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THE EFFECTS OF ALKALI METAL IONS ON ACTIVE Ca^{2+} TRANSPORT IN RECONSTITUTED GHOSTS FROM HUMAN RED CELLS *

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1. K^+, Rb^+ or Na^+ increase from 30 to 90% the maximum rate of Ca^{2+} transport from resealed ghosts, leaving unaltered the apparent affinity of the Ca^{2+} pump for Ca^{2+}. Li^+ is ineffective as activator of Ca^{2+} transport. 2. K^+ does not change the temperature dependence of Ca^{2+} transport. 3. The effects of K^+ and Na^+ are half-maximal at 4.6 mM and 24 mM, respectively. 4. The site(s) at which K^+ or Na^+ combine are accessible only from the cytoplasmic surface of the cell membrane. 5. Under conditions in which Na^+ activates the Ca^{2+} pump no Na^+ efflux coupled to Ca^{2+} efflux is apparent.

Introduction

In 1971 Schatzmann and Rossi [1] and Bond and Green [2] showed that alkali metal ions increase the Ca^{2+}-ATPase activity from human red cell membranes. Later on Rega et al. [3] showed that the Ca^{2+}-phosphatase activity of the Ca^{2+} pump is also highly dependent on monovalent cations.

Schatzmann and Rossi [1] reported that the rate of Ca^{2+} transport from resealed ghosts is unchanged by removal of Na^+ and/or K^+. This raised doubts on whether alkali ions did in fact interact with the Ca^{2+} pump [4] until Richards et al. [5] showed that K^+ or Na^+ protect Ca^{2+}-ATPase from inhibition by *N*-ethylmaleimide, and Sarkadi et al. [6] reported that Na^+ or K^+ significantly increases the rate of Ca^{2+} uptake in inside-out vesicles from human red cell membranes. Wierichs and Bader [7] and Romero [8] also showed activation of Ca^{2+} transport by Na^+ or K^+ in resealed red cell ghosts, ruling out the possibility of alterations in the molecular architecture of the membrane after vesiculation [9] as the cause of the activation of Ca^{2+} transport by alkali metal ions observed by Sarkadi. Furthermore, the finding that alkali metal ions increase the steady-state level and the turnover of the phosphoenzyme of the Ca^{2+} pump [10] and the ATPase activity of the purified enzyme [11] suggests rather strongly that all the observed effects of these cations are excerted through the Ca^{2+} pump mechanism.

However, it is not yet clear (cf. Refs. 6 and 8) on what surface of the cell membrane monovalent cations are effective as activators and whether they are transported or not during activation. Furthermore, it is not known whether monovalent cations act on the apparent affinity or on the maximum effect of Ca^{2+}. In this paper we report results of experiments in reconstituted ghosts designed to answer these questions.

Materials and Methods

Fresh blood collected on acid-citrate-dextrose solution was used. ATP disodium salt (substan-

* A preliminary account of the experiments in this paper has been presented at the International Conference on the Ca^{2+} Pump of Red Cells, Buenos Aires, 1982.

tially vanadium-free), phosphocreatine, creatine phosphokinase and ouabain were from Sigma Chem. Co. Other reagents were of analytical grade. The IS 561 Ca^{2+}-selective electrode was a kind gift from Dr. W. Simon from the Swiss Federal Institute of Technology.

Preparation of resealed ghosts

Red cells were washed three times with 10 vol. 15 mM sucrose/90 mM Tris-HCl (pH 7.7 at 25°C)/100 mM choline chloride. When used, alkali cations replaced an equimolar amount of choline. 1 ml of washed and packed red cells was squirted into 6 ml distilled water at 0°C in a thin-walled Erlenmeyer flask. After 2 min, 3 ml of a concentrated salt solution were added with rapid mixing to give the following final composition: 10 mM $MgCl_2$, 10 mM phosphocreatine di-Tris, 5 U/ml creatine phosphokinase, 2 mM ATP, 0.1 mM ouabain, 100 mM choline chloride or alkali metal chloride, 10 mM Tris-EDTA, 50 mM Tris-HCl (pH 7.7 at 25°C) and 1.3–7.5 mM $^{45}CaCl_2$ (0.4 mCi/mmol). Sealing was accomplished by incubating with gently shaking the ghosts suspension in a water-bath at 60°C until the suspension reached 37°C which, under these conditions, took about 10 s. This procedure reduced to a minimum the depletion of Ca^{2+} from the resealed ghosts and was used when sealing to alkali metal ions was not required. When sealing to these ions was required, incubation at 37°C had to be prolonged to 5 min. After incubation at 37°C 1 vol. of the ghosts suspension was mixed with 5 vol. incubation medium at 0°C containing 100 mM choline chloride or alkali metal chloride, 15 mM sucrose and 90 mM Tris-HCl (pH 7.7 at 25°C). The ghosts were spun down at 6000 × *g* during 5 min, washed twice at 4°C with 50 vol. of the incubation medium and at the end of the washing suspended in more of the same medium.

