdotoxin was added (50 nM, final concentration) to suspension C, and the three aliquots (A, B, and C) were preincubated in the low ionic strength ''binding medium'' to facilitate the binding of the toxin to the cells membrane [4]. After 20 min at 37°C, the cells were spun down and resuspended (5% Hct) in the flux medium (270 mOsm, pH 9.0, in mM): 100 $NaNO_3$, 20 BICINE, 0.35 $Ca(NO_3)_2$, 0.1 EGTA, 0.25% BSA, 70 Sucrose. A23187 (20 μM, final concentration) was added to aliquots B and C, while charybdotoxin was added to aliquot C only. $K^+$ efflux was then measured in aliquots A (Control, no increase in $Ca_i^{2+}$), B (increased $Ca_i^{2+}$), and C (increased $Ca_i^{2+}$ + charybdotoxin) as described above. (ii) In the second experimental protocol three aliquots of washed cells (D, E, F) were treated as follows: the cells were suspended (10% Hct) in a ''loading medium'' (270 mOsm, pH 9.0) containing (in mM): 50 $KNO_3$, 20 BICINE, 50 $NaNO_3$, 0.1 mM EGTA, 0.25% BSA, 70 Sucrose. EGTA (1 mM) was added to aliquot D, and 0.35 mM $Ca(NO_3)_2$ to aliquots E and F; the three aliquots were then incubated (20 min, 37°C) with A23187 (20 μM, final concentration) to modify $Ca_i^{2+}$. After this first preincubation the ionophore was removed by washing the cells twice in the medium used for loading (± Ca, as fitted) but with 1% BSA, and twice in ''binding medium.'' After the ionophore removal, the three cells aliquots were suspended in ''binding medium'' (*see above*) and preincubated for 30 min (37°C), with 50 nM charybdotoxin (final concentration) added to aliquot F only. The $K^+$ efflux was then measured as described above, with 1 mM EGTA added to aliquot D, and 20 nM charybdotoxin to F.

### Curve Fitting

In all figures shown here, symbols represent experimental data; the lines through them were generated using the computer program Origin (MicroCal Software, MA).

## Results

### Effect of pH on $K^+$ and $Na^+$ Efflux in Volume-Clamped LK SRBCs

To ascertain whether alkaline activation of $Na^+$ efflux occurs, and to confirm the reported alkaline activation of $K^+$ efflux, we examined the effect of several pHs on $Na^+$ and $K^+$ effluxes in LK SRBCs in $Na^+$-free and in 100 mM $Na^+$ solutions, respectively. Since LK SRBCs lack the Na:K:2Cl cotransport system, and have a very low Na:K pump activity, the fluxes were measured in the presence of 0.1 mM ouabain as the only inhibitor, and in $Cl^-$-free solutions [$Cl^-$ was replaced by $MSF^-$ (methane sulfonate)] to inhibit K-Cl cotransport [18]. To obviate the effect of pH on cell volume [12] the osmolality of the solutions was changed according to their pH as described in Materials and Methods: hypertonic at pH 6.5, isotonic at pH 7.4, and hypotonic at pH 9.0. Under the above conditions cellular volume is not modified in spite of the changes in external ($pH_o$) and internal ($pH_i$) pH [22], and the $H^+$ and $Cl^-$ distribution ratios [25].

Figure 1 *A* shows that in LK SRBCs the $Na^+$ efflux rate constant increased by about 260% as the pH was raised from 7.5 to 9.0, while the $K^+$ efflux rate constant at pH 9.0 was 350% of that at pH 6.5, and 260% of that at pH 7.4. The increase in the rate constant ($k$) of $Cl^-$-

