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ACTIVE CALCIUM TRANSPORT IN RED CELL GHOSTS RESEALED IN DEXTRAN SOLUTIONS

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1. Human erythrocytes when lysed and resealed to Ca in the presence of dextran can be readily separated from the suspending medium by low-speed centrifugation. 2. Ghosts trapped Ca and EGTA at the same ratio as present in the haemolytic medium and remained tight to Ca after washing and subsequent incubation for up to 90 min at 37°C. 3. Ca extrusion could be promoted by substrates other than ATP only from ghosts that had been loaded with low free Ca concentrations (1–22 μM). The order of activation by the various substrates employed was ATP > adenine + inosine > inosine. 4. The kinetics of extrusion depended markedly on internal free Ca. The system showed a high affinity state (K_{Ca} about 3 μM; V = 0.34 μmol Ca/ml ghosts per min) at low concentrations (1–22 μM) and a low affinity state (K_{Ca} about 250 μM; V = 0.17 μmol Ca/ml ghosts per min) at high concentrations (0.2–4.0 mM). 5. Both at low and at high free Ca, La-sensitive ATP hydrolysis was closely correlated with La-dependent Ca efflux, in keeping with an stoichiometry of 1. 6. The rate of extrusion was maximal in the presence of 160 mM KCl and decreased to various extents when K was fully replaced by different cations, following the order K > Na = choline > Mg. 7. The efflux rate of high-K ghosts, resealed to alkaline cations, was stimulated by external Na, whilst Mg and choline were practically without effect. 8. The results indicate that human red cells possess a powerful Ca extrusion mechanism, the activity of which can be modulated by alkaline cations.

Introduction

The presence of an outwardly directed active Ca transport is a well known fact in human red cells. It was described first in ghosts, loaded with Ca by reversal of haemolysis [1].

Although ghosting techniques are currently employed, their use is restricted by the low specific gravity of ghosts. This is a drawback to these techniques, specially when kinetic studies are involved. It seems of importance to overcome this problem by finding suitable means of increasing ghost specific gravity.

Dextrans are known to increase the sedimentation rate of red cells according to the molecular weight [2]. On the other hand, early work has shown that dextran reduces the loss of haemoglobin that occurs during osmotic lysis [3]. It thus seems possible to prepare ghosts with an increased specific gravity by lysing in the presence of high-molecular weight dextran.

The above possibility was investigated in the present work. It was found that after restoring isotonicity, human erythrocyte ghosts could be readily separated from the suspending medium by low-speed centrifugation. These ghosts were used to study some kinetic aspects of active Ca transport. The results showed that ghosts resealed in dextran solutions possess an extremely active Ca pump. Some results of this work were presented at the XXVIII International Congress of Physiological Sciences.

Methods

Analytical quality reagents were used whenever possible. EGTA and dextran (M_r 80 000) were purchased from Sigma, USA. ATP-Na_2, inosine and adenine were obtained from Boehringer, F.R.G.

Human blood was collected in acid citrate-dextrose solution and used within 1–3 days after collection.

The pH of all solutions was adjusted at room temperature within a range of ±0.01 unit.

Obtention of packed cells

About 30 ml blood were centrifuged at 3000 × *g* for 10 min at room temperature. After removing white cells, the erythrocytes were washed three times with a high-K medium, which contained (mM): KCl, 160; imidazole hydrochloride, 20; pH 7.0. Before each centrifugation glucose was allowed to equilibrate with the medium. Cells were finally packed by centrifuging for 15–20 min under the above conditions.

Preparation of ghosts

Packed cells (1 vol.) were lysed for 30 s at room temperature by stirring in 20–30 vol. of a medium containing (mM): $MgCl_2$, 4; imidazole-HCl, 20; pH 7.0; with the addition of 3% dextran (w/v). ATP-Na_2, when present, was added to a final concentration of 4 mM.

After lysis, isotonicity was restored with KCl and the suspension was kept for 1 h in an ice-cold water bath. Thereafter, ghosts were spun down at 3000 × *g* for 10 min, washed three times with a high-K medium and returned to the ice-bath. In some experiments K was substituted by Na, Mg or choline throughout the experimental procedure.

