Biochimica et Biophysica Acta 816 (1985) 365–378
Elsevier

BBA 72622

Two modes of inhibition of the Ca^{2+} pump in red cells by Ca^{2+}

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(Received January 16th, 1985)

Key words: Ca^{2+} pump; Ca^{2+} inhibition; Erythrocyte ghost; Mg^{2+}; Kinetics

(1) Two different and independent modes of inhibition of the Ca^{2+} pump by Ca^{2+} can be detected measuring active Ca^{2+} extrusion from resealed ghosts of human red cells: one requires extracellular and the other requires intracellular Ca^{2+}. (2) K_i for inhibition by extracellular Ca^{2+} is about 10 mM. (3) Extracellular Mg^{2+} replaces Ca^{2+} in inhibiting Ca^{2+} transport but with an apparent affinity for inhibition about 3-times less than that for Ca^{2+}. (4) Inhibition by external Ca^{2+} is not affected by Na^+ or K^+ at both surfaces of the cell membrane, external EGTA, internal Ca^{2+} or ATP. (5) The apparent affinity for external Ca^{2+} progressively raises as pH increases. (6) The effects of extracellular Ca^{2+} and Mg^{2+} are consistent with the idea that for Ca^{2+} pumping to proceed, external sites in the pump must be protonated and not occupied by extracellular Ca^{2+} or Mg^{2+} (6). Inhibition by intracellular Ca^{2+} takes place with a K_i of about 1 mM and is independent of external Ca^{2+}. (7). The inhibitory effects of intracellular Ca^{2+} can be accounted for if Ca^{2+} and CaATP were competitive inhibitors of the activation of the pump by Mg^{2+} and MgATP, respectively.

Introduction

It is now well-established that the very low concentration of cytosolic free Ca^{2+} in red cells is maintained by the active extrusion of Ca^{2+} through a Ca^{2+}-pumping ATPase located in the plasma membrane (for references see Ref. 1). This system is activated by micromolar concentrations of Ca^{2+} at the inner surface of the cell membrane. In disrupted membranes [2] and in the purified ATPase [3], Ca^{2+} at concentrations higher than those needed for activation inhibits the overall ATPase reaction and the dephosphorylation reaction [4].

In this paper, we report results of experiments in which the effect of high Ca^{2+} concentrations on the active efflux of Ca^{2+} from resealed ghosts was measured, varying the concentration of Ca^{2+} at both surfaces of the cell membrane. Results show that two modes of inhibition by high concentrations of Ca^{2+} can be identified in resealed red cell membranes: one requires extracellular Ca^{2+} and the other requires intracellular Ca^{2+}.

Materials and Methods

Materials. Fresh blood collected on acid/citrate/dextrose solution was always used. [γ-^{32}P]ATP was prepared following the method of Glynn and Chappell [5] except that no orthophosphate was added to the incubation medium. [^{32}P]P_i was from the Comisión Nacional de Energía Atómica, Argentina. $^{45}CaCl_2$ was from New England Nuclear (U.S.A.). ATP disodium salt (substantially vanadate-free), phosphocreatine, phosphocreatine kinase and cofactors and enzymes for the synthesis of labelled ATP, were from Sigma

(U.S.A.). All other reagents were of analytical reagent grade.

Methods. Red cells were washed three times at 4°C with 50 vol. of a solution containing: 75 mM sucrose, 60 mM Tris-HCl (pH 7.4 at 37°C) and 100 mM of either NaCl, KCl or choline chloride, depending on the final composition of the resealed ghosts. Resealed ghosts were prepared following a procedure essentially similar to that already described [6]. Except when otherwise indicated in Results, the solutions in which ghosts were sealed contained: 2 mM ATP, 10 mM phosphocreatine, 5 IU/ml creatine phosphokinase, 50 mM Tris-HCl (pH 7.4 at 37°C) and 100 mM of either NaCl, KCl or choline chloride. When only the effects of extracellular Ca^{2+} were studied, the resealing medium contained 7.5 mM $CaCl_2$ and 10 mM $MgCl_2$. This gave concentrations of free Ca^{2+} and free Mg^{2+} of about 0.1 and 5 mM, respectively. When internal Ca^{2+} was varied, the concentrations of $CaCl_2$ and $MgCl_2$ were adjusted to obtain the desired concentration of free Ca^{2+}, keeping the concentration of free Mg^{2+} constant at 5 mM. The specific activity of intracellular $CaCl_2$ was 0.3 mCi/mmol.

After isotonicity was restored, the ghosts that were used for studying the effects of extracellular Ca^{2+} were incubated during 5 min at 37°C for sealing. To reduce the uncertainties in the initial value of intracellular Ca^{2+} concentration caused by Ca^{2+} loss during preincubation, when the effects of intracellular Ca^{2+} were tested, sealing was attained by immersing the suspension for 10 s in a water-bath at 60°C. As described previously [6], this procedure allows the ghost suspension to reach 37°C at the end of the 10-s incubation period.

After sealing, the ghosts were washed in a solution of identical composition as that used to wash the red cells and suspended in more of this solution ready for use.

Ca^{2+} efflux was measured as described previously [6]. Hematocrit was 3–5% and temperature was 37°C. The composition of the incubation media depended on the experimental conditions and is given in the legends to figures. 0.1 mM ouabain was always present. Osmolarity was always kept at 0.365 ideal osmolar and unless otherwise indicated pH was kept at 7.4 (37°C) with Tris-HCl.

To measure Ca^{2+} influx, 0.5 ml of a 50% (v/v) suspension of resealed ghosts was added to 4.5 ml of a solution previously warmed at 37°C and containing: 100 mM NaCl, 15 mM sucrose, 90 mM Tris-HCl (pH 7.4 at 37°C). $CaCl_2$ labelled with ^{45}Ca (spec. act. 1 mCi/ml) replaced equiosmolar concentrations of Tris-HCl. After 1, 3, 5, 8 and 12 min incubation, duplicate 0.25-ml portions of the suspension were removed and transferred to a centrifuge tube containing 1 ml of an ice-cold solution of: 100 mM NaCl, 15 mM sucrose, 75 mM Tris-HCl (pH 7.4 at 37°C) and 10 mM EGTA (diTris salt). The ghosts were centrifuged for 1 min at 12000 rpm in an Eppendorf 5412 microcentrifuge and then washed twice at 4°C with a solution of composition similar to that described above except that 10 mM unlabelled $CaCl_2$ replaced EGTA. After this step, the pellet was suspended in 0.5 ml perchloric acid (7.8% v/v) and the precipitated material was eliminated by centrifugation. The supernatant was brought to neutrality with 2 M Tris and the radioactivity of ^{45}Ca was determined in a liquid scintillation counter. Ca^{2+} influx was calculated from the slope of the initial linear part of the plot of ^{45}Ca in the ghosts vs. incubation time.

To prepare disrupted membranes, red cells were washed and hemolyzed following a procedure identical to that used for the preparation of resealed ghosts. After hemolysis, the membranes were washed twice with distilled water and then frozen and thawed twice.

Ca^{2+}-ATPase activity of disrupted membranes was estimated from the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$ following the procedure described previously [7] except that the suspension was deproteinized with trichloroacetic acid (final concentration 6.25% w/v) before extraction of inorganic phosphate. The assay medium was similar to the medium in which ghosts were sealed except that ^{45}Ca and creatine phosphokinase were omitted and 0.1 mM ouabain was present. $CaCl_2$ and $MgCl_2$ concentrations were adjusted to give the desired concentrations of free Ca^{2+} and to keep free Mg^{2+} constant at 5 mM. Ca^{2+}-ATPase activity was taken as the difference between the activity measured in the above-mentioned media and in media with 1 mM orthovanadate.