The concentration of Ca^{2+} in the resealed ghosts was assumed to be identical to that in the sealing media. The fraction of ghosts that resealed to Ca^{2+} and ATP was calculated from the amounts of Ca^{2+} and adenine in the ghosts and in the sealing medium, assuming that 85% of the ghost volume is intracellular water and that the concentration of solutes in the intracellular water is equal to that in the sealing media. The volume of the ghosts was determined by the microhematocrit technique, centrifuging the suspension during 30 min at 13000 × *g*. The concentration of ATP was determined by its absorbance at 260 nm.

Measurement of Ca^{2+} efflux

The procedure of Rossi et al. [12] was modified as follows. 0.25 ml of a 50% (v/v) suspension of resealed ghosts was mixed with 4.75 ml of the incubation medium in a 50 ml plastic tube immersed in a water-bath at 37°C. Every minute 0.25 ml of the ghosts suspension was removed and mixed in a conical polyethylene tube kept at 0°C with 0.50 ml of the incubation medium containing 0.200 mM $LaCl_3$. The tubes were centrifuged for 1 min at 10000 × *g* in an Eppendorf centrifuge and the radioactivity in the supernatant was counted in a Beckman LS-100C liquid scintillation counter. The efflux of Ca^{2+} was estimated from the linear initial part of plots of the amount of $^{45}Ca^{2+}$ in the supernatant against incubation time. As judged from the extrapolation to zero time of these plots about 10% of the $^{45}Ca^{2+}$ was extracellular at the beginning of the incubation. Fluxes were expressed per ml of resealed ghosts.

2 mM ATP was chosen as the substrate concentration after measuring the rate of Ca^{2+} loss from ghosts resealed by 5 min incubation at 37°C in media containing 100 mM KCl and either 0.3, 2 or 5 mM ATP. Results showed that the rate of Ca^{2+} loss from the ghosts with 2 or 5 mM ATP were the same and remained constant during at least the first 5 min of the efflux experiment.

Measurement of Ca^{2+} concentration

The concentration of free Ca^{2+} in the medium in which the ghosts were sealed was determined with a IS 561 Ca^{2+}-selective electrode (W. Moller, Zurich) using a calomel reference electrode and a Corning model 121 pH meter. Within the range of Mg^{2+} concentrations used the electrode was insensitive to this cation. To take account of the effects of alkali metal salts from the sealing media on the activity of Ca^{2+} [13], the Ca^{2+} electrode was calibrated against solutions containing the components of the sealing media plus known amounts of Ca^{2+}.

Results

Since there is no practical way to measure the concentration of Ca^{2+} inside the ghosts except by using reagents that bind Ca^{2+} [14], we have assumed that the concentrations of Ca^{2+} in the resealed ghosts at the beginning of the efflux experiments were identical to those measured in the sealing media by means of the Ca^{2+} selective electrode. For this assumption to be close to reality it was necessary to reduce to a minimum the loss of Ca^{2+} from the ghosts during the sealing step. We measured the sealing of the ghosts to $^{45}Ca^{2+}$ and to ATP as a function of the time of incubation in a water-bath at 37°C. Results in Fig. 1 show that maximum sealing was attained after 60 s, which is the time that it takes for the ghosts suspension to reach 37°C. Prolonging the incubation at 37°C was without further effect. Results in Fig. 1 also show that sealing to ATP follows a pattern almost identical to that found with Ca^{2+}, reaching a value of 113%. Values larger than 100% for sealing to ATP were found in most of the experiments, indicating that after sealing the concentration of ATP in the intracellular water of the ghosts was higher than in the sealing medium. This fact has already been reported by other authors [15,16] and is the expected if the ghosts sealed to ATP at a volume