Ghosts were prepared to contain either high or low free Ca concentrations. High-Ca ghosts were obtained by lysing in the above medium, supplemented with different $CaCl_2$ concentrations up to 4 mM. Ghost-free Ca was calculated assuming not any great alteration in original cell content (33% Ca binding to cell buffers; see Ref. 4). Low-Ca ghosts, by contrast, were obtained by lysing in a similar medium but supplemented instead with 5 mM EGTA/imidazole (pH 7.0) and variable amounts of $CaCl_2$, to obtain 5–22 μM free Ca. Ca · EGTA buffers were calculated as described by Schatzmann [5].

In some experiments, ghosts were resealed to alkaline cations. Accordingly, ghosts were loaded in the presence of ATP, with an excess of Ca over EGTA and isotonicity was restored with KCl. They were centrifuged, resuspended in a fresh high-K medium and subsequently pre-incubated for 20–30 min at 37°C, in the presence of 4 mM inorganic phosphate, 10 mM inosine and 5 mM adenine. Under these conditions, ghosts pump out the excess Ca while resealing and at the same time ATP is regenerated from the substrates provided. The internal free Ca was generally about 20 μM after pre-incubation.

Incubation of ghosts

Ghosts (1 vol.) were usually incubated with 9 vol. high-K medium which contained (mM): KCl, 160; $CaCl_2$, 5; imidazole-HCl, 20 (pH 6.8 at 37°C). Inosine and adenine, when present, were at 10 and 5 mM, respectively. No phosphate was added to avoid any Ca precipitation.

Before incubating ghosts which had been resealed to alkaline cations, they were washed several times with isotonic solutions of NaCl, KCl, choline chloride or $MgCl_2$. Ghosts were adjusted to a haematocrit of 5% and finally incubated in one of the above media in the presence of adenine plus inosine, according to the method b specified below.

Two incubation procedures were employed, which are described as methods a and b.

Method a. Ghosts were pre-incubated for 15 min at 37°C with the above K-medium, supplemented with 0.5 mM $LaCl_3$. The incubation was started by adding 0.6 mM EGTA to remove free La ($K_{La} \cdot EGTA = 10^{15.8}$).

Method b. Ghosts were mixed with the incubation medium pre-warmed at 41°C and immediately incubated at 37°C. The temperature of the mixture fell to 37.5°C upon mixing.

Following either procedure, samples (1 ml) were withdrawn at zero time and then at intervals ranging from 1–5 min up to 30 min. They were squirted rapidly into an ice-cold medium, containing (mM): KCl or choline chloride, 160 (or 170, respectively); $CaCl_2$, 5; $LaCl_3$, 2; imidazole hydrochloride 20 (pH 7.0), in addition to 0.5 ml di-*n*-butyl phthalate (d = 1.042–1.045). The suspension was immediately spun down at 42 000 × *g* for 40 s and the pellet of ghosts which passed through the oil cushion was then lysed in water.

A sample of ghosts before incubation was thoroughly washed with a Ca-free high-K medium and finally packed by centrifuging for 10 min at 40 000 × *g*. The ghost Ca concentration was determined and used to correct the Ca content of incubated ghosts for the amount trapped in the extracellular space.

(Ca^{2+} + Mg^{2+})-ATPase activity of intact ghosts

In some experiments, ATPase activity was measured in the same batch of ghosts in which Ca transport was assessed. Accordingly, ghosts were incubated as described under method b in the presence of ouabain (0.17 mM), with and without addition of 250 μM $LaCl_3$.

The reaction was stopped at regular intervals by squirting samples of the incubation mixture into an ice-cold 10% trichloroacetic acid solution. The samples were filtered after standing for 20 min in an ice-cold water bath and immediately analysed for inorganic phosphate.

Analytical procedures

Haemoglobin. Haemoglobin (Hb) was determined as oxyhaemoglobin [6].

Measurements of Ca. These were done by atomic absorption flame photometry in trichloroacetic acid extracts of ghost lysates. La (50 mM) was added both to samples and standards.

Determinations of inorganic phosphate. Determinations of (P_i) were performed according to the method of Berenblum and Chain [7].