The concentration of free Ca^{2+} in sealing and incubation media was measured as described pre-

viously [6] using an IS 561 Ca^{2+}-sensitive membrane electrode. The total concentration of Ca^{2+} in the ghosts was calculated from the activity of the ghost suspensions and the specific activity of ^{45}Ca in the sealing media. The free Ca^{2+} concentration in sealed ghosts was calculated by interpolation in a plot of free Ca^{2+} vs. total Ca^{2+} obtained by measuring free Ca^{2+} with the membrane electrode in solutions of identical composition as those used for sealing. Free Mg^{2+}, free Ca^{2+}, CaATP and MgATP concentrations were calculated assuming dissociation constants of 54 and 45 μM for CaATP and MgATP [8] and of 0.014 and 1.565 μM for CaEDTA and MgEDTA [9]. The binding of Ca^{2+} and Mg^{2+} to phosphocreatine and to orthophosphate was neglected. These assumptions seem to give a good description of the binding properties of the sealing medium, since the concentration of free Ca^{2+} predicted by them agreed reasonably well with the values obtained by means of the Ca^{2+}-sensitive electrode. The concentration of ATP in the ghosts was estimated from the concentration of adenine in the ghost suspension. Adenine was measured from the difference between the absorbances at 260 nm and at 290 nm of the ghost suspension after deproteinization with 5% trichloroacetic acid. The molar extinction coefficient of adenine was taken as 15400 $M^{-1} \cdot cm^{-1}$. To calculate intracellular concentrations, the water content of the ghosts was assumed to be 85% of the ghost volume.

Theoretical equations were fitted to the experimental data by least-squares non-linear regression using the procedure of Gauss-Newton with optional damping (Rossi, R.C. and Garrahan, P.J., unpublished data). The velocity variable was assumed to be homoscedastic and the concentrations variable to have negligible error. Calculations were performed with a microcomputer having 14-digit precision.

Results

Inhibition of Ca^{2+} efflux by extracellular Ca^{2+}

Fig. 1 shows the initial linear part of the time-course of the loss of ^{45}Ca from resealed ghosts incubated at 37°C in media with and without 9.7 mM Ca^{2+}. In Ca^{2+}-free media, Ca^{2+} efflux, calculated from the slope of the plot of Ca^{2+} loss vs. time, was 27.6 mmol/l ghosts per h. When 9.7 mM Ca^{2+} was present, Ca^{2+} efflux was reduced in about 50%. This effect was not observed when the media contained 9.7 mM Ca^{2+} and 9.7 mM EDTA, showing that free Ca^{2+} is required. Addition of 9.7 mM EDTA to the Ca^{2+}-containing medium rapidly restored Ca^{2+} efflux to its control value, indicating that inhibition by external Ca^{2+} is reversible.

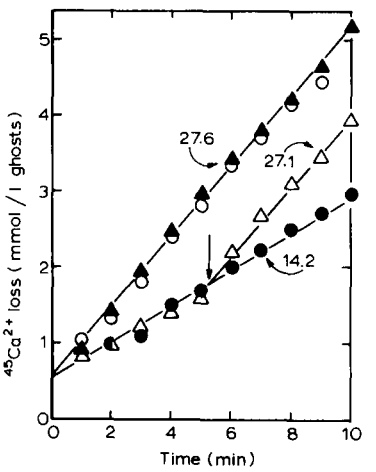

Fig. 1. The time-course of the loss of Ca^{2+} from resealed ghosts suspended in media without Ca^{2+} (○), or in media with either 9.7 mM $CaCl_2$ (●, △) or 9.7 mM $CaCl_2$ plus 9.7 mM EDTA (▲). When indicated by the vertical arrow, enough of a concentrated solution of EDTA was added to half of the tubes containing the ghosts suspended in the medium with 9.7 mM $CaCl_2$ as to give a final concentration of 9.7 mM EDTA (△). The medium without Ca^{2+} contained 100 mM NaCl, 60 mM Tris-HCl and 75 mM sucrose. $CaCl_2$ and EDTA replaced equiosmolar amounts of sucrose. Figures in brackets are fluxes (mmol/l ghosts per h).

Control experiments (not shown) in which 40–130 mM choline chloride were added to the incubation media keeping the osmolarity constant demonstrated that Ca^{2+} efflux is not modified by changes in ionic strength. Hence, the inhibition observed in the experiment in Fig. 1 cannot be attributed to the increase in ionic strength caused by the addition of Ca^{2+}.

Inhibition by Ca^{2+} in the incubation medium might result either from the effect of the cation at the external surface of the cell membrane or from the penetration of Ca^{2+} into the resealed ghosts. A significant increase in the concentration of intracellular Ca^{2+} might produce a direct inhibitory

effect. Even if the concentration of intracellular Ca^{2+} is not increased, dilution of the specific activity of $^{45}Ca^{2+}$ by inflowing Ca^{2+} would lead to the underestimation of Ca^{2+} efflux when this is calculated from the initial specific activity. Results in Fig. 1 make it unlikely that these factors are the cause of the inhibition of Ca^{2+} efflux by external Ca^{2+}, since if changes in intracellular Ca^{2+} were required, it is difficult to explain why in the presence of external Ca^{2+} the loss of Ca^{2+} from the ghosts is a linear function of time and why the removal of free Ca^{2+} from the external medium with EDTA leads to an almost instantaneous recovery of the efflux to its control value. In spite of this and in view of the importance that the elucidation of this point has in the interpretation of the results, control experiments were undertaken to measure in a more direct way the participation of changes in intracellular Ca^{2+} in the effects of external Ca^{2+}.

Fig. 2 shows the results of experiments in which the initial rate of $^{45}Ca^{2+}$ uptake by reconstituted ghosts was measured in media containing different concentrations of Ca^{2+}. It is clear that in a 0–20 mM concentration range, Ca^{2+} influx is a linear function of external Ca^{2+} concentration. A rate constant of 0.29 h^{-1} for the influx can be calculated from the slope of the line that gives best fit to the experimental points in Fig. 2. Hence, in media with 9.7 mM Ca^{2+}, the influx of the cation

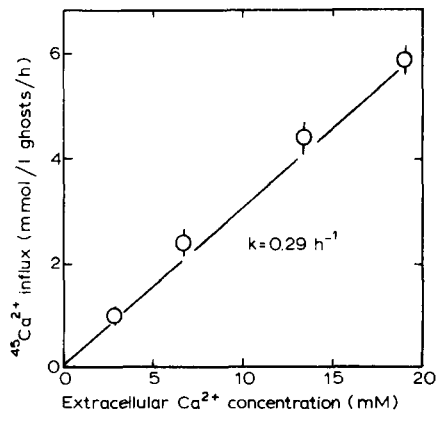

Fig. 2. The influx of Ca^{2+} into Na^+-rich ghosts as a function of the extracellular Ca^{2+} concentration. The vertical bars are ranges of the values of two experiments. For other details see Materials and Methods.

will be 2.7 mmol/l ghosts per h. This is about 20% of the efflux measured under the same conditions (Fig. 1), so that even in the presence of high external Ca^{2+} a net decrease in the concentration of intracellular Ca^{2+} will take place during experiments like those in Fig. 1. This allows to discard increases in the concentration of intracellular Ca^{2+} as the cause of the inhibition of Ca^{2+} efflux by extracellular Ca^{2+}.

