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Asymmetric Effects of Divalent Cations and Protons on Active Ca^{2+} Efflux and Ca^{2+}-ATPase in Intact Red Blood Cells

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Summary. The influence of the asymmetric addition of various divalent cations and protons on the properties of active Ca^{2+} transport have been examined in intact human red blood cells. Active Ca^{2+} efflux was determined from the initial rate of $^{45}Ca^{2+}$ loss after $CoCl_2$ was added to block Ca^{2+} loading via the ionophore A23187. Ca^{2+}-ATPase activity was measured as phosphate production over 5 min in cells equilibrated with EGTA-buffered free Ca^{2+} in the presence of A23187. The apparent Ca affinity of active Ca^{2+} efflux ($K_{0.5}$ = 30–40 μmol/liter cells) was significantly lower than that measured by the Ca^{2+}-ATPase assay ($K_{0.5}$ = 0.4 μM). Possible reasons for this apparent difference are considered. Both active Ca^{2+} efflux and Ca^{2+}-ATPase activity were reduced to less than 5% of maximal levels (20 mmol/liter cells · hr) in Mg^{2+}-depleted cells, and completely restored by reintroduction of intracellular Mg^{2+}. Active Ca^{2+} efflux was inhibited almost completely by raising external $CaCl_2$ (but not $MgCl_2$) to 20 mM, probably by interaction of Ca^{2-} at the externally oriented E_2P conformation of the pump. Cd^{2+} was more potent than Ca^{2+} in this inhibition, while Mn^{2+} was less potent and 10 mM Ba^{2+} was without effect. A Ca^{2+}: proton exchange mechanism for active Ca^{2+} efflux was supported by the results, as external protons (pH 6–6.5) stimulated active Ca^{2+} efflux at least twofold above the efflux rate at pH 7.8. Ca^{2+} transport was not affected by decreasing the membrane potential across the red cell.

Key Words Ca^{2+}-ATPase · Ca^{2+} efflux · erythrocytes · cations, divalent · proton asymmetry

Introduction

The physiological role of Ca^{2+} as a second messenger in signal transduction depends on the maintenance of a more than 10,000-fold electrochemical gradient of Ca^{2+} across the cell membrane, the resting intracellular Ca^{2+} levels being around 100 nmol/liter cells in most mammalian cells (Rasmussen, 1983). A number of cellular activation mechanisms involve the downhill movement of Ca^{2+} from outside to inside the cell. Many cells contain an ATP-fueled active Ca^{2+}-translocating ATPase in the plasma membrane which extrudes Ca^{2+} against its electrochemical gradient (Penniston, 1983). The most widely studied of these is the Ca^{2+}-translocating ATPase in the human red blood cell plasma membrane (Carafoli & Zurini, 1982). Most of the information on the Ca^{2+} translocation mechanisms has been derived from studies on isolated membranes, inside-out vesicles or reconstituted proteoliposomes (for review *see* Schatzmann, 1982). Fewer studies have been made, however, on the mechanism of active Ca^{2+} transport *in situ* in the environment of the intact red blood cell, largely because of the difficulty of maintaining and estimating the levels of cellular Ca^{2+} and other ligands in such studies. Complex interactions between the various ligands of the Ca^{2+}-translocating ATPase, including ATP, calmodulin, Ca^{2+}, Mg^{2+} and H^+, have been found in studies on the isolated systems (*see* Villalobo, Brown & Roufogalis, 1986). However, various potentially significant factors controlling the Ca^{2+}-pump activity may be lost or altered when cells are lysed during membrane isolation. In the present work we have studied Ca^{2+} efflux and the Ca^{2+}-translocating ATPase activity (Ca^{2+}-ATPase) in intact red blood cells in order to investigate the effect of the asymmetric alteration of the concentration of various ligands on the Ca^{2+} pump *in situ*.

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, ethyleneglycol *bis* (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; SITS, 4-acetamido-4′-isothiocyanostilbene-2′-disulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PPO, 2,5-diphenyloxazole.

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Materials and Methods

Chemicals

Ouabain, A23187, EGTA, dibutylphthalate, $CaCl_2$, bovine serum albumin, DIDS, amiloride, hexokinase, methazolamide and glucose-6-phosphate dehydrogenase were obtained from Sigma. All divalent cations and other salts were Analytical Reagent grade. Trichloroacetic acid and EDTA were from Ajax Chemicals. NADP was from Boehringer Mannheim. [^{45}Ca]$CaCl_2$ (1.67 Ci/mmol) was from Amersham.