Determinations of EGTA. EGTA was measured spectrophotometrically by microtitration [5].

^{45}Ca measurements. These were done by liquid scintillation counting using aquasol as scintillant.

Na and K. These cations were determined by flame emission.

Results

Activation by inosine of Ca extrusion

Pilot experiments have shown that when red cells are lysed in 3% dextran solutions, the amount of Hb trapped by ghosts is 2–3-times greater than that obtained in the absence of dextran. The possibility arises that some of the important enzymes for ATP synthesis may be also retained to a greater extent. In such a case, active Ca transport could be promoted by providing an adequate glycolytic substrate.

To test this possibility, erythrocytes were metabolically depleted by incubating for 17 h at 37°C in a high-K medium, containing 10 μg chloramphenicol/ml. Low-Ca ghosts (22 μM free Ca) were prepared from these cells and incubated with and without inosine, following method a.

The Ca content of ghost was not altered after 1 h incubation without substrate (Fig. 1). By contrast, when inosine was present, ghost Ca (in μmol Ca/ml ghosts) decreased from about 3.4 to 2.7 over the same period.

The results show that ghosts pump out Ca efficiently when an adequate supply of ATP is provided.

Lack of effect of substrates at high internal Ca

Early work has demonstrated that Ca ions inhibit glycolytic ATP synthesis by blocking both enolase- and puryvate kinase-catalysed reactions [8]. It was thus of interest to determine the maximal free Ca concentration at which active transport could be promoted by glycolytic substrates. Cells were lysed in media containing different Ca concentrations up to 2 mM and ghosts were subsequently incubated with both La and inosine according to method a.

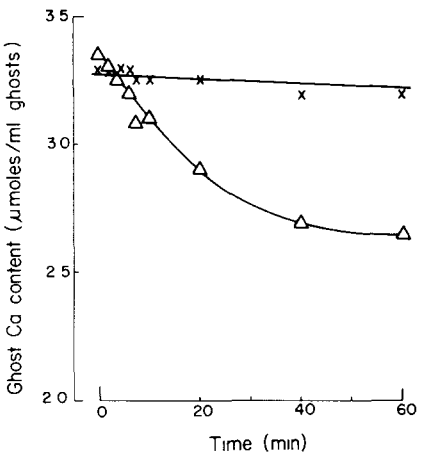


Fig. 1. Activation by inosine of Ca extrusion. Human red cells were incubated for 17 h at 37°C and a heamatocrit of 20% in a high-K medium, which contained (mM) KCl, 160, Tris-HCl, 20 (pH 7.4) and 10 μg chloramphenicol/ml. These cells were subsequently lysed in the presence of 3% dextran and 22 μM free Ca and ghosts pre-incubated for 15 min at 37°C in a medium containing (mM) KCl, 160, $CaCl_2$, 5, $LaCl_3$, 0.5, imidazole-HCl, 20 (pH 6.8 at 37°C), with and without addition of inosine (10 mM). After which, EGTA was added to a final concentration of 0.6 mM and samples were taken at the times indicated above. The graph shows the Ca concentration of ghosts which had been pre-incubated in the presence (△) and absence (X) of inosine. The results are given as the average of duplicate determinations from a single experiment. In the present and following figures, ghost Ca concentration was corrected for the amount of Ca trapped in the extracellular space.

The Ca content of ghosts prepared with 2 mM Ca did not change over the 15 min following addition of EGTA (Fig. 2). Essentially identical results were obtained with 0.5 and 0.1 mM $CaCl_2$. By contrast, active extrusion could be readily demonstrated when these ghosts were loaded with equimolar amounts of ATP and Mg, ghost Ca (in μmol/ml ghosts) decreasing from about 1.7 to 0.8 after 15 min incubation.

These results demonstrate that at high Ca concentrations, the pump becomes inactivated by a lack of ATP when the energy for transport is provided via glycolysis.

Effect of glycolytic substrates at low Ca

The effect of glycolytic substrates with different capacities of increasing cell ATP was studied at low free Ca to determine to what extent these metabolites stimulate active transport.