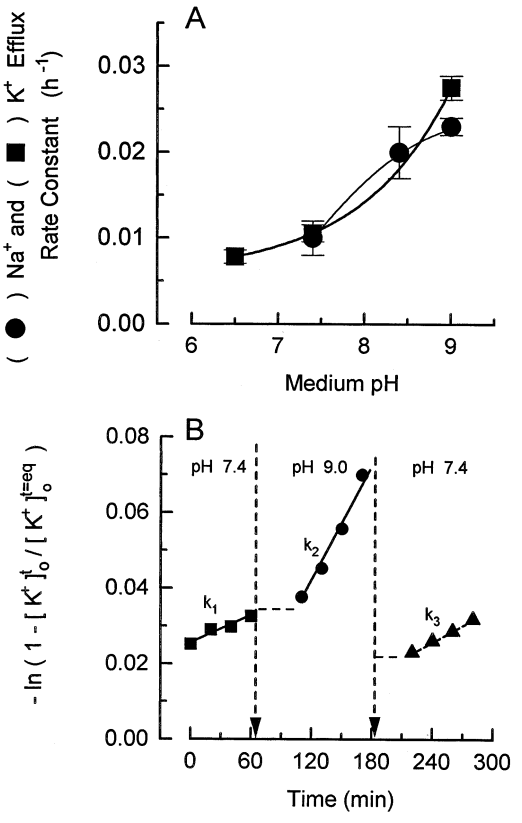


**Fig. 1.** Effect of medium pH on $Na^+$ and $K^+$ efflux rate constants and of consecutive pH changes on $K^+$ loss in volume clamped LK SRBCs (*A*) $Na^+$ and $K^+$ efflux rate constants were measured in $Cl^-$-free media (Na replaced by N-methyl-D-glucamine for determination of $Na^+$ efflux) as described in Materials and Methods. Symbols represent mean values and vertical bars standard errors. $Na^+$ efflux rate constants (●): $n = 3$; the solid line through the experimental points represent the equation: $k = \{(-0.005 - 0.025)/[1 + e^{(X - Xo)}/dX]\} + 0.025$, where $k$ = rate constant ($h^{-1}$), and $Xo$ = pH at center of lowest and highest pH values. $K^+$ efflux rate constant (■): $n = 7$–10; the solid line through the symbols describes the equation: $k = 0.006\ h^{-1} + 0.002\ e^{(X/1.06)}\ h^{-1}$, where $X$ = pH. (*B*) washed cells were equilibrated at pH 7.4 in medium ''BI'' and $K^+$ loss measured for 1 hr to determine $k_1$. After the last aliquot at time 60 min was removed, the cells were spun down and equilibrated for 30 min (broken horizontal lines) at pH 9.0 (medium ''CI''). The $K^+$ loss was then measured for 1 hr at pH 9.0 to obtain $k_2$, and then the remaining cells were spun down once more, equilibrated at pH 7.4 (medium ''BI'') for 30 min (broken horizontal lines), and the $K^+$ loss at pH 7.4 ($k_3$) determined once again. The slope of the lines ($k_1$, $k_2$, $k_3$) were obtained by linear regression analysis and were (mean ± SE; $n = 3$; $h^{-1}$): $k_1 = 0.007 \pm 0.001$, $k_2 = 0.030 \pm 0.002$, and $k_3 = 0.007 \pm 0.001$. Experimental details and media composition are described in Materials and Methods.

independent $K^+$ efflux in LK SRBCs at alkaline pH (pH > 7.4) did not cause significant cell dehydration, since the concentration of $K^+$ in these cells is low (about 22 mM [29]). On the other hand, alkaline activation of the $Na^+$ efflux rate constant in LK SRBCs modestly but definitely alters cell volume in low $Na^+$ media, since the cellular $Na^+$ concentration is about 140 mM [29]. At pH 9.0, with $k_{Na} \approx 0.025\ h^{-1}$, the $Na^+$ efflux was about 3.5 mmol × (L cell water × h)$^{-1}$, resulting in the loss of 7.0 mosmol × (L. cell water × h)$^{-1}$ when accompanied with a monovalent anion. Assuming an isotonic effluent, LK SRBCs were estimated to lose about 2.4% of the water content after 1 hr incubation in $Na^+$-free media at pH 9.0.

Figure 1*B* describes $K^+$ loss as a function of time in LK SRBCs consecutively incubated at pHs 7.4, pH 9.0, and 7.4. The activating effect of pH 9.0 on $K^+$ efflux was reversible as shown by the values of the rate constants (*see* values in legend to Fig. 1).

## Effect of External Divalent Cations and ATP Depletion on $K^+$ Efflux in LK SRBCs, and Effect of Quinine on both LK and HK SRBCs at pH 9.0

The membrane potentials ($E_m$) of LK SRBCs were calculated from the $H^+$ distribution ratios (equivalent to the $Cl^-$ ratios) recently reported by us [25]. Under experimental conditions equivalent to the used here, $E_m$ was (mean ± SE, n ⩾ 5 determinations): +9.6 ± 1.0, +0.3 ± 0.7, and −24 ± 2.0 mV, at pH 6.5, 7.4 and 9.0, respectively. Although the hyperpolarization at pH 9.0 should have reduced $K^+$ efflux through an electrodiffusional pathway, $K^+$ efflux actually increased with pH (Fig. 1). To test for the presence of putative $K^+$ channels unmasked by alkaline pH, we investigated the effect of external divalent cations ($Me_o^{2+}$: $Ca_o^{2+}$, $Mg_o^{2+}$, $Ba_o^{2+}$) and quinine on $K^+$ efflux rate constant in LK SRBCs at pH 9.0. All of the above agents are nonspecific external inhibitors of $K^+$ channels [7].