Active Ca^{2+} Efflux Measurement

Red blood cells were obtained from the Australian Red Cross Blood Bank one day after collection and used within 4 days of storage at 4°C. The packed cells were washed three times in an isotonic solution containing (in mM) 75 NaCl, 75 KCl, 10 Tris-HCl (pH 7.5 at 4°C) and 0.1 EGTA, and another three times in the same solution without EGTA. The buffy coat was carefully removed in each step. The cells were suspended at 10% hematocrit in a medium (henceforth referred to as the loading medium) containing (in mM) 60 NaCl, 75 KCl, 2.0 $MgCl_2$, 0.1 ouabain in 10 sodium-HEPES (pH 7.4 at 37°C) to which was added $CaCl_2$ at various concentrations and [^{45}Ca]$CaCl_2$ (2.5 μCi/ml). Suspensions were stirred vigorously with a small magnetic bar in thermostatted polyethylene tubes at 37°C. Ca^{2+} influx was initiated by the addition of the $Ca^{2+}:H^+$ exchanger A23187 (20 μM) in ethanol (the solvent being maintained at a final concentration of 1%). At various times (*see* figure legends) the Ca^{2+} influx was terminated by the addition of 0.25 mM $CoCl_2$, which exposes Ca^{2+} efflux according to the procedure of Tiffert, Garcia-Sancho and Lew (1984). We have confirmed previously that under these conditions ^{45}Ca uptake is completely blocked and ^{45}Ca efflux is exposed instantaneously (Xu & Roufogalis, 1988). The use of 75 mM KCl in the incubation medium avoids cell shrinkage due to Ca^{2+}-activated K^+ loss by maintaining electrochemical equilibrium of K^+ (Lew & Garcia-Sancho, 1988), while any shape changes induced by Ca^{2+} are isovolumic and should not influence the flux measurements (Sarkadi et al., 1977). Aliquots (0.1 ml) were added to Eppendorf tubes containing 0.4 ml of dibutylphthalate and 0.75 ml of "stop buffer" consisting of (in mM) 75 KCl, 75 NaCl, 5 EGTA, 0.2 $LaCl_3$ and 10 Tris-HCl (pH 7.55 at 4°C) on ice. The frequency of sampling depended on the extent of calcium loading and the subsequent rate of $^{45}Ca^{2+}$ loss. At very low levels of calcium loading (*see* Fig. 1*A*), the sample was taken up immediately after addition of $CoCl_2$ into a prewarmed plastic syringe at 37°C, and 0.1 ml aliquots dispensed into the stopping solution at one-second intervals, using a repetitive pipette and an electronic metronome to aid in the timing. Samples loaded at intermediate calcium levels (*see* Fig. 1*B*) were removed from the loading solution every 5 sec using a micropipettor, while longer sampling frequencies were used when cells were loaded with high intracellular calcium (*see* Fig. 1*C*). The tubes were immediately centrifuged for 10 sec in a microfuge (Lew & Garcia-Sancho, 1988). The supernatant was removed and the walls of the tube wiped with a cotton swab. The pellet was treated with 0.5 ml of 6% trichloroacetic acid and the radioactivity in 50 μl aliquots counted in 5 ml of scintillation fluid (2 : 1 toluene-Triton X-100 mixture containing 6 g/liter PPO). Intracellular calcium content in aliquots taken immediately after $CoCl_2$ addition ("zero time") was calculated from the specific activity of the tracer solution, neglecting the small calcium content originally present in the cells. The active Ca^{2+} efflux rate was estimated from the initial linear portion of the Ca^{2+} efflux curve. In some experiments ATP content was estimated at "zero time" by a coupled hexokinase/glucose-6-phosphate dehydrogenase assay (Beutler, 1984).

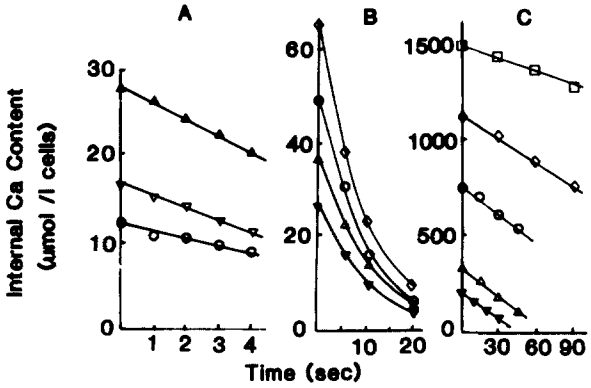

Fig. 1. Measurement of active Ca^{2+} efflux at different levels of calcium loading. Washed red cells were incubated in the loading medium with 20 μM A23187 as described in Materials and Methods. The concentration of $CaCl_2$ (with 2.5 μCi/ml ^{45}Ca tracer) and the time of incubation with A23187 before the addition of $CoCl_2$ (*see* Materials and Methods) were as follows: Panel *A*, 10 μM $CaCl_2$ for 2.0 sec (○), 3.0 sec (▽) and 5 sec (▲). Panel *B*, 50 μM $CaCl_2$ for 2 sec (▲), 5 sec (△)), 10 sec (○) and 20 sec (◇). Panel *C*, 500 μM $CaCl_2$ for 3 sec (▲), 6 sec (△), 10 sec (○), 20 sec (◇) and 30 sec (□). Ca^{2+} efflux was measured as in Materials and Methods

Ca^{2+}-ATPase Measurement

Washed red blood cells (*see above*) were suspended at a hematocrit of 10% in loading medium, containing in addition, 0.1 mM EGTA and various $CaCl_2$ concentrations required to buffer the free Ca^{2+} concentration from 10^{-7} to 10^{-4} M. The free Ca^{2+} concentrations were calculated by a computer program which solves equations from equilibria between Ca^{2+}, Mg^{2+}, H^+, EGTA and ATP (Goldstein, 1979), using an absolute equilibrium association constant of $10^{10.97}$ for the Ca-EGTA complex. Ca^{2+} equilibration was initiated by the addition of 20 μm A23187 in ethanol. We have shown previously that this ionophore concentration is optimal for rapid Ca^{2+} equilibration (Xu & Roufogalis, 1988). After 5 min with stirring at 37°C, 0.1 ml of cell suspension was lysed in 0.4 ml of 6.5% trichloroacetic acid with vigorous agitation in an Eppendorf tube. After centrifugation for 30 sec in a microfuge, a 0.2 ml aliquot of the clear supernatant was assayed for inorganic phosphate content according to the sensitive malachite green spectrophotometric method of Lanzetta et al. (1979).

Magnesium Depletion

Washed red blood cells were depleted of Mg^{2+} by incubating a suspension of cells at 10% hematocrit in the loading medium containing 4 mM EDTA with 5 μM A23187 for 15 min with stirring at 37°C. The incubated cells were then washed with buffer containing 0.5% bovine serum albumin (to remove the

ionophore) and the cells were then resuspended and loaded with calcium and/or magnesium with 20 μM A23187, as described in the figure legends.

Internal pH Manipulation

Internal pH was manipulated by procedures essentially as described by Escobales and Canessa (1986). Washed red blood cells (10% hematocrit) were preincubated for 10 min at 37°C with a medium containing (in mM) 75 NaCl, 75 KCl, 0.15 $MgCl_2$, 0.1 ouabain and adjusted to pH 6.0 with 10 MES-(Tris) buffer or to pH 7.4 and 8.0 with 10 MOPS-(Tris) buffer. The suspension was then treated with 125 μM DIDS, 0.4 mM methazolamide and 0.4 mM amiloride for 30 min at 37°C in the same buffers to reduce proton movements and lock the internal pH. The cells were separated by centrifugation and resuspended in the loading medium (pH 7.2) containing 100 μM $CaCl_2$. After 1 min at 37°C, 20 μM A23187 was added for a further 1 min and active Ca^{2+} efflux was then measured as described above.