Cells were divided into two lots. The first portion was lysed in the presence of 22 μM free Ca and the ghosts were incubated as described in method a, either with inosine or adenine plus inosine. As a control, La instead of EGTA was added to a portion of ghosts incubated with inosine.

The other portion of red cells was lysed in a medium containing the same amount of free Ca stated above and equimolar amounts of Mg and ATP. These ghosts were incubated according to method b.

Ghost Ca was not altered during 30 min incubation in the presence of inosine plus 1.1 mM La, thus showing a complete inhibition of the Ca pump by La (Fig. 3). By contrast, when La was chelated by EGTA, ghost Ca (in μmol/ml ghosts) was decreased

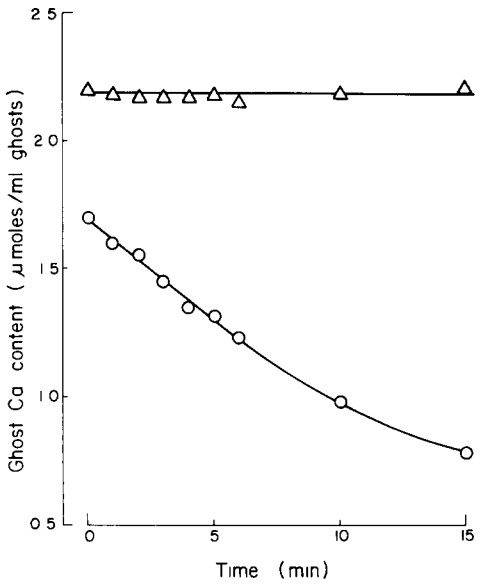


Fig. 2. Lack of effect of inosine at high free Ca. Red cells were lysed in the presence of 2 mM total Ca, with and without addition of equimolar amounts of Mg and ATP (4 mM). Ghosts were pre-incubated for 15 min at 37°C, in a high-K medium containing both 5 mM $CaCl_2$ and 0.5 mM $LaCl_3$. 10 mM inosine was added only to ghosts loaded with no ATP. After pre-incubation, EGTA was added as described in Fig. 1 and samples were taken at the time intervals shown above. The Ca concentration of ghosts loaded with (○) and without (△) ATP is shown in the graph as the mean value of two experiments.

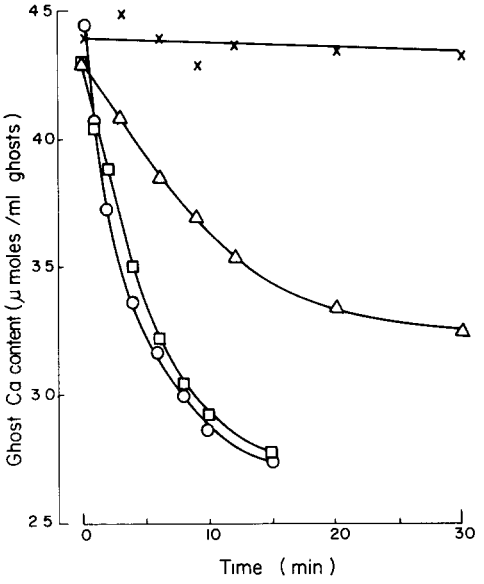


Fig. 3. Effect of substrates with different capacities of increasing cell ATP. Red cells were divided into two lots The first portion was lysed in the presence of 22 μM free Ca and ghosts were pre-incubated as described in Fig. 2, either in the presence of 10 mM inosine or 5 mM adenine plus 10 mM inosine. EGTA was added to start the incubation as described in Fig 1. 0.6 mM La instead of EGTA was also added to a portion of ghosts incubated with inosine. The second portion of red cells was lysed with the same Ca concentration stated above but in the presence of equimolar amounts of ATP and Mg. The incubation was started by mixing a cold suspension of ghosts with the incubation medium pre-warmed at 41°C, as described in the text under method b. The graph shows the Ca concentration of ghosts pre-incubated with inosine (△), inosine plus La throughout (X); adenine plus inosine (□) and of ghosts loaded with ATP (○). Results from a single experiment are presented.

from nearly 4.3 to 3.4 over the same incubation period. The addition of adenine together with inosine caused a marked enhancement of Ca efflux. Thus, ghost Ca was rapidly diminished from 4.3 to 2.8 after only 15 min incubation.