$K^+$ efflux was measured (*see* Materials and Methods) in cells containing 0.8 mmol ATP/Loc (Fed cells) incubated in medium ''CI'' without (Control), and with the addition of the inhibitors indicated on the abscissa in Fig. 2. In the latter, the columns represent the $K^+$ efflux rate constants in LK SRBCs incubated without addition (control; empty column), with the tested $Me_o^{2+}$ (7 mM), and with 1 mM quinine as indicated under each column. The black columns indicate the change in $K^+$ efflux rate constant (△) induced by the inhibitor. The three tested $Me^{2+}$ inhibited about 30% of $K^+$ efflux in LK SRBCs at pH 9.0; statistical significance and more experimental details are given in the Legend to Fig. 2.

Quinine (1 mM) inhibited 50% of $K^+$ efflux in LK SRBCs at pH 9.0, as shown in Fig. 2. The fraction of the flux inhibited by quinine was not increased by addition of any of the tested $Me_o^{2+}$ (7 mM) to the flux solution containing 1 mM quinine (*data not shown*). In contrast, the $K^+$ efflux rate constant of HK SRBCs at pH 9.0 was not significantly modified ($P = 0.3$) by 1 mM quinine as shown in Fig. 2 inset.

Since the inhibitory effect of $Me_o^{2+}$ and quinine (Fig. 2) suggested that the $K^+$ efflux occurred through trans-

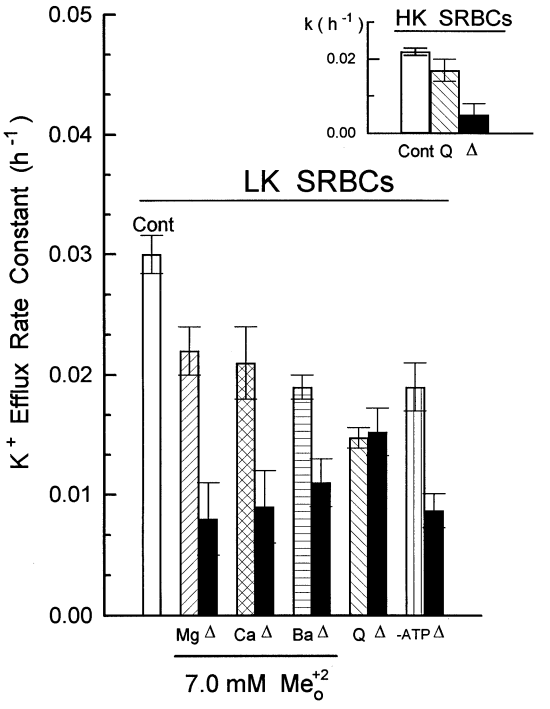


**Fig. 2.** Effect of external divalent cations and ATP depletion on $K^+$ efflux rate constant in volume-clamped LK SRBCs, and of quinine in both LK and HK SRBCs at pH 9.0. $K^+$ efflux rate constants were measured in medium ''CI'' as indicated in Materials and Methods. Black columns show the change (△) in the rate constant (with respect to the control) produced by the treatment indicated under the columns. Control (white column): glucose fed cells (0.8 mmol ATP/Loc) suspended in medium ''CI''. The effect of $Mg^{2+}$, $Ca^{2+}$, $Ba^{2+}$, and quinine (Q) was tested in glucose fed cells by addition of either the corresponding nitrate salt (7.0 mM) of the divalent cation or 1 mM quinine to medium ''CI''. Each column represents the average of 4 determinations ± SE. Cellular ATP was depleted by incubation with 2-deoxy-D-glucose (to 10 μmol/Loc) as indicated in Materials and Methods. $K^+$ efflux was measured in medium ''CI''; the average ± SE of 5 determinations is shown in column ''-ATP''. The inset in Fig. 2 describes the effect of 1 mM quinine on the $K^+$ efflux rate constant of HK SRBCs at pH 9.0. Student's *t*-test for paired values in LK SRBCs: the means of test and control are significantly different at the following levels: $Mg^{2+}$: $P < 0.005$; $Ca^{2+}$: $P < 0.05$; $Ba^{2+}$: $P < 0.001$; quinine (Q): $P < 0.001$; ATP-depleted: $P < 0.01$. In HK SRBCs: Q, $P = 0.3$.

porter/s or channel/s in LK SRBCs, we tested the ATP-dependency of this process. $K^+$ efflux was measured in control and ATP-depleted LK SRBCs (10 μmol ATP/Loc); the results are included in Fig. 2 which shows that the $K^+$ efflux rate constant was about 30% lower in ATP-depleted than in control cells. This difference was statistically significant ($P < 0.01$ for paired values; $n = 4$).