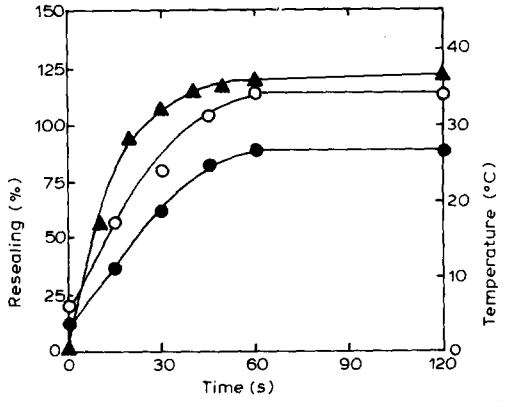

Fig. 1. Time course of the temperature (▲) and resealing of a suspension of red cell ghosts to Ca^{2+} (●) and ATP (○) in a water-bath at 37°C. 10 ml of the ghosts suspension in the sealing medium were placed in a 100 ml Erlenmeyer flask and kept at 0°C until transferred to the water-bath at 37°C. The flask was shaken gently and the temperature of the suspension measured by means of a YSI Tele-thermometer model 42SC with the probe immersed in the ghosts suspension.

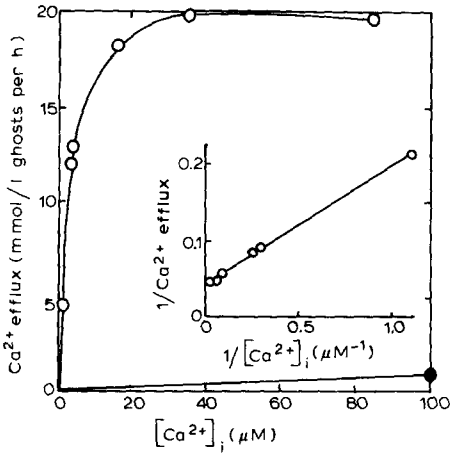

Fig. 2. $^{45}Ca^{2+}$ efflux from resealed ghosts containing 100 mM choline chloride with (●) and without (○) vanadate as a function of the intracellular Ca^{2+} concentration. The ghosts with vanadate were sealed and incubated in media with 0.2 mM vanadate. The inset represents a double reciprocal plot of the efflux in the absence of vanadate. Vanadate was added as NH_4VO_3.

greater than their final volume. The results in Fig. 1 demonstrate therefore that warming of the ghosts at 37°C is necessary for complete sealing. The rate of warming of the ghost suspension does not seem to affect sealing, since in experiments not shown it was observed that, when the temperature of the ghosts was raised from 4 to 37°C in less than 10 s, the sealing of the ghosts was at least equal to the maximum attained during the experiment of Fig. 1. After these findings the sealing procedure described in Materials and Methods was adopted. Under any of the conditions used in the experiments in this paper at least 85% of the ghosts have resealed to Ca^{2+} and ATP.

Fig. 2 shows the efflux of radioactive Ca^{2+} from ghosts containing 100 mM choline, various amounts of Ca^{2+} and either 0 or 0.2 mM vanadate suspended in choline medium with and without 0.2 mM vanadate. The Ca^{2+} efflux in the presence of vanadate can be taken as the passive leak of Ca^{2+} from the cells [12]. The passive leak was always very low, showing that the ghosts used are tightly sealed to Ca^{2+}. In this particular experiment the leak amounted to less than one-fiftieth the maximun vanadate-sensitive flux. For these reasons, in subsequent experiments the contribution of the passive leak to the total flux was neglected. A

double reciprocal plot of the total efflux (inset in Fig. 2) gives a straight line showing that the total efflux of Ca^{2+} changes with intracellular Ca^{2+} along a single Michaelis curve with $K_{0.5} = 3.3$ μM Ca^{2+} and a maximum value of 21 mmol/l ghosts per h.