On the other hand, ghosts loaded with ATP decreased their Ca content in a way similar to that of ghosts incubated with adenine plus inosine. However, Ca extrusion from the former ghosts was slightly increased at the beginning of incubation.

These findings show that the extent of active transport depends markedly on the substrate provided, decreasing in the order ATP > adenine + inosine ≫ inosine. The degree of stimulation found with substrates other than ATP is in keeping with their capacity to regenerate ATP in intact cells [9].

Trapping of EGTA by dextran-resealed ghosts

The free Ca concentration of ghosts loaded with Ca buffers depends on the final ratio of the buffer components. It was therefore of importance to assess whether ghosts trapped Ca and EGTA at the same ratio as present in the haemolytic medium.

Cells were divided into two lots. One portion was lysed in a medium containing ATP and Ca/EGTA at a ratio of 0.950, thus giving 22 μM free Ca at pH 6.8 and 37°C. The other portion was also lysed in the presence of both ATP and 22 μM free Ca but in the absence of EGTA. Allowance was made for binding of Ca to original cell buffers (33%) and to ATP (0.16%).

Ghosts were incubated for 1 h at 37°C in a high-K medium to which 5 mM $CaCl_2$ had been added. At the end of incubation the ghosts were divided into two identical portions. One was kept for EGTA analysis by microtitration. The other was incubated for 30 min at 37°C, in a high-K medium containing both 5 μM ionophore A23187 and free Ca concentrations ranging from $1 \cdot 10^{-9}$ to $5 \cdot 10^{-7}$ M, buffered with the same EGTA concentration added to the haemolytic medium. After incubation, the ghosts were spun down through an oil cushion and their Ca content was determined.

The initial Ca concentration (in μmol/ml ghosts) of ghosts loaded with both Ca and EGTA in three experiments was 3.34 ± 0.084 (mean value ± 1 S.D. of mean) and was decreased to 0.61 ± 0.035 after 1 h incubation. By contrast, the Ca concentration of ghosts lysed in the absence of EGTA was not appreciably modified, being in three experiments 0.098 ± 0.073 and 0.22 ± 0.015 at the beginning and end of incubation, respectively. The difference in Ca concentration after 1 h incubation between ghosts prepared with and without EGTA, corresponds to the amount of Ca that remains bound to EGTA after pumping.

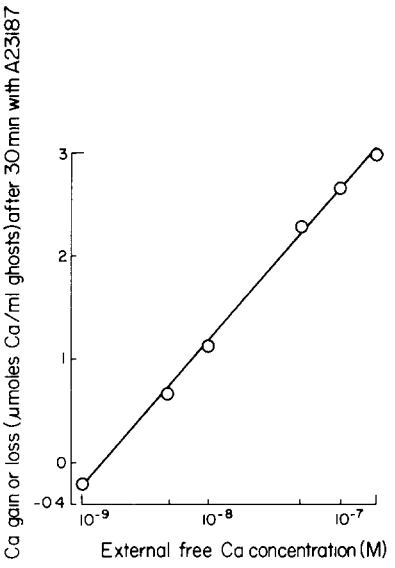
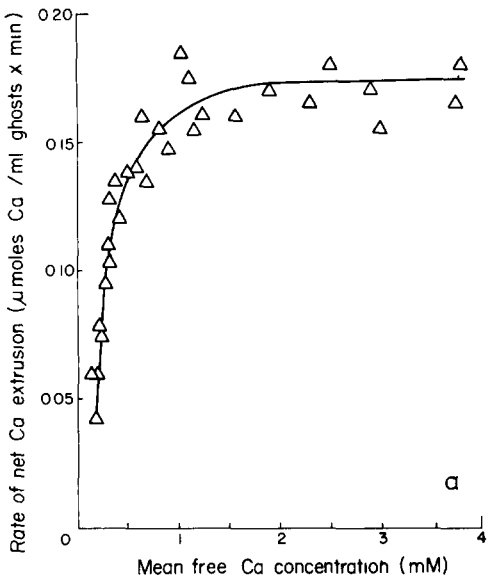