## Effect of Internal $Ca^{2+}$ on $Cl^-$-independent $K^+$ Efflux in LK SRBCs at pH 9.0

We examined the $Ca_i^{2+}$ effect on $K^+$ efflux under the present experimental conditions, at pH 9.0 (in $Cl^-$-free solutions to inhibit K-Cl cotransport) since at this pH the $K^+$ efflux is larger and therefore its determination more reliable. These results are summarized in Fig. 3 with experimental details given in the legend and in Materials and Methods. The experimental conditions are indicated on the abscissa. The columns in Fig. 3 indicate the $K^+$ efflux rate constants in LK SRBCs: in the control (*A*); in presence of the ionophore A23187 without $Ca^{2+}$ (*B*); with 0.2 mM $Ca_o^{2+}$ but without A23187 (*C*); and with both A23187 and 0.2 mM $Ca_o^{2+}$ (*D*). Figure 3 shows that a rise in $Ca_i^{2+}$ increased the $K^+$ efflux rate constant by 100% (*D*). The ionophore A23187 had no effect in $Ca^{2+}$-free solutions (*B*). The difference (*D* − *A*) was statistically significant ($P < 0.001$, $n = 6$). In HK SRBCs, however, a rise in $Ca_i^{2+}$ did not induce stimulation of $K^+$ efflux as shown in Fig. 3 inset. The activation seems to be specific for $Ca_i^{2+}$ since elevation of $Mg_i^{2+}$ (up to 1.2 mM $Mg_i^{2+}$, not shown) had no effect on $K^+$ efflux in LK SRBCs.

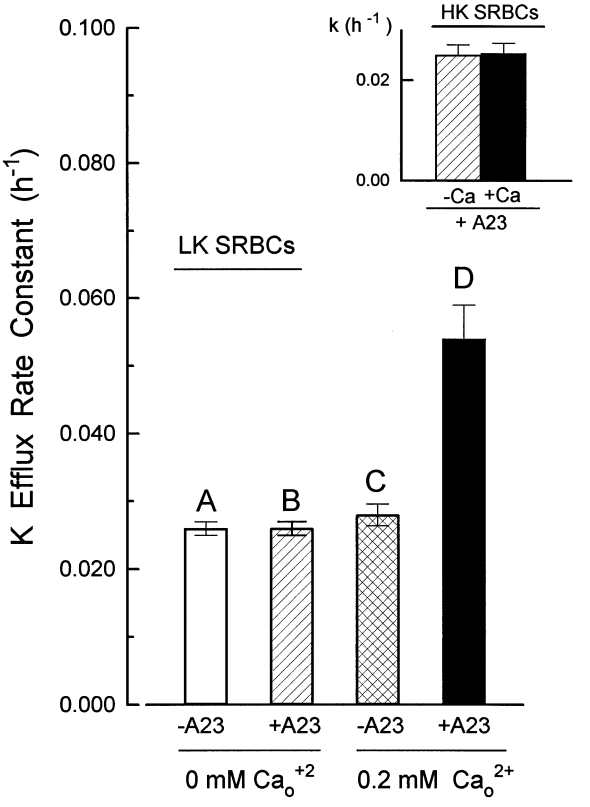


**Fig. 3.** Effect of external $Ca^{2+}$, in the presence and absence of A23187, on $K^+$ efflux in LK SRBCs incubated at pH 9.0. $K^+$ efflux rate constants were measured as indicated in Materials and Methods in $Ca^{2+}$-free medium ''CI'' (*A* and *B*) without (*A*) and with A23187 (*B*); and in medium ''CI'' containing 0.2 mM $Ca(NO_3)_2$ (*C* and *D*), without (*C*) and with A23187 (*D*). A23187: 10 μM (final in cell suspension). Columns represent average ± SE of 4–6 determinations. The inset in Fig. 3 describes the effect of $Ca_i^{2+}$ (0.2 mM $Ca_o^{2+}$ + 10 μM A23187) in HK SRBCs at pH 9.0. Student's *t*-test for independent values: *C–D*, the two means are significantly different at the $P < 0.001$ level ($n = 6$).