To check the actual intracellular pH achieved, the cells were washed twice with isotonic saline (unbuffered), hemolyzed in an equal volume of distilled water, and the pH was measured with a glass electrode, as described by Esocobales and Canessa (1986).

Manipulation of External pH

Washed red blood cells were incubated in the loading medium containing 100 μM $CaCl_2$ in the presence of DIDS, methazolamide and amiloride, as described above. Ca^{2+} loading was initiated by the addition of 20 μM A23187. After 1 min, active Ca^{2+} efflux was measured on the addition of 0.25 mM $CoCl_2$ alone (pH 7.2) or together with sufficient HCl or NaOH to achieve the required pH, which was measured with a pH electrode coupled to a pH meter and recorder.

Manipulation of Membrane Potential

The membrane potential of red blood cells was manipulated by taking advantage of the large increase in K^+ permeability that is stimulated by intracellular Ca^{2+} (Lew & Ferreira, 1977). Under normal conditions the permeability of the red blood cell membrane to chloride is much greater than that to either Na^+ or K^+, and the membrane potential is therefore very close to the chloride potential (Hladky, 1977). If the anion permeability is greatly reduced and the K^+ permeability is greatly enhanced the situation is reversed and the membrane potential is approximated by the K^+ potential. Anion permeability was reduced by a procedure described by Hoffman, Kaplan & Callahan (1979), whereby chloride was replaced by sulfate by incubating washed red cells (5% hematocrit) for 3 hr at 37°C in a medium containing either 95 mM sodium sulfate or 95 mM potassium sulfate, 0.2 mM $MgCl_2$, 5 mM glucose and 10 mM of either sodium or potassium-HEPES (pH 7.2), or combinations of these two buffers to give the required range of extracellular K^+ concentrations. After packing the cells they were again resuspended in the above sodium or potassium-sulfate media and incubated with 125 μM DIDS for 30 min at 37°C. Following centrifugation, the cells were again suspended in the above media to provide the required outward K^+ gradient. Ca^{2+} efflux was then measured after calcium loading in the presence of 100 μM $CaCl_2$ as described above. The final intra- and extracellular K contents of the red cells ($[K]_i$ and $[K]_o$, respectively) were measured by flame photometry. The membrane potential was calculated from the relationship

$$V_m = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i}.$$

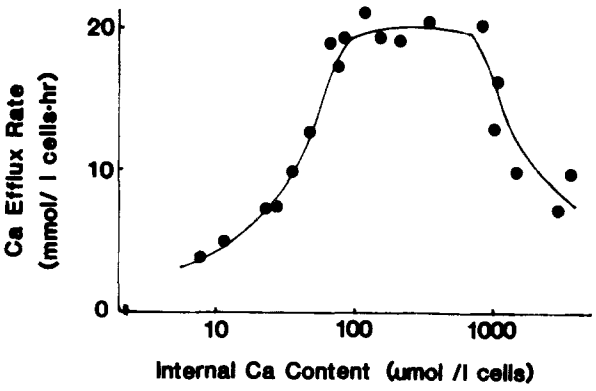

Fig. 2. Dependence of active Ca^{2+} efflux rate on cellular content of calcium. Ca^{2+} efflux rate was determined from experiments such as those shown in Fig. 1, and plotted as a function of the cellular calcium content at the beginning of the Ca^{2+} efflux measurement. The data were pooled from results obtained in three separate experiments

Results

Dependence of Active Ca^{2+} Efflux on Intracellular Ca Content

The dependence of the rate of active Ca^{2+} efflux on intracellular calcium levels was examined in washed red cells incubated in the presence of 20 μM A23187 with various concentrations of $CaCl_2$ for times ranging from 2 to 30 sec (Fig. 1). As the maximum pump rate is around 20 mmol/liter cells · hr, the ATP content, which was between 1.2 and 1.3 mmol/liter cells at the beginning of the experiment (Xu & Roufogalis, 1988), was decreased by less than 0.1 mmol/liter cells during loading in the presence of the ionophore A23187. Because the calcium pump rapidly depletes calcium at low intracellular calcium contents, short sampling intervals (1–5 sec) were necessary to accurately measure initial efflux rates (Fig. 1*A* & *B*). The Ca^{2+} efflux rate was independent of the extracellular $CaCl_2$ concentrations in the concentration range used in these experiments (*see* Fig. 3).

Figure 2 shows a plot of the active Ca^{2+} efflux rate as a function of the total intracellular calcium content of the cells. The Ca^{2+} efflux increased progressively, reaching a maximum rate of 20 mmol/

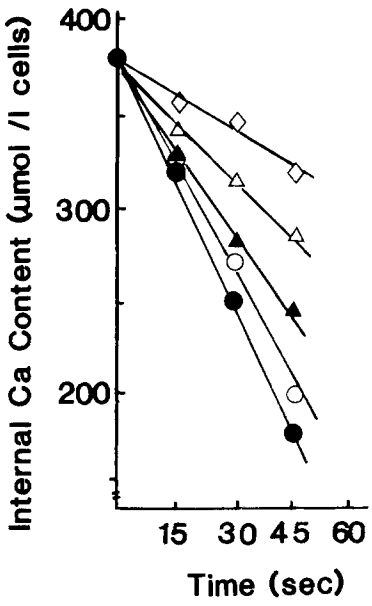

Fig. 3. Dependence of active Ca^{2+} efflux from intact red blood cells on extracellular $CaCl_2$. Washed red blood cells were incubated in the loading medium containing 20 μM A23187 and 100 μM $CaCl_2$ (with 2.5 μCi/ml of ^{45}Ca tracer) for 1 min at 37°C and Ca^{2+} efflux was exposed by the addition of $CoCl_2$ and various concentrations of $CaCl_2$, as follows: no added $CaCl_2$ (0.1 mM total external $CaCl_2$) (●), 2 mM $CaCl_2$ (○), 5 mM $CaCl_2$ (▲), 10 mM $CaCl_2$ (△) and 20 mM $CaCl_2$ (◇)