Fig. 4. Equilibration of Ca by A23187. Ghosts containing both Ca (22 μM) and ATP were pre-incubated for 1 h at 37°C in a high-K medium. After this, they were further incubated for 30 min at 37°C and haematocrit of 0.5%, in a 160 mM KCl/20 mM Tris-HCl (pH 7.8) medium, containing 5 μM A23187 and free Ca concentrations as indicated above. The graph shows the amount of Ca (in μmol/ml ghosts) gained or lost during incubation with the ionophore. Results from a single experiment are given

A net Ca movement was observed when ghosts that had been loaded with both Ca and EGTA and subsequently allowed to pump Ca, were further incubated in the presence of ionophore A23187. Fig. 4 shows the results from a typical experiment. It can be observed that the change in ghost Ca content (in μmol Ca/ml ghosts) increased linearly from about −0.3 to 3.0 when the external free Ca concentration was altered from 10^{-9} to nearly 10^{-7} M.

The ghost EGTA concentration can be calculated from the external free Ca concentration at which no net movement occurs and the amount of Ca that remains bound to EGTA after pumping.



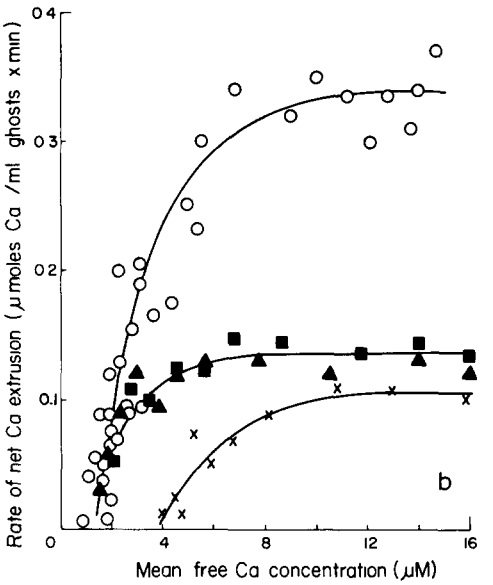


Fig. 5 The activation of Ca extrusion by internal Ca and the effect of other cations. Red cells were divided into two lots. One portion was lysed in the presence of ATP and Ca concentrations ranging from 0.5 to 4 mM (high-Ca ghosts). The other portion was lysed also in the presence of ATP and variable amounts of $CaCl_2$ plus EGTA (5 mM), to obtain free Ca concentrations between 5 and 22 μM (low-Ca ghosts). Both types of ghost were incubated at 37°C in a high-K medium containing 5 mM $CaCl_2$, after restoring isotonicity with KCl. Samples were withdrawn at 1 (low-Ca ghosts) or 2 min intervals (high-Ca ghosts). The rate of extrusion was calculated from the decrease in ghost Ca at each time interval and was plotted against the mean free Ca concentration during the corresponding interval. Collected results from high-Ca (part a) and low-Ca ghosts (part b) from at least four experiments are shown. Part b also shows collected results from ghosts prepared and incubated in the presence of Na^+(▲), choline (■) or Mg (X).

Ghost EGTA in three experiments was 3.55 ± 0.033 μmol/ml ghosts (mean value ± 1 S.D.), which is practically the same EGTA concentration measured by microtitration, namely 3.58 ± 0.056. the results lead to an initial Ca/EGTA ratio of about 0.940, not very different from that of 0.950 expected.

These findings clearly demonstrate that the ghosts can be effectively loaded with both Ca and EGTA, as they are trapped at the same ratio as present in the haemolytic medium.

Effect of low and high levels of free Ca on active extrusion

In view of the above results, Ca extrusion was studied in ghosts loaded with both ATP and either low or relatively high free Ca concentrations. They were incubated according to method b and samples were taken at 1–2 min intervals to determine the initial rate of extrusion.

The Ca concentration that produced half maximal activation of Ca efflux (K_{Ca}) from high-Ca ghosts was about 250 μM and the maximal efflux rate was 0.174 μmol Ca/ml ghosts per min (Fig. 5a). A Hill plot of these results showed a straight line (r^2 = 0.724) of slope 1.9 ± 0.12 (S.E.), significantly different from a slope of 1 ($t = 3.37$; $P < 0.005$).