The effects of alkali metals

Fig. 3 shows double reciprocal plots of Ca^{2+} efflux as a function of the intracellular Ca^{2+} concentration in ghosts containing 100 mM of either K^+, Na^+, Rb^+ or Li^+ and suspended in media with 100 mM of K^+, Na^+, Rb^+ or Li^+, respectively. Within the range of Ca^{2+} concentrations tested all the plots are straight lines, indicating that the activation of Ca^{2+} transport by intracellular Ca^{2+} in the presence of monovalent cations can be expressed by single Michaelis equations. K^+, Na^+ and Rb^+ stimulate the Ca^{2+} efflux to about the same extent. This confirms previous findings by Sarkadi et al. [6] and Wierichs and Bader [7]. Li^+ is uneffective as activator of the Ca^{2+} efflux. For all the cations tested, the $K_{0.5}$ for Ca^{2+} remains almost unchanged. In eight experiments with

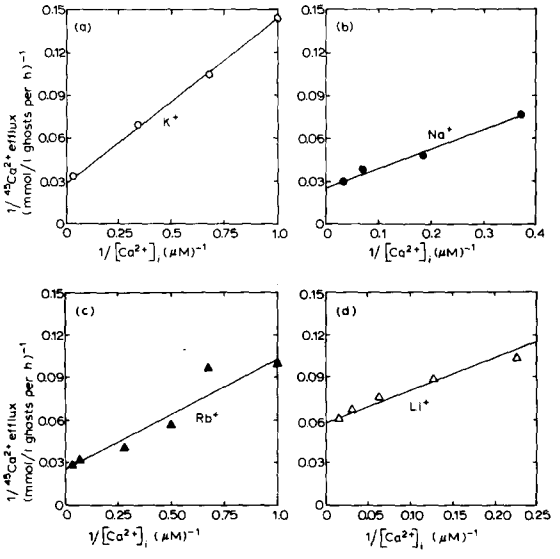

Fig. 3. Double reciprocal plots of $^{45}Ca^{2+}$ efflux against internal Ca^{2+} concentration from resealed ghosts in the presence of 100 mM of either K^+ (a), Na^+ (b), Rb (c) or Li^+ (d) on both sides of the membrane. (a) $J_{max} = 37$ mmol/l ghosts per h; $K_{0.5} = 4.3$ μM; (b) $J_{max} = 39$ mmol/l ghosts per h; $K_{0.5} = 5.7$ μM; (c) $J_{max} = 39$ mmol/l ghosts per h; $K_{0.5} = 3.2$ μM; (d) $J_{max} = 17.5$ mmol/l ghosts per h; $K_{0.5} = 4.0$ μM.

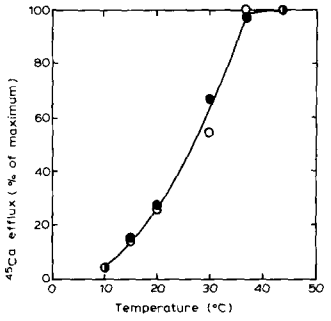

Fig. 4. The effect of temperature on $^{45}Ca^{2+}$ efflux from ghosts with (○) and without (●) 100 mM K^+. The K-containing ghosts were incubated in media with 100 mM K^+. The maximal effluxes were 23.6 mmol/l ghosts per h for the ghosts without K and 31.0 mmol/l ghosts per h for the ghosts with K^+.

100 mM K^+ the maximum efflux (±S.E.) was 32.9 ± 2.6 mmol Ca^{2+}/l ghosts per h and the $K_{0.5}$ 5.5 ± 0.6 μM Ca^{2+}, and in three experiments with 100 mM Na^+ the maximum efflux (±S.E.) was 30.2 ± 4.9 mmol/l ghosts per h and the $K_{0.5}$ 6.3 ± 0.3 μM Ca^{2+}.

Temperature dependence

Fig. 4 shows a plot of Ca^{2+} efflux from resealed ghosts with and without 100 mM K^+ at both sides of the membrane incubated at temperatures ranging from 10 to 44°C. The curves are superimposable, indicating that, within the range of temperature tested, activation by K^+ does not change the temperature dependence of the Ca^{2+} efflux.

Activation by K^+ or Na^+

Ca^{2+} efflux was measured in resealed ghosts containing 100 μM Ca^{2+} plus different amounts of either K^+ or Na^+ in media containing the alkali

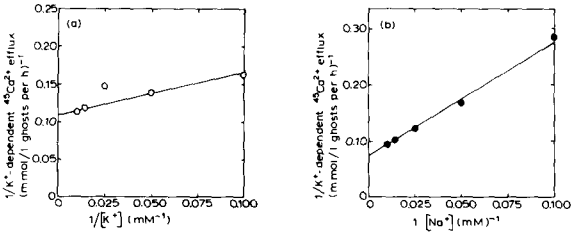

Fig. 5. Double reciprocal plots of K^+-dependent $^{45}Ca^{2+}$ efflux as a function of K^+ concentration (a) or Na^+-dependent $^{45}Ca^{2+}$ efflux as a function of Na^{2+} concentration (b) on both sides of the membrane.