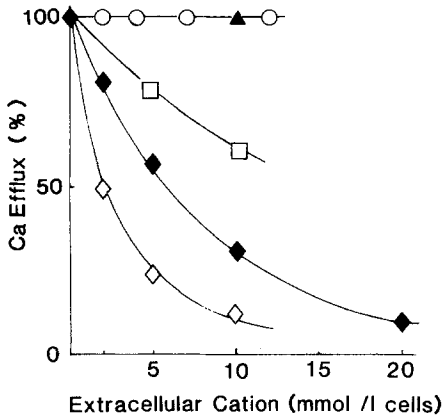

Fig. 4. Dependence of active Ca^{2-} efflux on several divalent cations in the extracellular medium. Red blood cells were incubated with 100 μM $CaCl_2$ (including 2.5 μCi/ml of ^{45}Ca tracer) and 20 μM A23187 for 1 min before $CoCl_2$ addition. The initial Ca^{2+} efflux rate was normalized to 100%, which represents Ca^{2-} efflux rates from 19–22 mmol/liter cells · hr in the various experiments. ^{45}Ca efflux rate was determined in the presence of increasing concentrations of divalent cations added to the cell suspension together with $CoCl_2$, as described in Fig. 3. The divalent cations used were $CdCl_2$ (◇), $CaCl_2$ (◆), $MnSO_4$ (□), $BaCl_2$ (▲) and $MgCl_2$ (○). The results are the average of five experiments for Ca^{2+} and duplicate or triplicate experiments for the other cations

liter cells · hr, with half-maximal calcium activation occurring at 30–40 μmol/liter cells. Ca^{2+} efflux remained constant at Ca contents up to 900 μmol/liter cells, and thereafter decreased as Ca was increased further. The half-maximal concentration of calcium for inhibition was around 1.5 mmol/liter cells.

Influence of Extracellular Cations on Active Ca^{2+} Efflux

The active Ca^{2+} efflux rate was progressively inhibited as the extracellular calcium concentration was increased from 0.1 to 20 mM (Fig. 3), with half-maximal inhibition occurring at 6 mM. Other divalent cations were also found to inhibit Ca^{2+} efflux with various potencies, as illustrated in Fig. 4. Cadmium was more potent than extracellular calcium ($K_{0.5}$ = 2 mM), whereas manganese appeared to be less potent, and magnesium and barium were without inhibitory effect over a similar concentration range.

Dependence of the Calcium Pump on Intracellular Magnesium

The dependence of both the Ca^{2+}-ATPase activity and active Ca^{2+} efflux on intracellular magnesium was examined in washed red blood cells depleted of magnesium by preincubation in the presence of EDTA and A23187, as described in Materials and Methods. Inorganic phosphate release in the first 5 min of incubation of cells with 20 μM A23187 at various Ca^{2+} concentrations has been shown in a previous study from our laboratory to be a reliable estimate of the Ca^{2+}-stimulated ATPase activity (Xu & Roufogalis, 1988). This is further supported in the present study, where it is shown that Ca^{2+}-stimulated inorganic phosphate production is absolutely dependent on the presence of intracellular magnesium (Fig. 5). In normal red blood cells inorganic phosphate production is progressively increased by Ca^{2+}, with half-maximum effect at 0.2 μM and maximum activation reaching a plateau above 10 μM. By contrast, in magnesium-depleted red blood cells, little stimulation (less than 5% of maximum) of inorganic phosphate production was observed at any of the Ca^{2+} concentrations examined (Fig. 5). Ca^{2+}-stimulated ATPase activity could be rapidly and fully restored by the introduction of magnesium to previously magnesium-depleted cells, with half-maximal recovery occurring between 0.1 and 1 mM added $MgCl_2$ (Fig. 6).

A similar dependence on intracellular magnesium was observed for active Ca^{2+} efflux, further supporting the close coupling between the ATPase and efflux activities. Active Ca^{2+} efflux was at the

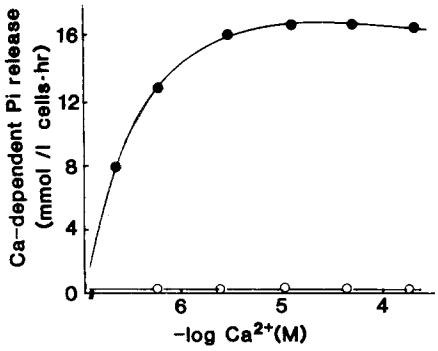

Fig. 5. Dependence of Ca^{2+}-stimulated inorganic phosphate production in intact cells on intracellular magnesium. Washed red blood cells, either normal (●) or depleted of magnesium (*see* Materials and Methods) (○) were incubated for 5 min at 37°C in loading medium containing 0.1 mM EGTA and various concentrations of $CaCl_2$ required to give the indicated free Ca^{2+} concentrations. The cells were lysed and their inorganic phosphate content was determined as described in Materials and Methods. The result shown is the average of two experiments

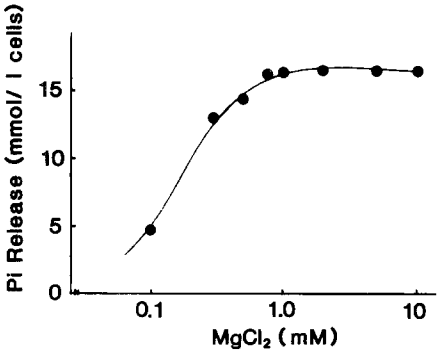

Fig. 6. Effect of added Mg^{2+} on Ca^{2+}-stimulated inorganic phosphate production in Mg^{2+}-depleted intact red blood cells as a function of $MgCl_2$ reintroduction. Washed red blood cells were depleted of Mg^{2+}, as described in Materials and Methods, and incubated in the loading medium containing 35 μM free Ca^{2+} (buffered with 0.1 mM EGTA) and various concentrations of $MgCl_2$. The Ca^{2+}-ATPase reaction was initiated by the addition of 20 μM A23187. Inorganic phosphate was determined after 5 min at 37°C as described in Materials and Methods. The result shown is one of two similar experiments performed in duplicate

detection limit of the assay in magnesium-depleted red blood cells, whereas it was restored to normal levels by the progressive reintroduction of magnesium from 0.2 to 2.0 mM (Fig. 7). The dependence of Ca^{2+} efflux on the magnesium concentration present during the loading of calcium was also examined in normal (nondepleted) cells (Fig. 8). Maximum Ca^{2+} efflux was reached when the $MgCl_2$ concentration in the loading (and efflux) medium was 0.2 mM, and it remained constant at $MgCl_2$ concentrations as high as 2 mM. As expected from the