On the other hand, the apparent K_{Ca} of low-Ca ghosts was about 2.7 μM, which is almost two orders of magnitude smaller than that found in the absence of EGTA (Fig. 5b; upper curve). The maximal extrusion rate was 0.340 μmol Ca/ml ghosts per min, nearly 2-times greater than that obtained in the presence of high Ca levels. A Hill analysis of the results showed a straight line ($r^2 = 0.827$), with a slope of 2.5 ± 0.21 (S.E.). This value is highly different from a slope of 1 ($t = 7.08$; $P < 0.005$).

The above findings clearly show that both the affinity and the maximal extrusion rate of the Ca pump are considerably decreased when ghosts are exposed to high free Ca levels.

Tightness to Ca during and after pumping

In the preceding experiments, Ca extrusion was measured against a large gradient. The possibility exists that the efflux rate was underestimated due to leakage of Ca into the ghosts.

To investigate this possibility, ghosts were loaded with ATP and either 22 μM free Ca or 2 mM total Ca. They were divided into two lots. One portion was incubated for periods up to 6 min at 37°C in a high-K medium, containing 5 mM $CaCl_2$ and trace amounts of ^{45}Ca.

The other portion was allowed to pump Ca by incubating at 37°C in a medium similar to that described above, but containing no tracer. After 1 h, ^{45}Ca was added and the incubation continued for periods up to 30 min. At the end of incubation, ghosts were spun down through an oil cushion and the radioactivity of the supernatant solutions was determined. This value was used to calculate the haematocrit of the ghost suspension at each time interval.

The haematocrit at the beginning of incubation was also measured by both the micro-haematocrit method and by relating the total Hb content of the ghost suspension to the Hb concentration of a known volume of packed ghosts. Comparison between these values would give an estimate of the degree of tightness to external Ca.

In every case but one, the haematocrit determined by means of tracer Ca at the beginning of incubation of low- and high-Ca ghosts, which had been pre-incubated or not, was slightly higher (about 2%) than

TABLE I

TIGHTNESS OF GHOSTS TO Ca

High- and low-Ca ghosts, prepared to contain the Ca concentrations indicated above, were incubated in the presence of ^{45}Ca, with and without preliminary incubation. After this, ghosts were centrifuged and the radioactivity remaining in the supernatant solutions was measured to calculate the haematocrit (Ht) of the ghost suspension, according to the following equation $Ht = (1 - (V_m A_m / V_t A_t))\ 100$ where. A_m and A_t correspond to the radioactivity in the supernatant solution (in cpm/ml) before and after adding the ghosts at t min, respectively, V_m = volume of tracer solution added, V_t = volume of ghost suspension plus tracer solution. Ghost haematocrit was also determined by microcentrifugation (Micro-Ht) and by assessing the ghost haemoglobin concentration (Hb A). The Table shows ghost haematocrit at zero time (part a) and during incubation with ^{45}Ca (part b). Results from a single experiment are presented.

Part a

Cells loaded with	Pre-incubation	Haematocrit (%)				
		^{45}Ca (a)	Micro-Ht (b)	Hb A. (c)	(a − b)	(a − c)
22 μM free Ca	None	44 9	43.0	42.8	1.9	2 1
	1 h, 37°C	43.6	43.8	44.0	−0.2	−0.4
2 mM total Ca	None	46 3	44.0	43 9	2.3	2.4
	1 h, 37°C	46.7	44.0	43 9	2.7	2.8

Part b.