Regarding the dilution of the specific activity of intracellular $^{45}Ca^{2+}$, if all the intracellular Ca^{2+} were available for quick exchange with inflowing Ca^{2+}, which seems likely in view of the very quick rate reported for the association and dissociation of divalent cations from EDTA [10], the linear dependence of Ca^{2+} influx with external Ca^{2+} would predict that the intracellular specific activity at a given incubation time ($SA(t=t)$) will be related to the initial specific activity ($SA(t=0)$) by the following equation:

$$\frac{SA(t=0)-SA(t=t)}{SA(t=0)} = \frac{1}{1+\frac{[Ca^{2+}\,tot]_i}{k[Ca^{2+}]_e t}} \qquad (1)$$

where $[Ca^{2+}tot]_i$ is the total content of calcium (free plus complexed) in the ghosts at the beginning of the incubation, $[Ca^{2+}]_e$ the extracellular Ca^{2+} concentration, k the rate constant for Ca^{2+} influx (0.29 h^{-1}) and t the incubation time. Eqn. 1 permits to calculate that at the end of the experiment in Fig. 1, the specific activity of intracellular Ca^{2+} in the ghosts suspended in media with 9.7 mM Ca^{2+} will be 94.6% of the initial specific activity. The systematic error introduced in the estimation of Ca^{2+} efflux by this change in specific activity falls well within the experimental error.

Since none of the effects of inflowing Ca^{2+} seem to be able to explain the reduction of Ca^{2+} efflux observed upon the addition of Ca^{2+} to the incubation medium, it seems reasonable to conclude that Ca^{2+} acting at the external surface of the cell is the cause of the inhibition of active transport of Ca^{2+}.

The kinetics of inhibition by extracellular Ca^{2+}

In the experiments shown in Fig. 3, the effects of increasing concentrations of external Ca^{2+} on

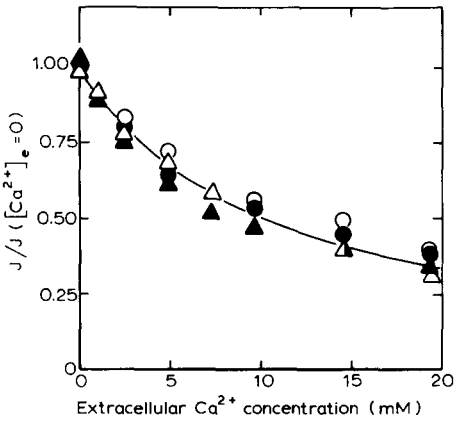

Fig. 3. The effect of extracellular Ca^{2+} on the efflux of Ca^{2+} from ghosts resealed in solutions containing 100 mM of either KCl (▲), NaCl (●, ○), or choline chloride (△). The nominally Ca^{2+}-free incubation media contained: 100 mM of the same chloride salt as that in the solution in which the ghosts were sealed, 60 mM Tris-HCl and 75 mM sucrose with (○) and without (▲, △, ●) 5 mM $CaCl_2$ plus 5 mM EGTA. Excess Ca^{2+} was added as $CaCl_2$ and replaced equiosmolar amounts of sucrose. Ca^{2+} concentration was assumed to be equal to the concentration of $CaCl_2$ minus the concentration of EGTA. For each of the experimental condition tested, Eqn. 2 was fitted to the data assuming $J_{([Ca^{2+}]_e=\infty)} = 0$. Fluxes are expressed as the ratio between the experimental flux and the corresponding calculated value of $J_{([Ca^{2+}]_e=0)}$, which in mmol/l ghosts per h was: 29.5 for K^+-containing ghosts, 25.6 for choline-containing ghosts, 30.2 for Na^+-containing ghosts suspended in the medium without EGTA, and 36.5 in the ghosts suspended in the medium with EGTA. The continuous line is the solution of Eqn. 2 for: $J_{([Ca^{2+}]_e=0)} = 1$, $J_{([Ca^{2+}]_e=\infty)} = 0$ and $K_{i_{Ex}} = 10.7$ mM which is the weighted average of the best-fitting values of $K_{i_{Ex}}$ for each experimental condition.

Ca^{2+} efflux were measured in ghosts resealed in media containing 100 mM of either KCl, NaCl or choline chloride and incubated in media with 100 mM of the chloride salt that was present in the sealing medium. In the case of the Na^+-containing ghosts, the effects of external Ca^{2+} were also tested in media with 5 mM EGTA. It can be seen that the efflux of Ca^{2+} progressively decreases as external Ca^{2+} increases and that this effect is independent of the presence of EGTA in the suspending medium and of the ionic composition of the ghosts and the media.

Six additional independent experiments like those in Fig. 3 were performed using Na^+-containing ghosts suspended in Na^+-rich media. A non-linear regression procedure was applied to fit the following equation to the experimental points:

$$J = \frac{J_{([Ca^{2+}]_e=0)}}{1+\frac{[Ca^{2+}]_e}{K_{i_{Ex}}}} + J_{([Ca^{2+}]_e=\infty)} \quad (2)$$

where $J_{([Ca^{2+}]_e=0)} + J_{([Ca^{2+}]_e=\infty)}$ is the efflux in media without Ca^{2+}, $J_{([Ca^{2+}]_e=\infty)}$ the efflux when the concentration of external Ca^{2+} tends to infinity and $K_{i_{Ex}}$ the concentration of Ca^{2+} required to reduce to one-half the inhibitable fraction of the efflux. Eqn. 2 implies that Ca^{2+} inhibits following simple hyperbolic kinetics and allows for the possible existence of a fraction of the efflux that is not inhibitable by Ca^{2+}.

In none of the seven experiments the best-fitting value of $J_{([Ca^{2+}]_e=\infty)}$ was significantly different from zero, strongly suggesting that the Ca^{2+} pump can be fully blocked by external Ca^2.

The weighted averages ($\pm$ standard error) over the seven independent experiments of the parameters of Eqn. 2 that gave best fit when $J_{([Ca^{2+}]_e=\infty)} = 0$, were: $J_{([Ca^{2+}]_e=0)} = 26.6 \pm 0.3$ mmol/l ghosts per h; $K_{i_{Ex}} = 9.5 \pm 0.3$ mM.

The effects of external Mg^{2+}

Fig. 4 shows the results of one of four separate experiments in which Ca^{2+} efflux was measured in resealed ghosts suspended in Ca^{2+}-free media containing Mg^{2+} at different concentrations. It can be seen that as Mg^{2+} concentration increases, Ca^{2+} efflux progressively decreases. Eqn. 2 was adjusted to the experimental points by non-linear regression. In all experiments, $J_{([Mg^{2+}]_e=\infty)}$ was not significantly different from zero. The weighted averages ($\pm$ standard error) of the parameters that gave best fit, taking $J_{([Mg^{2+}]_e=\infty)} = 0$ were: $K_{i_{Ex}} = 34.2 \pm 2.4$ mM; $J_{([Mg^{2+}]_e=0)} = 22.7 \pm 0.3$ mmol/l ghosts per h.

These results indicate that inhibition by external Mg^{2+} follows the same kinetic pattern as inhibition by external Ca^{2+} except that the apparent affinity for inhibition of Ca^{2+} efflux by Mg^{2+} is at least 3-times less than that for inhibition by Ca^{2+}.