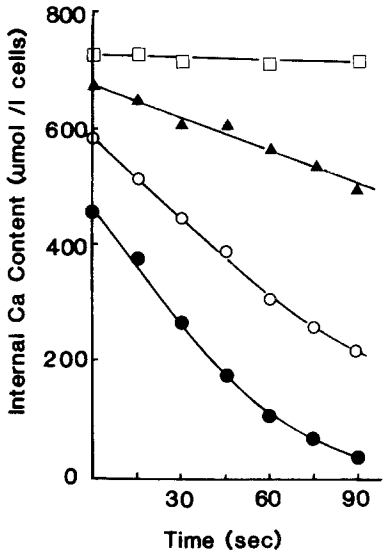

Fig. 7. Effect of intracellular Mg^{2+} on active Ca^{2+} efflux from Mg^{2+}-depleted intact red blood cells. Mg^{2+}-depleted red blood cells (*see* Materials and Methods) were incubated for 15 min at 10% hematocrit in loading medium containing 20 μM A23187 and various $MgCl_2$ concentrations as follows: no added $MgCl_2$ (□), 0.2 mM $MgCl_2$ (▲), 1.0 mM $MgCl_2$ (○) and 2.0 mM $MgCl_2$ (●). Calcium influx was then initiated by addition of 100 μM $CaCl_2$ (containing 2.5 μCi/ml of ^{45}Ca tracer) for 1 min, after which Ca^{2+} efflux was exposed by the addition of $CoCl_2$. The initial rate of Ca^{2+} efflux in mmol/liter cells · hr was 0.5 (0 $MgCl_2$), 6.0 (0.2 mM $MgCl_2$), 16.3 (1 mM $MgCl_2$) and 22.1 (2 mM $MgCl_2$). The results are typical of three similar experiments

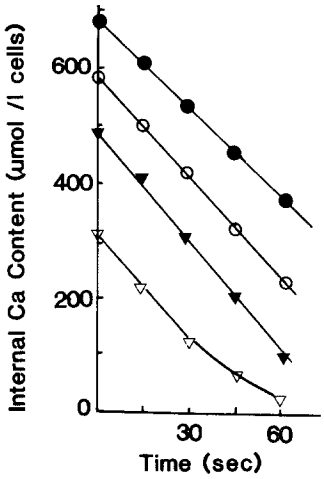

Fig. 8. Effect of extracellular magnesium in the loading and efflux medium on active Ca^{2+} efflux from normal intact red blood cells. Washed red cells were loaded with 100 μM $CaCl_2$ containing ^{45}Ca tracer in loading medium containing 20 μM A23187 and various $MgCl_2$ concentrations. Ca^{2+} efflux was exposed with $CoCl_2$ in the same medium. The $MgCl_2$ concentrations (with Ca^{2+} efflux rates in units of mmol/liter cells · hr in parentheses) were as follows: 0 mM $MgCl_2$ (17.8) (●), 0.2 mM (21.9) (○), 0.5 mM (22.7) (▼) and 2.0 mM (20.8) (▽). Note that as the $MgCl_2$ concentration was increased in the loading medium less calcium was taken up by the cells

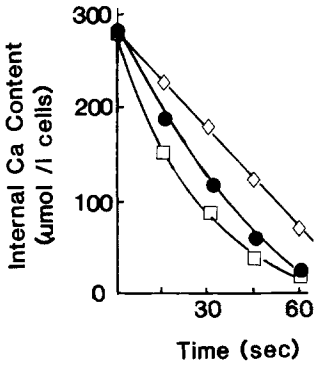

Fig. 9. Active Ca^{2+} efflux from red blood cells at various extracellular pH values. Washed red cells, pretreated with DIDS, methazolamide and amiloride pH-lock solution (*see* Materials and Methods), were loaded with 100 μM $CaCl_2$ and A23187 as indicated in Materials and Methods. Ca^{2+} efflux was measured 1 min after the addition of 0.25 mM $CoCl_2$ alone (pH 7.2) (●), or followed immediately with sufficient 2 M NaOH (◇) or 4 M HCl (□) to give the required pH. The result is typical of results obtained in three experiments

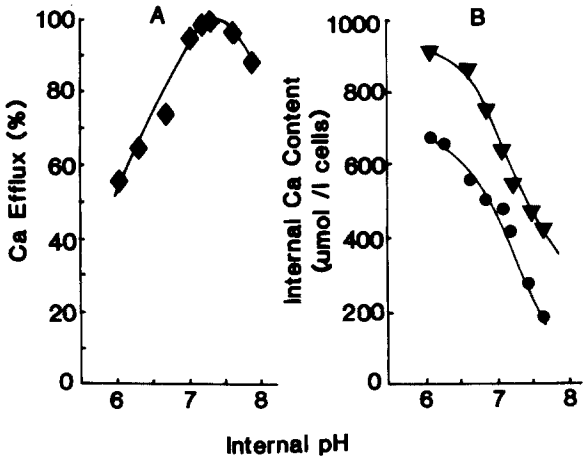

Fig. 10. (*A*) Active Ca^{2+} efflux rate from intact red blood cells as a function of intracellular pH. pH-locked red blood cells were prepared as described in Materials and Methods, and loaded in the presence of 100 μM $CaCl_2$ as in Fig. 9. The maximum efflux rate (normalized to 100%) corresponds to 16.0 mmol/liter cells · hr. The result is the average of three separate experiments performed in duplicate. (*B*) The dependence of total cell calcium content on internal pH and extracellular $MgCl_2$ concentration. Washed red blood cells were suspended in loading medium (10% hematocrit) containing 0.1 mM $CaCl_2$ (plus ^{45}Ca tracer 2.5 μCi/ml) and $MgCl_2$ 2.5 mM (●) or 0.2 mM (▼). Calcium loading was initiated by 20 μM A23187 for 1 min before $CoCl_2$ addition. The result is one of two similar experiments performed in duplicate

known divalent cation specificity of ionophore A23187, calcium loading of the intact cells was progressively decreased as the $MgCl_2$ concentration increased in the calcium-loading medium (Figs. 7 and 8).