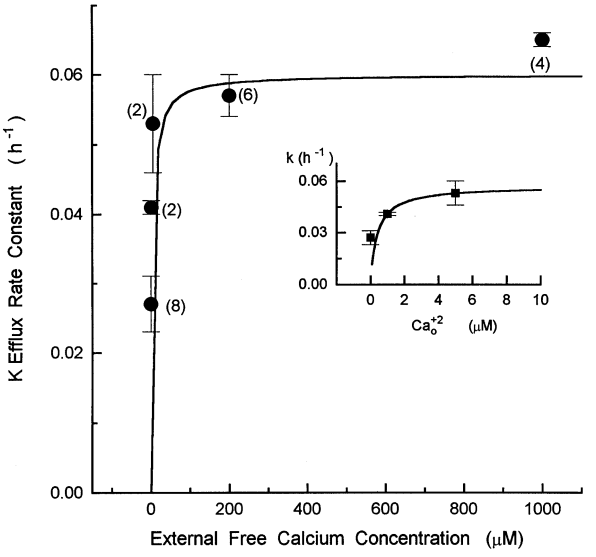


**Fig. 4.** $K^+$ efflux rate constant in LK SRBCs at pH 9.0 as a function of $Ca_o^{2+}$ concentration in the presence of A23187. $K^+$ efflux was measured (at pH 9.0) in $Me_i^{2+}$-depleted LK SRBCs suspended in medium ''CI'' with 50 μM EGTA and 10 μM A23187 without (0 μM $Ca^{2+}$) and with $Ca(NO_3)_2$ to give the $Ca_o^{2+}$ concentrations indicated at the abscissa. $Ca_i^{2+}$ was about 4 times $Ca_o^{2+}$ (*see text*). Experimental details are indicated in Materials and Methods. The solid line through the experimental points was obtained by least mean square fit of the data. Symbols represent mean values and vertical bars SE (range for $n = 2$). Numbers between parenthesis indicate number of determinations. Figure 4 inset shows the data with expanded abscissa in the range 0–5 μM $Ca^{2+}$; the solid line through the experimental points represents the equation: $k = (0.057\ h^{-1}\ [Ca^{2+}]_o\ \mu M)/(0.4 + [Ca^{2+}]_o)\ \mu M$.

Figure 4 describes the $K^+$ efflux rate constant in LK SRBCs at pH 9.0 (with 10 μM A23187) as a function of the $Ca_o^{2+}$ concentration in the range 0–1,000 μM. The experiments summarized in Fig. 4 were carried out in $Me^{2+}$-extracted cells (*see* Materials and Methods and Fig. 4 legend). The $Ca_o^{2+}$ concentration (in μM), calculated from the total concentration of Ca and EGTA in the medium using the program ''Chelator'' [28], is shown on the abscissa (Fig. 4). $K^+$ efflux (in the presence of 10 μM A23187 in suspension) was a saturating function of the $Ca_o^{2+}$ concentration. In the presence of this high A23187 concentration (196 μmol/Loc) $Ca^{2+}$ should have equilibrated across the red cell membrane independently of the pH [8]. In human erythrocytes, the ionophore content and ionophore-induced $Me^{2+}$ permeability are uniform [13]. Furthermore, at 10 μM A23187, the ionophore-induced calcium fluxes are much greater than the Ca pump fluxes in human and ferret red cells [9, 10]. Because of both the reported low Ca pump activity in low $K^+$ red cells of mature ruminant mammals [31], and the uniform A23187 solubilization in membrane lipids of the whole cell population, we calculated the $Ca_i^{2+}$ concentration by equation [10]:

$$Ca_i^{2+} = \left(\frac{H_o^+}{H_i^+}\right)^2 \times Ca_o^{2+} = \left(\frac{Cl_i^-}{Cl_o^-}\right)^2 \times Ca_o^{2+}$$

The $H^+$ distribution ratio in LK SRBCs at pH 9.0 is 2.2 ± 0.04 (Mean ± SEM, $n = 5$) and not significantly different in both $Cl^-$ and $MSF^-$ media [25]. Thus, the $Ca_i^{2+}$ concentrations in the experiments described in Fig. 4 must have been more than four times the external concentrations. In Fig. 4 inset, the data are shown with the abscissa expanded in the range 0–5 μM $Ca_o^{2+}$; the symbols represent experimental data and the solid line represents a hyperbola with $K_{0.5} = 0.4$ μM $Ca_o^{2+}$, and $V_m$ ($k_{max}$) = 0.057 $h^{-1}$. Although more experimental data would be required for a precise determination of $K_{0.5}$, the high $Ca_i^{2+}$ affinity of the system is evident. Since, as discussed above, LK SRBCs at pH 9.0 are hyperpolarized (compared to pH 7.4), the $K^+$ efflux rate constants described in Fig. 4 are lower than they would be if the membrane potential at pH 9.0 remained equal to that at pH 7.4.

## Properties of the $Ca_i^{2+}$-activated $K^+$ Efflux in LK SRBCs

The $Ca_i^{2+}$-activated $K^+$ efflux rate constants (rate constant in 0.2 mM $Ca_o^{2+}$ minus that in $Ca^{2+}$-free media) in LK SRBCs were measured, in the presence of A23187, at several pHs. The values ($h^{-1}$) found were: (mean ± SE; $n = 3$): 0.013 ± 0.003; 0.017 ± 0.04; 0.013 ± 0.006, and 0.015 ± 0.005 at pHs 6.7, 7.4, 8.0, and 9.0, respectively. Thus, the result suggest that the activating effect of $Ca_i^{2+}$, at saturating $Ca_o^{2+}$ concentration, was independent of the pH.