The interaction between Ca^{2+} and Mg^{2+}

If inhibition by external Ca^{2+} and Mg^{2+} were exerted at the same class of sites, the observed

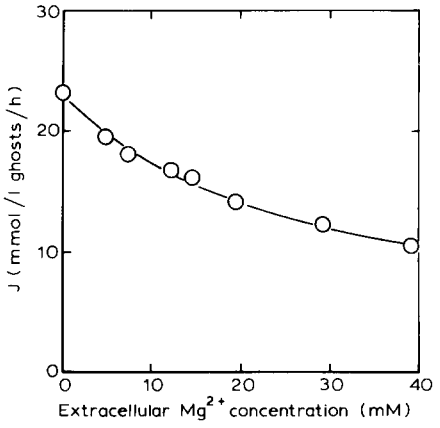

Fig. 4. The effect of increasing concentrations of Mg^{2+} in the incubation medium on the efflux of Ca^{2+}. Ghosts were resealed in a Na^+-rich medium. The Mg^{2+}-free incubation medium contained 100 mM NaCl, 15 mM sucrose and 90 Tris-HCl. $MgCl_2$ replaced equiosmolar amounts of Tris-HCl. The concentration of free Mg^{2+} was assumed to be equal to that of $MgCl_2$. The continuous line is the solution of Eqn. 2 for $J_{([Mg^{2+}]_e=0)} = 23.0$ mmol/l ghosts per h, $J_{([Mg^{2+}]_e=\infty)} = 0$ and $K_{i_{Ex}} = 32.1$ mM.

individual effects of each cation predict that the following equation would describe inhibition by a mixture of both cations:

$$J = \frac{J_{([Me^{2+}]_e=0)}}{1 + \frac{[Ca^{2+}]_e}{K_{i_{CaEx}}} + \frac{[Mg^{2+}]_e}{K_{i_{MgEx}}}} \tag{3}$$

where $J_{([Me^{2+}]_e=0)}$ is the efflux in the absence of external Ca^{2+} and Mg^{2+} and $K_{i_{CaEx}}$ and $K_{i_{MgEx}}$ are the apparent dissociation constants of Ca^{2+} and Mg^{2+}, respectively, from the site from which they exert their inhibitory effects.

If the sum of the concentrations of external Ca^{2+} and Mg^{2+} were kept constant ($Ct = [Mg^{2+}]_e + [Ca^{2+}]_e$), Eqn. 3 can be rearranged to yield:

$$\frac{1}{J} = \frac{1}{J'_{([Ca^{2+}]_e=0)}} + \frac{K'_{i_{Ca}}}{J'_{([Ca^{2+}]_e=0)}}[Ca^{2+}]_e \tag{4}$$

where:

$$J'_{([Ca^{2+}]_e=0)} = J_{([Me^{2+}]_e=0)}/\left(1 + Ct/K_{i_{MgEx}}\right) \tag{5}$$

and

$$K'_{i_{Ca}} = \left(K_{i_{MgEx}} - K_{i_{CaEx}}\right)/\left(K_{i_{CaEx}}\left(K_{i_{MgEx}} + Ct\right)\right) \tag{6}$$

On the other hand, if external Ca^{2+} and Mg^{2+} inhibited acting at two classes of independent sites, the effect of a mixture of both cations on Ca^{2+} efflux would be described by:

$$J = \frac{J_{([Me^{2+}]_e=0)}}{\left(1 + \frac{[Ca^{2+}]_e}{K_{i_{CaEx}}}\right)\left(1 + \frac{[Mg^{2+}]_e}{K_{i_{MgEx}}}\right)} \tag{7}$$

where the parameters have the same meaning as in Eqn. 3. When the sum of external Mg^{2+} and Ca^{2+} is kept constant, Eqn. 7 can be rearranged to yield:

$$\frac{1}{J} = \frac{1}{J'_{([Ca^{2+}]_e=0)}} + \frac{K_1[Ca^{2+}]_e}{J'_{([Ca^{2+}]_e=0)}} - \frac{K_2[Ca^{2+}]_e^2}{J'_{([Ca^{2+}]_e=0)}} \tag{8}$$

where

$$K_1 = K_2\left(K_{i_{MgEx}} - K_{i_{CaEx}} + Ct\right) \tag{9}$$

$$K_2 = 1/\left(K_{i_{CaEx}}\left(K_{i_{MgEx}} + Ct\right)\right) \tag{10}$$

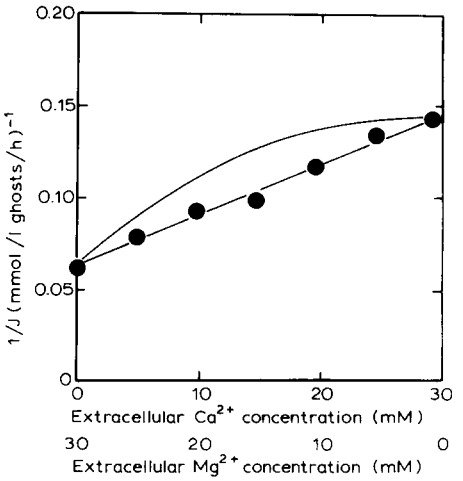

Fig. 5. A plot of the reciprocal of Ca^{2+} efflux vs. the concentration of Ca^{2+} in media in which the sum of the concentration of Ca^{2+} and Mg^{2+} was kept constant. Ghosts were sealed in Na^+-rich media and suspended in media containing: 100 mM NaCl, 45 mM Tris-HCl, 10 mM sucrose and 29.3 mM ($CaCl_2 + MgCl_2$). The continuous straight line is the solution of Eqn. 4 and the continuous curve is the solution of Eqn. 8 for: $K_{i_{CaEx}} = 9.5$ mM, $K_{i_{MgEx}} = 34.2$ mM, $Ct = 29.3$ mM and $J_{([Me^{2+}]_e=0)} = 28.6$ mmol/l ghosts per h.

and $J'_{([Ca^{2+}]_e=0)}$ has the same meaning as in Eqn. 5. To see which of the two models analyzed above provides a better description of the interaction between external Ca^{2+} and Mg^{2+} during inhibition of Ca^{2+} efflux, the effect of increasing concentrations of Ca^{2+} on Ca^{2+} efflux was measured in media in which the sum of the concentration of Ca^{2+} and Mg^{2+} was kept constant at 29.3 mM. A plot of the reciprocal of the efflux against the concentration of Ca^{2+} is shown in Fig. 5 together with the solutions of Eqns. 4 and 8 for the already mentioned mean values of $K_{i_{CaEx}}$ (9.5 mM) and $K_{i_{MgEx}}$ (34.2 mM). The value of $J_{([Me^{2+}]_e=0)}$ was determined on an aliquot of the same preparation of ghosts. The experimental points can be fitted by a straight line which is adequately described by Eqn. 4 but not by Eqn. 8. Results in Fig. 5 therefore support the idea that external Ca^{2+} and Mg^{2+} share the same class of sites to inhibit Ca^{2+} efflux.

Effects of pH

In the experiment shown in Fig. 6A, the efflux of Ca^{2+} from resealed ghosts into Ca^{2+}-free media was measured at different pH values. It can be seen that, in agreement with findings by other authors [11–13], Ca^{2+} efflux varies with pH along a bell-shaped curve with a maximum around pH 7.5. To see if the Ca^{2+}-buffering capacity of the intracellular medium varied with pH, free Ca^{2+} concentration in media of identical composition as those in which the ghosts were sealed was measured with a Ca^{2+}-sensitive electrode. Results (not shown) demonstrated that in agreement with the calculations of Wolf [9], the level of free Ca^{2+} was independent of pH between 6 and 9. The effects of pH on Ca^{2+} efflux therefore cannot be attributed to changes in the intracellular concentration of free Ca^{2+} caused by variations in the buffering capacity of the intracellular medium.