Effect of Extracellular H^+ Concentration on Active Ca^{2+} Efflux

To examine the effect of extracellular H^+ on the rate of active Ca^{2+} efflux, proton movements through the anion channel (band 3) and carbonic anhydrase (the Jacobs-Stewart cycle) were inhibited with DIDS and methazolamide pretreatment, respectively, and through the $Na^+:H^+$ exchanger with amiloride (*see* Materials and Methods). Ca^{2+} efflux was increased from 21 mmol/liter cells · hr at pH 7.4 to 26 mmol/liter cells per hr by the addition of protons (pH 6–6.5) and decreased to 12 mmol/liter cells · hr by depletion of protons from the extracellular medium (pH 7.8) (Fig. 9). During the initial phase of active Ca^{2+} efflux (approx. 15 sec) the measured extracellular pH did not change by more than 0.25 pH units. A similar effect of extracellular H^+ concentration was found when active Ca^{2+} efflux was measured at 10 mM extracellular $CaCl_2$, where the Ca^{2+} pump rate was inhibited by 60% (*results not shown*).

Effect of Intracellular pH on Active Ca^{2+} Efflux

The dependence of the initial Ca^{2+} efflux rate on the measured intracellular pH in DIDS, methazolamide and amiloride pH-locked cells (*see above* and Materials and Methods) was found to reach a maximal active Ca^{2+} transport rate at around pH 7.2, the rate decreasing above this value (Fig. 10*A*). The initial intracellular calcium content of pH-locked cells decreased progressively with increasing intracellular pH, as expected for a $Ca^{2+}:H^+$ ionophore (Fig. 10*B*). This confirms that the intracellular pH had been successfully altered. Intracellular calcium content was also decreased in cells loaded in 2.5 mM $MgCl_2$-containing loading medium compared to one containing 0.2 mM $MgCl_2$. This occurred at all intracellular pH values examined, probably due to Mg^{2+} competition for Ca^{2+} entry, again as expected for the A23187 ionophore.

Effect of Membrane Potential on Ca^{2+} Efflux

Ca^{2+} efflux was examined in DIDS-treated cells in which Cl^- was replaced with SO_4^{2-}. The membrane potential was altered by varying the K^+ gradient in K^+-permeable cells and was calculated from the measured K^+ gradient (*see* Materials and Methods). The Ca^{2+} efflux rate was unchanged when the membrane potential was decreased from +5 to −100 mV (Fig. 11).

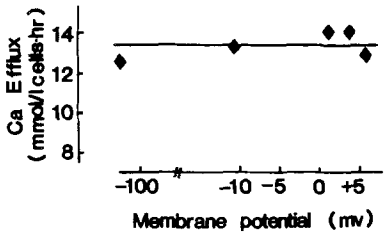

Fig. 11. Influence of membrane potential on active Ca^{2+} efflux from intact red blood cells. The membrane potential was altered by substitution of Cl^- with SO_4^- and incubation of cells with 125 μM DIDS, in the presence of various K^+ gradients, as described in Materials and Methods. Ca^{2+} efflux was measured after loading cells in 100 μM $CaCl_2$ for 1 min, as described in Materials and Methods. The result is one of two similar experiments

Discussion

In the present study we have investigated a number of properties of active Ca^{2+} efflux coupled to the Ca^{2+}-ATPase in the intact human erythrocyte. The recently described method of Tiffert et al. (1984) was used to instantaneously block all Ca^{2+} uptake through the $Ca^{2+}:H^+$ exchanger A23187 and expose active efflux of Ca^{2+} by the Ca^{2+} pump, while changes in cell volume were avoided by maintaining electrochemical equilibrium of K^+ across the membrane (Brown & Lew, 1983). The operation of the Jacobs-Stewart cycle normally ensures rapid restoration of the proton gradient during A23187 loading. The use of cobalt to rapidly expose active Ca^{2+} efflux avoids significant ATP-depletion that invalidates the measurement of Ca^{2+} efflux rates at steady state, where a significant heterogeneity occurs and a population of ATP-depleted cells reaches equilibrium with extracellular calcium (Lew & Garcia-Sancho, 1985). The validity of the method used to measure active Ca^{2+} efflux in this study is supported by the finding that cells loaded with calcium (plus A23187) for short intervals (up to 1 min) were able to lower their calcium to normal basal levels of 1–2 μmol/liter cells when cobalt was added. Mean ATP levels remained greater than 1 mmol/liter cells during the Ca^{2+} efflux experiments, which has been previously shown to be saturating for active Ca^{2+} efflux (Xu & Roufogalis, 1988).