TABLE I

ASYMMETRIC ACTIVATION OF Ca^{2+} EFFLUX BY K^+ OR Na^+ IN RESEALED GHOSTS

The ghosts were sealed by incubation at 37°C during 5 min. The ghosts from Expt. 1 contained 6 mmol Na^+/l ghosts and 5 mmol K^+/l ghosts brought in by the component salts of the sealing media and the red cells. The ghosts from Expt. 2 contained less than 0.8 mmol Na^+/l ghosts and 1 mmol K^+/l ghosts. To allow dilution of Na^+ and K^+ leaking from the ghosts, 1 vol. of ghosts was suspended in 40 vol. incubating medium during the efflux experiment. The incubation media of Expt. 1 contained 5 mM quinine-HCl.

	Intracellular medium with 100 mM	Incubation medium with 100 mM	Ca^{2+} efflux (mmol/l ghost per h)
Expt. 1	Choline	Choline	5.2
	K^+	K^+	7.5
	K^+	Choline	8.6
	Choline	K^+	5.6
Expt. 2	Choline	Choline	29.6
	Na^+	Na^+	39.0
	Na^+	Choline	40.0
	Choline	Na^+	30.4

cations in concentrations equal to those in the ghosts.

A double reciprocal plot of the K^+-dependent fraction of the efflux vs. K^+ concentration (Fig. 5a) give a straight line with $K_{0.5} = 4.6$ mM and a maximum value of 9.2 mmol/l ghosts per h. Likewise, the effect of Na^+ can be represented by a straight line with a $K_{0.5}$ of 24.1 mM and a maximum value of 13.3 mmol/l ghosts per h (Fig. 5b).

The sidedness of the activation by alkali metals

Ca^{2+} efflux from K^+-containing ghosts was measured in media with and without K^+, and Ca^{2+} efflux from Na^+-containing ghosts was measured in media with and without Na^+ and compared with Ca^{2+} efflux from choline-containing ghosts. Results in Table I show that the increment in Ca^{2+} efflux elicited by K^+ at both sides of the membrane remains when K^+ is present at the intracellular medium only and is lost when K^+ is in the incubation medium only. The effects of Na^+ are similar to those of K^+ in the sense that only Na^+ in the intracellular medium is effective as activator of Ca^{2+} transport. The low rate of Ca^{2+} efflux observed in the K^+ experiment is

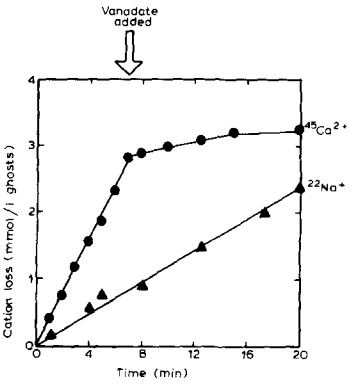

Fig. 6. The time course of the loss of ^{45}Ca (●) and ^{22}Na (▲) from resealed ghosts. The fluxes were measured in parallel on two preparations of ghosts from a single batch of red cells. The ghosts contained 7 mM $CaCl_2$ and 90 mM NaCl at the beginning of the experiment. Vanadate was added as a concentrated solution to give a final concentration of 0.8 mM in the incubation medium.

probably a consequence of the presence of quinine, since in a parallel experiment (not shown) about 65% of the Ca^{2+}-ATPase activity in fragmented membranes was inhibited by 5 mM quinine-HCl. Quinine was added to diminish the increase in K^+ permeability elicited by intracellular Ca^{2+} [17].

Effect of Ca^{2+} transport on Na^+ efflux

To see whether or not the activating monovalent cation is transported by the Ca^{2+} pump we have measured effluxes of $^{45}Ca^{2+}$ and $^{22}Na^+$ from resealed ghosts, with the results shown in Fig. 6. It can be seen that upon inhibition of the pump with vanadate the efflux of $^{45}Ca^{2+}$ drops from 24.0 to 3.0 or less mmol Ca^{2+}/l ghosts per h. The efflux of $^{22}Na^+$, on the other hand, shows no sign of change in magnitude, remaining constant in 7.2 mmol/l ghosts per h throughout the experiment.