In human red blood cells, $K^+$ efflux through the $K_{Ca}$ channels is activated by external $K^+$, which is optimal at 2–3 mM [16]. In contrast, the magnitude of the $Ca_i^{2+}$-activated $K^+$ efflux in LK SRBCs at pH 9.0 appeared to be independent of the external $K^+(Rb^+)$ concentration in the range 0.06–2 mM. This observation is based on the results of two experiments (at pH 9.0) in which the medium $Rb^+(K^+)$ concentration was varied between 0.06 and 2.0 mM [0.06 ($K^+$ contaminant), 0.1, 0.26, 0.46, 0.86, 1.06, and 2.06], while $Ca_o^{2+}$ (0.2 mM) and A23187 (10 μM) were kept constant. The $K^+$ efflux rate constants in LK SRBCs at any of the tested $Rb^+$ concentrations were similar (mean and range, $n = 2$): 0.037 ± 0.002, and 0.039 ± 0.002 $h^{-1}$, at 0.06 mM $K_o^+$ and 2.06 mM $Rb_o^+$, respectively.

Quinine is one of the agents that inhibit $K_{Ca}$ channels in human erythrocytes [23]; 1 mM quinine, however, had no statistically significant effect on $Ca_i^{2+}$-stimulated $K^+$ efflux in LK SRBCs (*data not shown*).

Charybdotoxin is a specific blocker of $Ca_i^{2+}$-activated K channels in human red blood cells [4, 30].

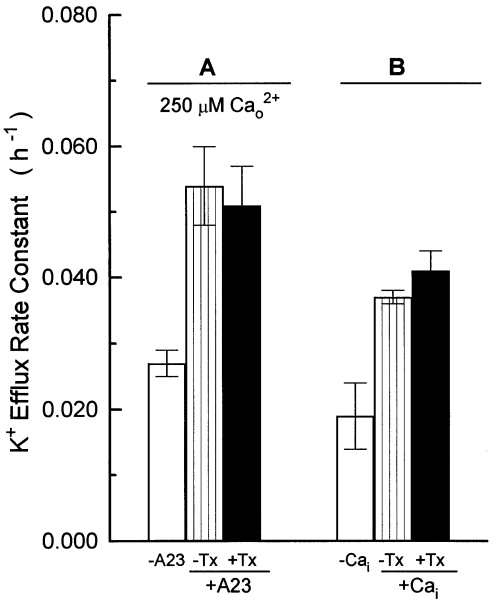


**Fig. 5.** Effect of charybdotoxin on the $Ca_i^{2+}$-activated $K^+$ efflux in LK SRBCs. The columns describe the mean values ± SE (vertical bars) of $K^+$ efflux rate constants in LK SRBCs at pH 9.0 under the experimental conditions indicated on the abscissa. Data from three original samples (one experiment). Experimental details are described in Materials and Methods. *Group ''A'':* $K^+$ efflux was measured in a medium containing 250 μM $Ca_o^{2+}$: without A23187 (empty column), with A23187 present during fluxing (both lined and black columns), and with 20 nM charybdotoxin (after preincubation with 50 nM charybdotoxin, black column). *Group ''B'':* Before the flux, the cells were preincubated as follows: first, cytoplasmic $Ca^{2+}$ was modified by treatment with A23187 and, either 1 mM EGTA (empty column, $-Ca_i$), or 250 μM $Ca_o^{2+}$ (both lined and black columns, $+Ca_i$). Second, the ionophore was removed by washing the cells with 1% bovine serum albumin (in the same medium as for A23187 treatment but without the ionophore). Third, an aliquot of the high $Ca_i^{2+}$ cells (black column, +Tx) was preincubated with 50 nM charybdotoxin; charybdotoxin was also present (20 nM) during the flux of this aliquot.