In the experiment in Fig. 6B, the inhibitory effect of three different concentrations of external Ca^{2+} (4.8, 9.7, and 19.4 mM) was measured as a function of pH in the incubation media. It can be seen that as pH raises, the inhibitory effect of each of the concentrations of Ca^{2+} assayed increases so that at pH 9 even 4.8 mM external Ca^{2+} becomes able to inhibit completely Ca^{2+} efflux. The possible participation of Ca^{2+} influx on the effects of alkaline pH was investigated, since it has been reported that passive permeability to Ca^{2+} increases with pH [14,15]. Results (not shown) indicated that at pH 9 the rate constant for Ca^{2+} influx is about 20% higher than at neutral pH. Using Eqn. 1 it is easy to demonsrate that changes of this magnitude cannot account for the observed enhancement of the inhibition of Ca^{2+} efflux by external Ca^{2+} at alkaline pH.

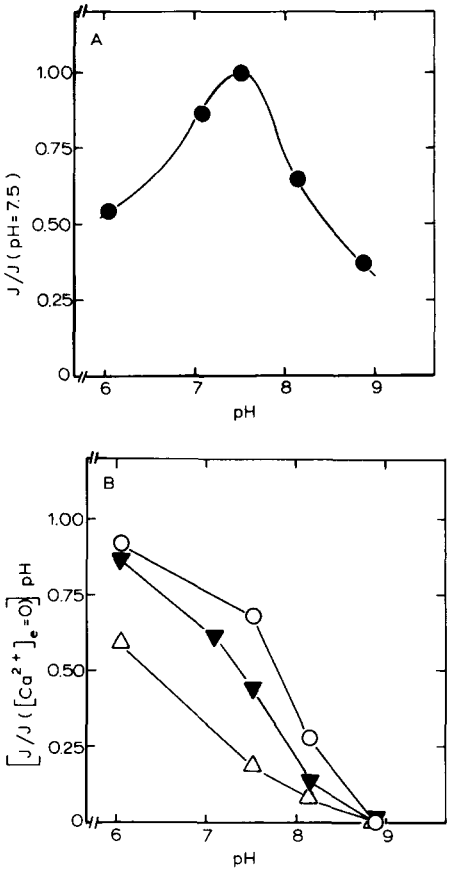

Fig. 6. (A) The effect of pH on Ca^{2+} efflux from ghosts suspended in Ca^{2+}-free media. All fluxes were measured simultaneously on the same batch of Na^{+}-rich resealed ghosts. The incubation media contained 100 mM NaCl, 15 mM sucrose and either imidazole-HCl (for pH 6–7.5) or Tris-HCl (for pH 7.5–9). (B) The effect of pH on the inhibition of Ca^{2+} efflux by 4.8 (○), 9.7 (▼) and 19.4 (△) mM external Ca^{2+}. Ca^{2+} was added as $CaCl_2$ replacing an equiosmolar amount of the corresponding buffer. The ordinate is the ratio between the measured flux and the flux measured at the same pH but in media without Ca^{2+}.

The interaction between the sites for activation by Ca^{2+} and ATP and the sites for inhibition by external Ca^{2+}

The inhibition of Ca^{2+} efflux by external Ca^{2+} was studied in ghosts containing either 2, 4, 15 or 40 μM free Ca^{2+}. Results in Fig. 7 show that for the four kind of ghosts, external Ca^{2+} inhibits efflux along curves that can be adjusted by rectangular hyperbolae that tend to zero and reach their half-maximal values at concentrations of Ca^{2+} that are not significantly different.

Fig. 7 also shows that for all the concentrations of external Ca^{2+} tested, Ca^{2+} efflux increases with internal Ca^{2+}. This effect can be described by a Michaelis-like equation with $K_{0.5} \simeq 3$ μM (results not plotted), showing that the main effect of raising from 2 to 40 μM the intracellular concentration of Ca^{2+} is to increase the degree of saturation of the transport sites of the Ca^{2+} pump. Therefore, the results in Fig. 7 indicate that the apparent affinity for inhibition by external Ca^{2+} is not affected by the state of occupation of the high-affinity intracellular transport sites of the Ca^{2+} pump.

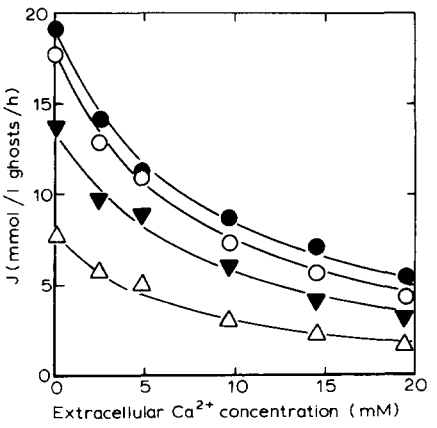

Fig. 7. The effect of external Ca^{2+} on the efflux of Ca^{2+} from ghosts containing 2 (△), 4 (▼), 15 (○) and 40 (●) μM intracellular free Ca^{2+} at the beginning of the experiment. The ghosts were sealed in Na^+-rich media. The Ca^{2+}-free incubation medium contained: 100 mM NaCl, 60 mM Tris-HCl and 75 mM sucrose. Ca^{2+} was added as $CaCl_2$, replacing equiosmolar amounts of sucrose. Eqn. 2 was adjusted to each set of experimental data. The continuous lines are solutions of this equation for $J_{([Ca^{2+}]_e=\infty)} = 0$, $J_{([Ca^{2+}]_e=0)} = 7.8$, 13.7, 18.0 and 18.9 mmol/l ghosts per h and $K_{i_{Ex}} = 6.2$, 7.2, 6.9 and 7.5 mM for the ghosts containing 2, 4, 15 and 40 μM Ca^{2+}, respectively.

Table I compares the kinetic parameters for inhibition by external Ca^{2+} in ghosts containing either 4.0, 2.7 or 0.25 mM ATP. It can be seen that a reduction in the concentration of ATP that significantly decreases Ca^{2+} efflux has no effect on the apparent affinity for external Ca^{2+} during inhibition.

Inhibition by intracellular Ca^{2+}

As it has already been mentioned in Introduction, the response of the Ca^{2+}-ATPase to Ca^{2+} in disrupted red cell membranes, in which Ca^{2+} has access to both surfaces of the membrane, is biphasic so that activation by low Ca^{2+} concentrations is followed by inhibition at higher Ca^{2+} concentrations. In view of this, it seemed pertinent to see whether the latter could be explained by the inhibitory effects of external Ca^{2+} reported in this paper. To do this, the effect of increasing Ca^{2+} concentrations on the Ca^{2+}-ATPase activity of disrupted membranes was compared with the effect of increasing Ca^{2+} concentrations on the efflux of Ca^{2+} from ghosts in which internal and external Ca^{2+} concentration was increased simultaneously and from ghosts in which only external Ca^{2+} was increased. In the ATPase experiments and in those efflux experiments in which internal Ca^{2+} concentration was varied, the total $MgCl_2$ in the medium or in the sealing solution, respectively, was adjusted to keep the concentration of free Mg^{2+} constant at about 5 mM. Results in Fig. 8 make clear that inhibition of the ATPase activity of disrupted membranes takes place with considerably higher apparent affinity than inhibition of Ca^{2+} efflux by external Ca^{2+} only, but that when Ca^{2+} concentration is raised simultaneously at both surfaces of the cell membrane the pattern of inhibition of Ca^{2+} efflux becomes equal to that of the Ca^{2+}-ATPase.

Hence, increases in the concentration of Ca^{2+} at the inner surface of the cell membrane are required for Ca^{2+} to exert fully its inhibitory effect. This might be caused either by a direct effect of intracellular Ca^{2+} or by a positive interaction in affinity between inhibitory extracellular sites and intracellular sites of lower affinity than those involved in activation of the Ca^{2+} pump. In the first case, inhibition by intracellular Ca^{2+} would not require external Ca^{2+}; in the second, inhibition

TABLE I

EFFECT OF THE INTRACELLULAR CONCENTRATION OF ATP ON THE KINETIC PARAMETERS FOR INHIBITION OF Ca^{2+} EFFLUX BY EXTERNAL Ca^{2+}

The ghosts were sealed in Na^+-rich media with and without added ATP. The effect of extracellular Ca^{2+} was measured in 0–20 mM concentration range. Kinetic parameters were estimated adjusting Eqn. 2 to the experimental data.