Calcium Sensitivity of Active Calcium Efflux and Ca^{2+}-ATPase

The estimated concentration of calcium for half-maximal activation of active Ca^{2+} efflux found in the present study (30–40 μmol/liter cells) is somewhat higher than values reported from experiments in intact cells performed at steady state (0.7–0.9 μmol/liter cells) (Ferreira & Lew, 1976) or in resealed ghosts (Schatzmann, 1973; Muallem & Karlish, 1979), but similar to the values of 7.7–36.5 μmol free Ca^{2+}/liter cells calculated recently from Ca^{2+} efflux *vs.* time curves in intact human red blood cells (Dagher, Amar & Khelif, 1987). The $K_{0.5}$ values found are nevertheless considerably lower than values of 300–1000 μmol/liter cells reported in early experiments (Romero & Whittam, 1971; Sarkadi et al., 1977). However, even after correcting for the fraction of total calcium which is free, reported to be between 0.15 to 0.4 at steady state (Ferreira & Lew, 1976; Dagher et al., 1987; Lew & Garcia-Sancho, 1988), the resulting $K_{0.5}$ for free Ca^{2+} of 4.5 to 16 μmol/liter cells is more than one order of magnitude greater than the $K_{0.5}$ of 0.2 μM obtained by measuring inorganic phosphate (P_i) production in intact cells (Fig. 5). Unlike the active Ca^{2+} efflux experiments, Ca^{2+}-ATPase activity was determined over 5 min in cells in which free Ca^{2+} in the medium (buffered with EGTA) had equilibrated with intracellular Ca^{2+}. Assuming a membrane potential of -8 to -10 mV in the intact cell, the adjusted $K_{0.5}$ for free Ca^{2+} activation of the Ca^{2+}-translocating ATPase (around 0.4 μM) is similar to values obtained previously for the Ca^{2+} affinity of the ATPase in Ca^{2+}-permeabilized intact cells (Muallem & Karlish, 1982), resealed ghosts (Muallem & Karlish, 1979), inside-out vesicles (Enyedi et al., 1987) and in the purified Ca^{2+}-ATPase (Villalobo et al., 1986).

The difference between the estimate of Ca^{2+} sensitivity of the Ca^{2+}-ATPase and of active Ca^{2+} efflux is difficult to interpret at the present time, until free Ca^{2+} can be measured under the conditions of the efflux experiments. A number of possible explanations can be advanced. The estimate of the fraction of free Ca^{2+} in the Ca^{2+} efflux experiments, determined mainly from steady-state experiments in ATP-depleted cells (Ferreira & Lew, 1976), may not be valid under the nonequilibrium conditions of the active Ca^{2+} efflux experiment, and hence free ionized Ca^{2+} may have been overestimated. This possibility gains support from recent studies in which the resting free intracellular Ca^{2+} concentration of cells containing 1–2 μmol Ca/liter cells was found to be only 10–30 nM (Lew & Garcia-Sancho 1988). No difference was found in the rate of active Ca^{2+} efflux in cells loaded to a similar Ca content (60–70 μmol/liter cells) in the presence or absence of 0.1 mM EGTA, indicating that extracellular EGTA used in the Ca^{2+}-ATPase measurements did not alter the apparent calcium affinity of the active Ca^{2+} efflux mechanism (Xu & Roufogalis, *unpublished results*). Secondly, despite the known uniform distribution of A23187 among the red cell population (Simonsen, Gomme & Lew, 1982), at

low levels of calcium loading a heterogeneous distribution of Ca^{2+} may occur (Lew & Garcia-Sancho, 1988), resulting in an overestimate of the calcium content of the population of cells actively effluxing calcium. Nevertheless, it is unlikely that a large proportion of the cell population was unable to actively efflux calcium due to ATP-depletion in our experimental conditions (*see above*), which are clearly different from the "steady-state" conditions described by Lew and Garcia-Sancho (1985). Thirdly, the active Ca^{2+} efflux rates in cells loaded with calcium below 100 μM may be abnormally low because the short loading times used (2–60 sec) may be insufficient for calmodulin to maximally bind and activate the Ca^{2+} pump. A time-dependent hysteretic activation of the Ca^{2+} pump by calmodulin described by Scharff and associates (Scharff, Foder & Skibsted, 1983; Scharff & Foder, 1986) is consistent with this possibility. This interpretation may also account for the S-shaped calcium dependence of the active Ca^{2+} efflux curve, compared to the apparently hyperbolic Ca^{2+} dependence of the Ca^{2+}-ATPase activity (Fig. 5), which was determined over a period of 5 min.

Other possible explanations for the difference in apparent calcium sensitivity were considered, but are less likely. Ca^{2+} efflux may require a higher intracellular calcium content than Ca^{2+}-ATPase activity; however, such uncoupling has not been observed in inside-out vesicles (Quist & Roufogalis, 1977; Akyempon & Roufogalis, 1982). Alternatively, the Ca^{2+} pump may progressively lower its Ca^{2+} sensitivity when exposed to the relatively high calcium contents used in the Ca^{2+} efflux experiments (Lew et al., 1980), perhaps by disrupting a Ca^{2+} diffusion barrier between the cytosol and Ca^{2+} pump sites (Schatzmann & Bürgin, 1978), but a molecular basis for this mechanism is unknown. On the other hand, the high apparent Ca^{2+} affinity in the Ca^{2+}-ATPase studies may be the result of the prolonged exposure of the cells to A23187 in the Ca^{2+}-ATPase measurements, an effect which may also occur in membranes subjected to a lysis procedure, which again may result from disruption of a Ca^{2+} diffusion barrier. The difference between the apparent Ca^{2+} sensitivities of the Ca^{2+} pump as measured by the active Ca^{2+} efflux and Ca^{2+}-ATPase method remains an apparent anomaly to be addressed in future work.

Effect of Divalent Cations

Studies on other properties of the Ca^{2+} pump in intact red blood cells have been made at intracellular calcium levels sufficient to saturate active Ca^{2+} efflux (i.e. between 100–800 μmol/liter cells). Active Ca^{2+} efflux and Ca^{2+}-ATPase activities in intact red blood cells were almost totally dependent on the presence of intracellular Mg^{2+} (Figs. 5 and 7). In normal red blood cells maximum active Ca^{2+} efflux rate was reached at about 0.2 mM $MgCl_2$ in the Ca^{2+} and A23187 containing loading medium, which corresponds to the concentration of $MgCl_2$ previously found to preserve the intracellular free Mg^{2+} concentration at around 0.4 mM (Flatman & Lew, 1980). However, active Ca^{2+} efflux was not inhibited when cells were loaded in the presence of 2 mM $MgCl_2$, where total intracellular magnesium may be as high as 8 mM (or around 2 mM free Mg^{2+}) (Flatman & Lew, 1980). Similarly, Ca^{2+}-ATPase activity was not inhibited when Mg^{2+} was reintroduced to Mg^{2+}-depleted cells at concentrations 10-fold higher than the optimal concentration (Fig. 6). Overall, the data indicate that the Ca^{2+} pump is maximally active at free intracellular Mg^{2+} concentrations around the normal resting free Mg^{2+} concentration of 0.4 mM and that the Ca^{2+} pump is relatively insensitive to Mg^{2+} at concentrations some 10-fold higher than the optimum. This contrasts to the membrane bound and purified Ca^{2+}-ATPase, where Mg^{2+} in excess of ATP in the millimolar concentration range inhibits the enzyme activity (Caride, Rega & Garrahan, 1986; Villalobo et al., 1986).