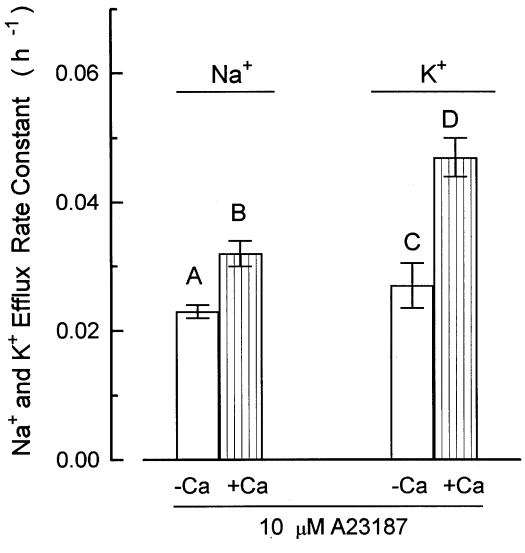


**Fig. 6.** Effect of $Ca_i^{2+}$ on $Na^+$ and $K^+$ efflux rate constants in LK SRBCs at pH 9.0. $Na^+$ and $K^+$ effluxes were measured in LK SRBCs incubated in medium ''GI'' ($Na^+$-free, pH 9.0) with 10 μM A23187, 1 mM $RbNO_3$, and with [$+Ca^{2+}$; 0.2 mM $Ca(NO_3)_2$] and without $Ca^{2+}$ ($-Ca^{2+}$; 50 μM EGTA). Empty ($-Ca^{2+}$) and vertical lined ($+Ca^{2+}$) columns represent $Na^+$ and $K^+$ efflux rate constants (mean ± SE; $n$ = 4–7). For experimental details *see* Materials and Methods. Student's *t*-test for independent values: The differences A−B ($P < 0.05$, $n = 4$) and C−D ($P < 0.0001$, $n = 7$) were statistically significant.

In experiments carried out to test the effect of the toxin (*not shown*) on $K_{Ca}$ channels in LK SRBCs, a small percentage (4%) of the cells lysed when incubated simultaneously with A23187 and charybdotoxin, invalidating the measure of the minute $K^+$ effluxes reported here. To circumvent this difficulty, two experimental approaches, described in detail in Materials and Methods, were used. In one of them, the flux media contained bovine serum albumin (BSA) to reduce lysis and binding of charybdotoxin to the tube walls, and A23187 was present during the $K^+$ efflux measurement. In the other, the ionophore was removed before exposure of the cells to the toxin. In Fig. 5, Group ''A'' shows the $K^+$ efflux rate constants in cells incubated with 250 μM $Ca_o^{2+}$ without A23187 (empty column), with A23187 present during the flux (both lined and black columns), and with 20 nM charybdotoxin (after preincubation with 50 nM charybdotoxin, black column). Group ''B'' shows the values of the $K^+$ efflux rate constants in cells in which after modifying $Ca_i^{2+}$ with A23187 (nominally $Ca_i^{2+}$-free cells, empty column, $-Ca_i$; high $Ca_i^{2+}$ cells, both lined and black columns, $+Ca_i$) the ionophore was removed. Before the flux, an aliquot of high $Ca_i^{2+}$ cells was preincubated with the toxin; the latter was also present during the flux of this aliquot (black column). The activating effect of a rise in $Ca_i^{2+}$ was observed in both groups (Fig. 5 *A* and *B*, compare empty and lined columns). Charybdotoxin did not inhibit the $Ca_i^{2+}$-stimulated $K^+$ efflux in LK SRBCs either in the presence or in the absence of A23187 (groups A and B, compare lined and black columns).

## Effect of a Rise in $Ca_i^{2+}$ on $Na^+$ and $K^+$ Effluxes in LK SRBCs

Figure 6 describes the effect of increased $Ca_i^{2+}$ on both $Na^+$ and $K^+$ efflux rate constants in LK SRBCs incubated in Na-free solutions with 10 μM A23187 (columns A, B, C, and D) without (A and C), and with 0.2 mM $Ca^{2+}$ (columns B and D). For experimental details *see* Materials and Methods and Fig. 6 legend. The data described in Fig. 6 show that a rise in $Ca_i^{2+}$ activated $K^+$ efflux (74%, D) even in the absence of external $Na^+$, thus, a

Na:K exchange can be ruled out. The activating effect of $Ca_i^{2+}$, however, was not selective for $K^+$, since the $Na^+$ efflux rate constant was also higher (about 40%) in the presence of $Ca_i^{2+}$ (*B*).

## Discussion

The increase in the passive $K^+$ and $Na^+$ effluxes induced by alkalinization, reported here in LK SRBCs (Fig. 1), has also been observed in human erythrocytes [2, 5]. The increase in the outward movement of cellular cations took place despite the membrane hyperpolarization associated with a higher external pH, suggesting that the effect is due to the change in pH and independent of $E_m$. Because a rise in $pH_o$ is accompanied by a rise in $pH_i$, we can not define, at present, the sidedness of the pH effect. In human red cells, however, Chipperfield and Shennan [5] demonstrated that a higher $pH_i$, rather than $pH_o$, is involved in the increased cation effluxes caused by alkalinization.