Discussion

Results in this paper confirm previous findings of other authors showing that alkali metals such as K^+, Na^+ and Rb^+ stimulate the active transport of Ca^{2+} from human red cells. Li^+, which is a poor activator of the Ca^{2+}-ATPase [2], did not activate Ca^{2+} transport. On raising the temperature of the ghosts suspension from 10 to 44°C the rate of Ca^{2+} efflux increases more than 20-times in a

manner which is independent of the presence or absence of 100 mM K^+. Results also show that monovalent cations activate Ca^{2+} efflux, increasing the maximum rate of transport and leaving unaltered the apparent affinity of the Ca^{2+} pump for Ca^{2+}. This agrees with previous findings from our laboratory on the turnover of the Ca^{2+}-ATPase from disrupted membranes of red cells [10].

The value of the $K_{0.5}$ for K^+ and Na^+ varied from preparation to preparation. Nevertheless, in agreement with Romero's findings [18], when both cations were tested on the same preparation of ghosts, the $K_{0.5}$ for Na^+ was 4–5-times larger than that for K^+. A relationship of this sort between apparent affinities for Na^+ and K^+ has also been reported for the Ca^{2+}-ATPase [1,11] and the Ca^{2+}-phosphatase [3] activities of red cell. It has been suggested by Scharff [19] that the difference in affinities between Na^+ and K^+ depends on calmodulin. Since the resealed ghosts used for the experiments reported here have not been washed with chelators, they might have retained enough calmodulin to activate most of the Ca^{2+} pump units in the ghosts.

Results are clear in demonstrating that the alkali metals are effective as activators of the Ca^{2+} pump from the inner surface of the cell membrane, being ineffective at the external surface. Accumulations in the incubation media of cations leaking from the ghosts during the experiment can be disregarded as the cause of the results shown, since even if the monovalent cations had reached equilibrium between intra- and extracellular media their concentrations would have been 2 mM as a maximum, a value that in view of the apparent affinities of Na^+ and K^+ is too low to reproduce the activating effect of 100 mM Na^+ or K^+. It can be concluded therefore that the site(s) at which these ions combine to activate the Ca^{2+} pump are located on the inner surface of the cell membrane and hence that the Ca^{2+} pump of red cells is asymmetric to monovalent cations. This conclusion agrees with those of Sarkadi et al. [6] and Wierichs and Bader [7] and with the findings of Rossi et al. [12] on the sidedness of the potentiation by K^+ of the inhibition of Ca^{2+} efflux by vanadate, but is at variance with the proposal of Romero [8,18] on activation of Ca^{2+} efflux from resealed ghosts by Na^+ and K^+ from the outer surface of the membrane. Furthermore, Wierichs and Bader [7] reported that ghosts containing 35 mM K^+ incubated in medium with 130 mM Na^+ show Ca^{2+} efflux and Ca^{2+}-ATPase activity higher than ghosts containing 38 mM Na^+ in medium with 130 mM K^+. These results are expected from the lower affinity of the intracellular sites for Na^+ compared to K^+ and the absence of effect of extracellular monovalent cations reported here.

The sites for monovalent cations being on the inner surface of the cell membrane, if Ca^{2+} transport were coupled to movements of alkali metals it would be expected that Na^+ would be transported outward from the ghosts. We have shown that a drop of 8-fold and more in the rate of Ca^{2+} transport is without effect on Na^+ efflux. There is no correspondence therefore between Ca^{2+} pumping and Na^+ efflux under conditions in which this cation activates the Ca^{2+} pump, suggesting rather strongly that the activating alkali metal ion is not transported by the Ca^{2+} pump.

Monovalent cations also activate Ca^{2+} transport across sarcoplasmic reticulum membranes from skeletal muscle [20,21]. As shown here for the red cell, in sarcoplasmic reticulum the effect is mainly exerted on the rate of Ca^{2+} transport [20,21].

Under normal conditions the cytoplasmic fluid bathing the inner surface of the red cell membrane contains enough potassium ions to activate fully the Ca^{2+} pump. Hence, the effect of K^+ on Ca^{2+} transport across the red cell membrane has to be taken into account when analyzing the physiological behaviour of the Ca^{2+} pump.

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