Expt. No.	[ATP] (mM)	$J_{([Ca^{2+}]_e=0)}$ (mmol/l ghosts per h)	$J_{([Ca^{2+}]_e=\infty)}$ (mmol/l ghosts per h)	$K_{i_{Ex}}$ (mM)
1	4.03	27.1 ± 0.4	−1.1 ± 1.1	8.9 ± 0.4
	0.26	16.6 ± 0.3	1.6 ± 1.3	10.2 ± 0.6
2	2.74	26.8 ± 1.0	−4.7 ± 4.0	9.0 ± 1.1
	0.24	20.3 ± 0.3	−1.9 ± 0.9	9.0 ± 0.5

would be totally dependent on external Ca^{2+}.

To discriminate between these two alternatives, Ca^{2+} efflux was measured in resealed ghosts containing different concentrations of Ca^{2+} and suspended in media either without Ca^{2+} or with a similar Ca^{2+} concentration as that of the media in which the ghosts were sealed. Results in Fig. 9 demonstrate that inhibition persists when ghosts are suspended in the Ca^{2+}-free medium and that, under these conditions, external Ca^{2+} produces only a small additional effect. Internal Ca^{2+} therefore is able to exert a direct inhibitory effect which is independent of external Ca^{2+}.

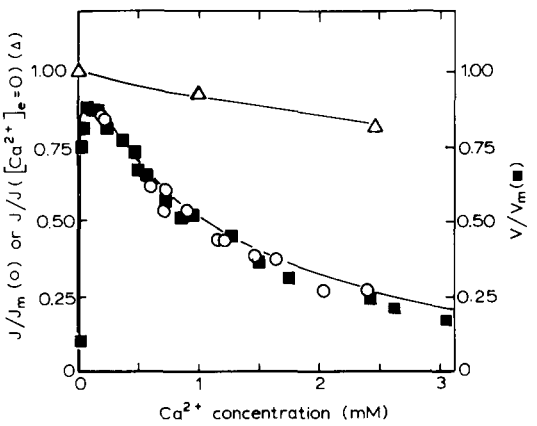

Fig. 8. The effects of Ca^{2+} on the Ca^{2+}-ATPase activity of disrupted red cell membranes (■) (two experiments), on the efflux of Ca^{2+} from resealed ghosts in which Ca^{2+} was increased in the incubation media only (△) (one experiment) and on the efflux of Ca^{2+} from ghosts in which Ca^{2+} was increased simultaneously in the intracellular and extracellular media (○) (two experiments). The ghosts were sealed in Na^+-rich media. In the efflux experiments, the Ca^{2+}-free incubation medium contained: 100 mM NaCl, 60 mM Tris-HCl and 75 mM sucrose. Ca^{2+} was added as $CaCl_2$ replacing equiosmolar amounts of sucrose. For the conditions in which only external Ca^{2+} was increased, the fluxes are expressed as the ratio: $J/J_{([Ca^{2+}]_e=0)}$, $J_{([Ca^{2+}]_e=0)} = 29.5$ (mmol/l ghosts per h) was calculated by fitting Eqn. 2. For the other two experimental conditions, activities or fluxes are expressed as the ratios v/V_m or J/J_m, respectively. $V_m = 0.8$ and 0.3 μmol/mg protein per h and $J_m = 36.5$ and 27.9 mmol/l ghosts per h were calculated fitting Eqn. 12 to the experimental data, assuming $K_{Ca} = 0.005$ mM and $K_{i_{Ex}} = 9.5$ mM.

The relation between inhibition by intracellular and extracellular Ca^{2+}

Assuming a combination of simple hyperbolic activation plus simple hyperbolic inhibition by internal Ca^{2+}, the following equation would describe the response of Ca^{2+} efflux to internal Ca^{2+} of ghosts suspended in Ca^{2+}-free media:

$$J = \frac{J_m}{1 + \frac{K_{Ca}}{[Ca^{2+}]_i} + \frac{[Ca^{2+}]_i}{K_{i_{In}}}} \quad (11)$$

where K_{Ca} is the value of the apparent dissociation constant of Ca^{2+} from the intracellular activating site, $K_{i_{In}}$ has the same meaning for the intracellular inhibitory site and J_m is the flux that would be attained at non-limiting $[Ca^{2+}]_i$ in the absence of inhibitory effects. Eqn. 11 implies that J will tend to zero as $[Ca^{2+}]_i$ tends to infinity. This is justified because when equations that allow for the existence of a residual non-inhibitable flux were adjusted to experimental data, the value of residual flux that gave best fit was always not significantly different from zero (results not shown).

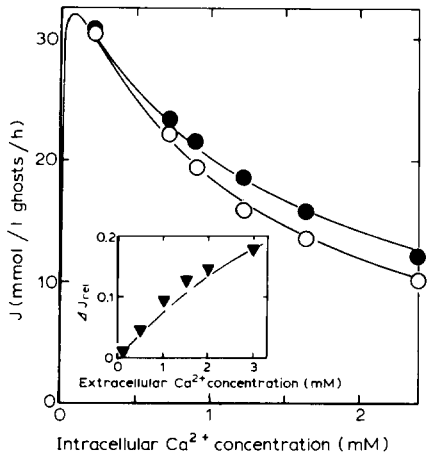

Fig. 9. The effect of internal Ca^{2+} on the efflux of Ca^{2+} from resealed ghosts suspended in a Ca^{2+}-free medium (●) or in media in which external Ca^{2+} was adjusted to keep it approximately equal to the internal Ca^{2+} concentration (○). Ghosts were sealed in Na^{+}-rich media. The Ca^{2+}-free incubation medium contained: 100 mM NaCl, 60 mM Tris-HCl and 75 mM sucrose. External Ca^{2+} was added as $CaCl_2$ replacing equiosmolar amounts of sucrose. The continuous line that fits the points of the experiment performed in the absence of external Ca^{2+} is the solution of Eqn. 11 for $J_m = 36.0$ mmol/l ghosts per h, $K_{Ca} = 0.005$ mM and $K_{i_{In}} = 1.31$ mM. The continuous line that fits the points of the experiment performed in the presence of external Ca^{2+} is the solution of Eqn. 12 for $J_m = 36.4$ mmol/l ghosts per h, $K_{Ca} = 0.005$ mM, $K_{i_{In}} = 1.29$ mM and $K_{i_{Ex}} = 13.8$ mM. The inset is a plot of the ratio in the left-hand side of Eqn. 13 against the external Ca^{2+} concentration. The continuous line in the inset is the solution of Eqn. 13 for $K_{i_{Ex}} = 13.8$ mM.

Eqn. 11 was adjusted by non-linear regression to the experimental results. Since no points describing the ascending part of the curve were available, K_{Ca} was fixed at 0.005 mM on the basis of previous data obtained in ghosts prepared essentially as those used for the experiments described here [6]. Best fit was reached with $J_m = 36.0 \pm 0.5$ mmol/l ghosts per h and $K_{i_{In}} = 1.292 \pm 0.05$ mM. Hence, the apparent affinity for inhibition by internal Ca^{2+} seems to be about 7-times higher than that for inhibition by external Ca^{2+}.