The partial inhibition by external divalent cations and quinine, nonspecific $K^+$ channel blockers [7], of $K^+$ efflux in LK SRBCs at pH 9.0 (Fig. 2), and the reversibility of the alkalinization effect (Fig. 1*B*), argue against a disruptive effect of alkaline pH on the lipid bilayer of the SRBCs membrane, and suggest that alkalinization opens discrete transport pathways in the cell membrane. Quinidine, but not quinine, has been reported to inhibit the $Cl^-$-independent $K^+$ efflux in LK SRBCs at pH 7.4 [1]. The fact that quinine was inhibitory in the present study could be due, either to the different anion used as $Cl^-$ replacement ($NO_3^-$ in that report, and $MSF^-$ in this one), or, to the difference in the pH at which the $K^+$ efflux was measured. The lack of effect of quinine on $K^+$ efflux in HK SRBCs at pH 9.0 points at another difference in the erythrocyte membrane of both phenotypes.

The results presented here also give evidence of another pathway for $K^+$ and $Na^+$ transport which was induced by a rise in $Ca_i^{2+}$ and which was present in LK, but not in HK SRBCs (Figs. 3 and 6). This pathway appears to be selective for activation by $Ca_i^{2+}$, but not for $K^+$ transport. The lack of selectivity for $K^+$ over $Na^+$ has been reported also in the nucleated erythrocyte of fish and chicken [24]. Whether the lack of selectivity for $K^+$ over $Na^+$ is due to molecularly different $K_{Ca}$ channels among species (and between the two sheep phenotypes), or to different regulatory mechanisms on the same channels has yet to be determined.

The $Ca_i^{2+}$-activated pathway for $K^+$ efflux in LK SRBCs was a saturable function, in the presence of A23187, of the $Ca_o^{2+}$ concentration (Fig. 4). The $Ca^{2+}$ affinity appears to be high since $K^+$ efflux was saturated at about 5 μM $Ca_o^{2+}$. As stated in Results, $Ca_i^{2+}$ must have been about fourfold $Ca_o^{2+}$; the precise characterization of the $Ca_i^{2+}$ affinity requires further studies. The present findings differ from previous reports that mature LK SRBCs lack $K_{Ca}$ channels [3, 17]. The experimental conditions in which K($^{86}$Rb) uptake was measured in those reports [3, 17], however, were different from the ones described here. Because K-Cl cotransport is active in LK SRBCs [18], the reported K($^{86}$Rb) influx measured in $Cl^-$ was the sum of all $K^+$ fluxes. Since $Ca_i^{2+}$ inhibits K-Cl cotransport [19], the effect of a rise in $Ca_i^{2+}$ on $K^+$ efflux depends on the relative magnitude of the putative $K^+$ pathways. Furthermore, the increase in $K^+$ permeability induced by $Ca_i^{2+}$ in LK SRBCs (2-fold) differed in magnitude from that reported in human red cells (8-fold) [16, 23, 27]. This difference could be due to, either a lower density of $K_{Ca}$ channels or heterogeneity in the LK SRBCs population, with channels present in only a small number of them. Alternatively, the high internal $Na^+$ in LK SRBCs could inhibit the $K_{Ca}$ channels as it does in human red cells [16].

The quinine and charybdotoxin insensitivity of $K_{Ca}$ channels in LK SRBCs points to another difference among species: while in human erythrocytes $K_{Ca}$ channels are inhibited by quinine, quinidine [16, 23, 27] and charybdotoxin [4, 30], they are insensitive to quinidine (and probably to quinine and charybdotoxin) in chicken red cells [24]. Thus, quinine appears to inhibit $Ca_i^{2+}$-activated channels which are selective for $K^+$ [16, 23, 27], but not those in LK sheep and chicken red cells [24], which carry both $K^+$ and $Na^+$.

Although in human erythrocytes the activation of the $K_{Ca}$ channels is maximal at about 2 mM external $K^+$ [16], the $Ca_i^{2+}$-activated $K^+$ efflux in LK SRBCs was independent of the concentration of external $Rb^+$. Perhaps this lack of sensitivity to external $Rb^+$ was due to the high pH at which the fluxes were measured. In human erythrocytes, the dependency on external $K^+$ decreases sharply when the pH is raised from 6.15 to 8.15 [16]. Alternatively, the lack of activation by low external $Rb^+$ could indicate that, although $Rb^+$ can permeate $Ca_i^{2+}$-activated $K^+$ channels [27], it may not replace $K^+$ in the activation of the external sites.

In human red cells, the $K_{Ca}$ channels are inhibited by quinine and charybdotoxin, require low concentrations of external $K^+$ for activation by internal $Ca^{2+}$, and have a $K^+/Na^+$ selectivity of 17:1 to 100:1 [27]. Since none of these traits was apparent in the $Ca_i^{2+}$-activated $K^+$ efflux described in the present study, the $Ca_i^{2+}$-activated $K^+$ transport in LK SRBCs must be either a different molecular entity, or subject to different modulation, with functional characteristics closer to the $Ca_i^{2+}$-activated $K^+$ transport described in the nucleated red cell of chicken [24].

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