Similarly, Mg^{2+} did not affect Ca^{2+} efflux when it was increased up to 10 mM in the external medium. By contrast, Ca^{2+} efflux was inhibited by extracellular Ca^{2+} ($K_{0.5}$ = 6 mM), with near complete inhibition occurring at 20 mM. This result differs from studies on the resealed ghost, where both extracellular Ca^{2+} and Mg^{2+} were found to inhibit Ca^{2+} efflux in a competitive manner (Kratje, Garrahan & Rega, 1985). If we accept that the inhibition by extracellular Ca^{2+} is due to the binding of Ca^{2+} to the outwardly oriented E_2P form of the enzyme, thereby inhibiting Ca^{2+} dissociation and allowing the accumulation of E_2P conformation (*see* Allen, Katz & Roufogalis, 1987), it is apparent that this low affinity Ca^{2+} binding site exposed during translocation of Ca^{2+} from the intracellular high affinity site is selective for Ca^{2+} over Mg^{2+}. This interpretation is consistent with the observation that Ca^{2+} alone is sufficient for phosphoprotein formation (Rega & Garrahan, 1975; Schatzmann & Bürgin, 1978; Szász et al., 1978; Allen et al., 1987), while Mg^{2+} accelerates the rates of both phosphorylation and dephosphorylation, probably at an allosteric site (Caride et al., 1986). Other divalent cations also inhibited active Ca^{2+} efflux from the extracellular medium (Fig. 4). Manganese was less potent than Ca^{2+} ($K_{0.5}$ = 12 mM), while Ba^{2+} was without effect at similar concentrations. On the other hand, Cd^{2+}

was more potent than Ca^{2+} ($K_{0.5}$ = 2 mM), and it achieved almost the same maximum inhibition as that with Ca^{2+}, suggesting that it may occupy the same site as Ca^{2+} on the E_2P orientation of the pump. The inhibition of Ca^{2+}-ATPase activity by Cd^{2+} is also greater than was expected from its ionic radius (Pfleger & Wolf, 1975).

Influence of Protons

To study the pH dependence of Ca^{2+} efflux in intact cells, proton equilibration through band 3 and carbonic anhydrase (the Jacobs-Stewart cycle) and proton movements via the $Na^+ : H^+$ exchanger were reduced with selective inhibitors. It is of interest that the active Ca^{2+} efflux rate was not decreased after treatment of red blood cells with 125 μM DIDS, 0.4 mM methazolamide and 0.4 mM amiloride, which might have limited the dissipation of any proton gradient generated during Ca^{2+} transport. This contrasts with results in inside-out vesicles, where active Ca^{2+} uptake was nearly abolished when band 3 was blocked by 5 μM DIDS (Minocherhomjee & Roufogalis, 1982) or 100 μM SITS (Waisman et al., 1981). This inhibition was most probably due to the H^+ gradient generated by $Ca^{2+} : H^+$ countertransport during Ca^{2+} translocation by the Ca^{2+}-transport ATPase (Niggli, Sigel & Carafoli, 1982; Smallwood et al., 1983). However, the failure of DIDS to inhibit active Ca^{2+} efflux in intact red cells in the present work may be due to the large buffering capacity of hemoglobin (Dalmark, 1975), or to the presence of other remaining H^+ conductance pathways. Alternatively, the active Ca^{2+} translocation pathway may not in fact occur by exchange of Ca^{2+} for H^+ in the intact cells. In this case the Ca^{2+} pump may translocate Ca^{2+} without the countertransport of another cation, being electrogenic rather than electroneutral. This would contrast with the observations on the reconstituted enzyme (Niggli et al., 1982; Smallwood et al., 1983; Villalobo & Roufogalis, 1986). This mechanism, however, is unlikely from our experiments on the dependence of Ca^{2+} efflux on extracellular H^+ concentration. In these experiments H^+ movement from outside to the inside of intact cells was reduced by treatment of the cells with the pH-lock solution, as confirmed by measurement of external pH in response to the addition of H^+ which showed that the external pH was maintained essentially constant during the time course of active Ca^{2+} efflux experiments. Ca^{2+} efflux was enhanced by 100% when the pH of the external medium was decreased from pH 7.8 to pH 6.0–6.5 (Fig. 9). This increase was not due only to the displacement of extracellular Ca^{2+} from low-affinity Ca^{2+} inhibitory sites (*see* Kratje et al., 1985), as it occurred to a similar extent at 0.1 mM external $CaCl_2$ (Fig. 9) as well as at 10 mM $CaCl_2$ (*results not shown*). Furthermore, more direct measurement made it unlikely that active Ca^{2+} efflux was electrogenic mechanism in the intact cell, as progressively decreasing the membrane potential of the red cell to around −100 mV did not alter the active Ca^{2+} efflux rate (Fig. 11). Therefore, these results are in agreement with previous studies on isolated inside-out vesicles and proteoliposomes, and provide evidence for a $Ca^{2+} : H^+$ countertransport mechanism for active Ca^{2+} translocation in the intact red blood cell.

Similar protocols used to lock intracellular pH of the red cell revealed a maximum in the curve of intracellular pH dependence of Ca^{2+} efflux, consistent with the participation of acidic and basic groups in the Ca^{2+} translocation mechanism. This is similar to the pH profiles found in the nonvectorial membrane system. The pH optimum (around pH 7.2) falls within the physiological range of intracellular pH in the intact red blood cell.

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