If the inhibitory effects of extracellular Ca^{2+} took place independently of those of intracellular Ca^{2+}, the following modification of Eqn. 11 would account for the effect of Ca^{2+} at both surfaces of the cell membrane:

$$J = \frac{J_m}{\left(1 + \frac{K_{Ca}}{[Ca^{2+}]_i} + \frac{[Ca^{2+}]_i}{K_{i_{In}}}\right)\left(1 + \frac{[Ca^{2+}]_e}{K_{i_{Ex}}}\right)} \quad (12)$$

in which the parameters have the same meaning as in Eqns. 2 and 11.

Eqns. 11 and 12 were fitted to the data of the experiment in which Ca^{2+} was increased simultaneously at both surfaces of the cell membrane (Fig. 9). The standard error of the regression was 0.4941 for Eqn. 11 and 0.2456 for Eqn. 12, indicating that when the effects of external Ca^{2+} are taken into account a better description of the experimental results is attained. The parameters in Eqn. 12 that gave best fit were: $J_m = 36.4 \pm 0.45$ mmol/l ghosts per h, $K_{i_{In}} = 1.31 \pm 0.05$ mM, and $K_{i_{Ex}} = 13.8 \pm 4.5$ mM. These values suggest that the apparent affinity for inhibition by intracellular Ca^{2+} is not affected by extracellular Ca^{2+}, and that when internal Ca^{2+} is increased, inhibition by extracellular Ca^{2+} persists with an apparent affinity which is not significantly different from that measurable varying external Ca^{2+} at constant low internal Ca^{2+} concentration (Fig. 3).

A more direct visualization of what seems to be the additive inhibitory effects of external and internal Ca^{2+} can be obtained considering that if Eqns. 11 and 12 described the fluxes in the absence ($J_{([Ca^{2+}]_e = 0)}$) and in the presence ($J_{([Ca^{2+}]_e)}$) of external Ca^{2+}, respectively, the extra inhibition by external Ca^{2+} would be expressable as:

$$\Delta J_{rel} = \frac{J_{([Ca^{2+}]_e = 0)} - J_{([Ca^{2+}]_e)}}{J_{([Ca^{2+}]_e = 0)}} = \frac{1}{1 + \frac{K_{i_{Ex}}}{[Ca^{2+}]_e}} \quad (13)$$

The continuous curve in the inset to Fig. 9 is the solution to Eqn. 12 using the value of $K_{i_{Ex}}$ obtained by adjusting Eqn. 13 to the data in the experiment in Fig. 9. It can be seen that Eqn. 13 gives a reasonable description of the extra inhibition observed when internal Ca^{2+} is increased together with external Ca^{2+}.

Discussion

The experiments described in this paper show that there are two modes of inhibition of the Ca^{2+}

pump of red cells by Ca^{2+}: one requires Ca^{2+} at the outer surface and the other requires Ca^{2+} at the inner surface of the cell membrane. Inhibition by external and internal Ca^{2+} seems to be mediated by two separate and independent mechanisms since: (i) the apparent affinities are different, the K_i for external Ca^{2+} being about 7-times higher than that for internal Ca^{2+}, (ii) the kinetic parameters of the inhibition at a given surface are not modified by the degree of inhibition at the opposite surface of the cell membrane and (iii) the inhibition by intracellular and extracellular Ca^{2+} acting simultaneously can be accounted for by the sum of the effect of Ca^{2+} at each surface of the cell membrane.

Inhibition by extracellular Ca^{2+}

Free ionic Ca^{2+} is the inhibitory species. The kinetic parameters of inhibition are not modified by Na^+ and K^+ or by EGTA. This contrasts with the 30–90% increase that Na^+ or K^+ induce in the activating effect of intracellular Ca^{2+} [6] and with the large increase in the apparent affinity for activation by Ca^{2+} induced by EGTA [16].

The concentration-dependence of inhibition can be described by a rectangular hyperbola that tends to zero and is half-maximal at about 10 mM Ca^{2+}. This suggests that inhibition is caused by the combination of Ca^{2+} at a single class of identical and non-interacting sites and that when these sites are fully occupied Ca^{2+} pumping stops. Although the results presented here do not provide experimental evidence that the sites for external Ca^{2+} are intrinsic components of the pump, it is tempting to identify them with extracellular low-affinity sites of the pump like those that have been described for the Ca^{2+} pump of sarcoplasmic reticulum (for references see Ref. 17). If this were the case, the kinetics of inhibition would indicate that active transport only takes place in those pumps whose extracellular sites are empty.

The effects of Ca^{2+} are mimicked, albeit with lesser apparent affinity, by Mg^{2+}, other divalent cations were not tested. The ratio between the apparent affinities for inhibition by extracellular Ca^{2+} and Mg^{2+} is about 3, indicating that the selectivity for Ca^{2+} of these sites is low. The intracellular transport sites of the pump also seem to bind Mg^{2+}; however, in this case Mg^{2+} cannot replace Ca^{2+} and the selectivity for Ca^{2+} with respect to Mg^{2+} is very high since the ratio between apparent affinities is about 200 (Caride, A.J., Garrahan, P.J. and Rega, A.F., unpublished observations). It has been proposed that high selectivity for Ca^{2+} is a property of intracellular Ca^{2+}-binding proteins, which have to bind Ca^{2+} in the presence of a large excess of Mg^{2+}, and not of extracellular proteins that have to bind Ca^{2+} in media in which its concentration is usually higher than that of Mg^{2+} [18]. It may be that the difference in selectivity for Ca^{2+} between the intracellular and extracellular sites is a particular example of this general phenomenon.

Regardless of its meaning, inhibition by extracellular Ca^{2+} and Mg^{2+} is relevant when studying the kinetics of the pump. In fact: if the measured values of the apparent affinities for Ca^{2+} and Mg^{2+} are inserted into Eqn. 3, it can be calculated that at the usual concentrations of these cations in the extracellular medium (1 mM Mg^{2+} and 2 mM Ca^{2+}) the rate of Ca^{2+} pumping will be about 88% of that in the absence of external Ca^{2+} and Mg^{2+}. Hence, inhibitory effects of external cations are present under physiological conditions. These effects will become even more relevant when using inside-out vesicles in which Ca^{2+} concentration builds-up rapidly at the extracellular surface of the cell membrane.

Experiments in this paper show that the kinetic parameters for inhibition by extracellular Ca^{2+} are independent of the intracellular concentration of Ca^{2+}. On their face value, these results ould indicate that the sites for inhibition by external Ca^{2+} are physically distinct and coexist with those for internal Ca^{2+}. However, in view of the above-mentioned uncertainties concerning the relation of the sites with the pump and of the very complex reaction mechanism of this system alternative explanations cannot be discarded.

The apparent affinity for external Ca^{2+} is also independent of variations in the intracellular ATP concentrations within a range in which significant changes in the state of occupation of the low-affinity regulatory site of the pump [7] will take place. This suggests that there are no interactions between the sites for Ca^{2+} and the regulatory sites for ATP of the pump.

One of the salient features of inhibition by

external Ca^{2+} is its strong dependence on pH. When pH raises, Ca^{2+} becomes much more effective as an inhibitor. Since, as it has been already discussed, at sufficiently high Ca^{2+} concentrations Ca^{2+} efflux tends to zero the effect of pH has to be exerted on the apparent affinity of the site at which Ca^{2+} combines to inhibit active Ca^{2+} transport. An effect of pH on inhibition by external Ca^{2+} similar to that found here has been reported by Dipolo and Beaugé [19] in squid giant axons. These authors also demonstrated that enhancement of inhibition appears only when extracellular pH is increased. The sideness of the effects of pH cannot be studied in resealed ghosts, since intracellular and extracellular hydrogen ions quickly reach equilibrium across the red cell membrane [20] and no procedure for altering the pH only at one of the surfaces of the cell membrane is available. If the apparent affinity for external Ca^{2+} in red cells depended on external pH, the observed effects suggest that deprotonation of the sites for Ca^{2+} favors binding. This may be of little or no physiological significance, but more speculative explanations that relate directly the effects of pH to the process of Ca^{2+} translocation merit consideration. In fact, experimental evidence suggests that the Ca^{2+} pump of red cells catalyzes the exchange of internal Ca^{2+} for external protons [21,22]. According to this view, Ca^{2+} transport would require the protonation of externally-facing sites of the pump. It is tempting to propose that the sites for protons are the same sites at which divalent cations bind to inhibit transport and that binding of Ca^{2+} or Mg^{2+} promotes deprotonation of these sites. Such a mechanism would imply that external Ca^{2+} or Mg^{2+} inhibit not only because they block the release of Ca^{2+} that has been transported but also because they impede the binding of the protons that have to be transported to complete a pump cycle. This hypothesis predicts a competitive interaction between external Ca^{2+} and protons. The increase in the apparent affinity for inhibition by Ca^{2+} when pH is raised is in qualitative agreement with this prediction. However, additional experimental work is necessary to make this proposal more than a mere speculation.

Inhibition by intracellular Ca^{2+}

Results in this paper show that K_i for inhibition by internal Ca^{2+} is around 1–1.5 mM Ca^{2+}, that is about 7-times less than that for extracellular Ca^{2+}. For this reason, when disrupted membrane preparations are used, the effect of Ca^{2+} at the internal surface predominates and becomes the main cause of the overall inhibition. Nevertheless, inhibition by internal Ca^{2+} has less physiological significance than that by external Ca^{2+}. This is so because the value of K_i for internal Ca^{2+} is at least 10000-times higher than the physiological intracellular Ca^{2+} concentration whereas the value of K_i for external Ca^{2+} is only about 5-times higher than the physiological extracellular Ca^{2+} concentration.

Three different mechanisms may be proposed for the inhibition by intracellular Ca^{2+} namely: (i) binding of Ca^{2+} to an inhibitory site, (ii) competitive displacement of Mg^{2+} from sites in the Ca^{2+} pump and, (iii) competitive inhibition by CaATP of the proposed activating effects of MgATP [23]. If the sites involved in the first two mechanisms were non-interacting, inhibition would be a hyperbolic function of the concentration of Ca^{2+}. If Ca^{2+} acted forming CaATP, hyperbolic responses to free Ca^{2+} are only to be expected if the concentration of CaATP is a linear function of the concentration of Ca^{2+}. This is possible only if the concentration of Ca^{2+} is substantially lower than the value of the dissociation constant for CaATP. This requirement is fulfilled for the range of internal Ca^{2+} concentrations (0–3 mM) used in the experiments of this paper since, in media with the concentration of Mg^{2+} used (5 mM), the dissociation constant for CaATP can be calculated to be about 6.07 mM. It would seem therefore that the three proposed mechanisms are consistent with the observed kinetics of inhibition.

The complex composition of the intracellular medium makes it very difficult to test directly the hypothesis that inhibition is caused by the binding of Ca^{2+} to low-affinity internal sites. This question can however be approached indirectly by seeing to which extent displacement of Mg^{2+} by Ca^{2+} from a site in the pump or from MgATP is able to account for the apparent affinity for inhibition by internal Ca^{2+}. To do this, we may proceed as follows: the conventional treatment of competitive inhibition by a ligand I on the activation of an enzymic reaction by a ligand A predicts that I will

reduce the activity to one-half its control value when its concentration is such that the following equation is satisfied:

$$1 + \frac{K_{0.5}}{[A]} = \frac{K_{0.5}[I]}{K_i[A]} \quad (14)$$

where [A] is the concentration of the activating ligand [Mg^{2+} or MgATP in our case), [I] the concentration of the competitive inhibitor (Ca^{2+} or CaATP in our case), and $K_{0.5}$ and K_i the apparent constants for the dissociation of A and I, respectively, from their site in the enzyme.

In the experiments reported in this paper, the concentration of MgATP and of free Mg^{2+} were 5–10- or 100-times larger, respectively, than the corresponding $K_{0.5}$ values (Ref. 23 and Caride, A.J., Garrahan, P.J. and Rega, A.F., unpublished observations). Under these conditions, only a negligible error will be introduced if the ratio $K_{0.5}/[A]$ is neglected and Eqn. 14 is simplified to:

$$1 = \frac{K_{0.5}[I]}{K_i[A]} \quad (15)$$

In the experiments in which the effects of internal Ca^{2+} were tested, internal free Mg^{2+} was kept constant at 5 mM. Hence, if Ca^{2+} acted displacing Mg^{2+}, when the concentration of Ca^{2+} is that needed for half-maximal inhibition (1 mM), the ratio [I]/[A] will be about 0.2. Therefore, to satisfy Eqn. 15, the ratio $K_{0.5}/K_i$ should be about 5. Results from our laboratory (Caride A.J., Garrahan, P.J. and Rega, A.F., unpublished observations) show that in disrupted red cell membranes free Mg^{2+} activates with a $K_{0.5}$ of about 0.05 mM and that free Ca^{2+} competes with Mg^{2+} with a K_i of about 0.025 mM which gives a $K_{0.5}/K_i$ ratio of about 2. On the other hand, if Ca^{2+} acted through the formation of CaATP, and since the concentration of total ATP was 2 mM, when the concentrations of free Ca^{2+} and Mg^{2+} are 1 and 5 mM, respectively, the calculated concentration of MgATP and of CaATP will be about 1.7 and 0.28 mM, respectively. In this case, to satisfy Eqn. 15, the ratio $K_{0.5}/K_i$ should be about 6. Muallem and Karlish [23] have reported that in disrupted red cell membranes $K_{0.5}$ for MgATP is 0.360 mM and that K_i for CaATP is 0.150 mM, so that the ratio of these constants is about 2.4.

Therefore, for each pair of ligands, the experimentally measured value of the ratio $K_{0.5}/K_i$ seems to be from 2- to 3-times lower than that required to explain the observed K_i for inhibition of Ca^{2+} efflux by free internal Ca^{2+}. On their face value, these results suggest that when considered separately, displacement of Mg^{2+} or formation of CaATP account only for a part of the inhibitory effect of internal Ca^{2+}. It must be pointed out, however, that the validity of this conclusion rests on the yet unproven assumption that the values for $K_{0.5}$ and K_i measured in disrupted membranes are the same as those in resealed ghosts. In view of this, and since the discrepancy between theory and experiment is not very large, our results cannot yet be taken as a conclusive proof against the possibility that one of the above-mentioned mechanisms is the only cause of inhibition by internal Ca^{2+}.

Another way of analyzing the mechanism of inhibition by internal Ca^{2+} is to look at the predictions that can be derived from mechanisms that imply that activation of Ca^{2+} transport requires the concurrent interaction of both MgATP and free Mg^{2+} at sites from which they are competitively displaced by CaATP and Ca^{2+}, respectively. Eqn. 15 can be adapted to analyze mechanisms of this kind. When this is done it is easy to show that if Ca^{2+} displaced Mg^{2+} from a site in the pump as well as from MgATP the predicted K_i for internal Ca^{2+} becomes very close to that observed experimentally. Hence, until further evidence is provided there appears to be no need to postulate the existence of separate inhibitory sites to account for the inhibition of Ca^{2+} efflux by intracellular Ca^{2+}.

Acknowledgements

This work was supported by grants from CONICET, SUBCYT and Universidad de Buenos Aires, Argentina. R.B.K. is a Research Fellow from CONICET. A.F.R. and P.J.G. are established investigators from CONICET.

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