Cells loaded with	Pre-incubation	Haematocrit (%) after incubating for t (min) at 37°C with ^{45}Ca					
		0	2	4	6	15	30
22 μM free Ca	None	44.9	46.3	46.3	44.9	–	–
	1 h, 37°C	43.6	42.4	43.6	43.6	44.9	43.6
2 mM total Ca	None	46.3	47.7	47.7	46.3	–	–
	1 h, 37°C	46.7	46.7	45.8	44.0	45.8	46.7

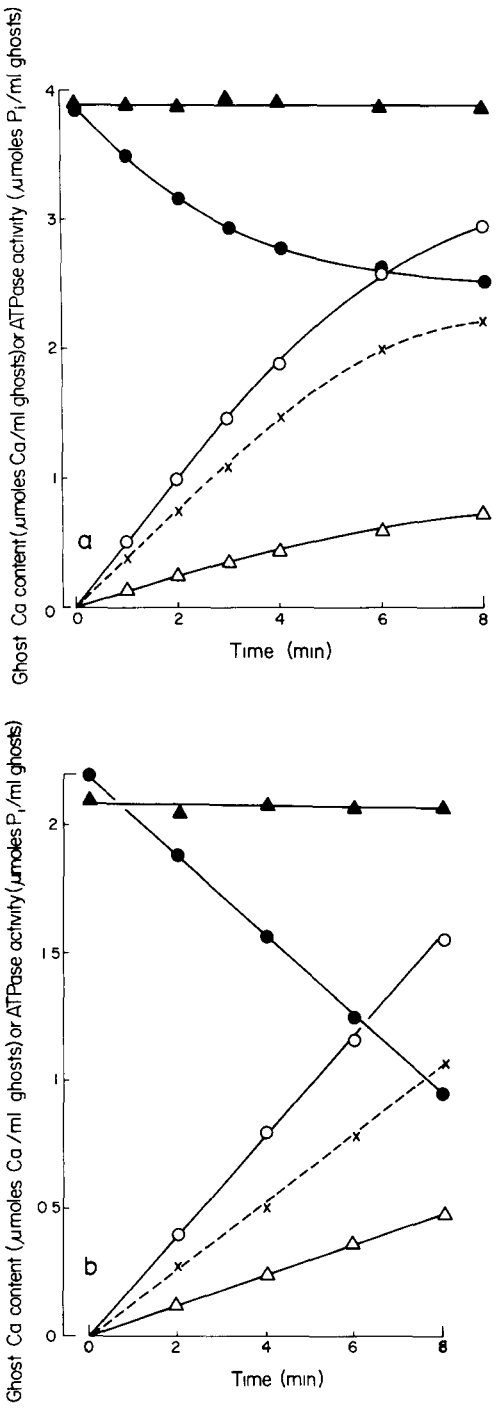


Fig 6. Correspondence between La-sensitive ATP hydrolysis and La-dependent Ca extrusion. High- and low-Ca ghosts were prepared in the presence of K as described in Fig. 5. They were incubated in a high-K medium containing 0.17 mM ouabain, with and without $LaCl_3$ (250 μM). Samples were taken at times indicated above to determine ghost Ca concentration and ATPase activity. The graphs show the Ca concentration (filled symbols) and the ATPase activity (open symbols) of low-Ca (part a) and high-Ca ghosts (part b), incubated in the presence (△, ▲) and in the absence of La (○, ●). Dotted lines represent La-sensitive ATPase activity. Results from a single experiment are shown.

that obtained by the two other methods (Table I, part a), thus indicating the absence of a fraction of ghosts readily permeable to Ca. Those ghosts that were loaded with 22 μM free Ca and pre-incubated for 1 h at 37°C, showed practically identical haematocrit values regardless of the method employed.

Virtually no change in haematocrit during incubation with ^{45}Ca was found with either type of ghosts, irrespective of whether they were pre-incubated for 1 h or not (Table I, part b). These results show that Ca permeability of ghosts loaded with either 22 μM free Ca or 2 mM total Ca, is not increased after incubating for up to 90 min at 37°C. The findings clearly demonstrate that the ghosts are tight to Ca.

$(Ca^{2+} + Mg^{2+})$-ATPase activities of dextran-resealed ghosts

It was of interest to determine the rate of ATP hydrolysis of low-Ca ghosts in view of the high pumping rate found in these ghosts. On the other hand, preceding results showed that the pumping rate can be markedly decreased by high internal Ca. ATPase activity was also studied in high-Ca ghosts to investigate if it was correspondingly altered.

Ghosts containing either a low (22 μM) or a high free Ca concentration (about 1 mM) were divided into two lots and incubated with and without La (250 μM), according to method b. One portion was used to assess active transport whilst ATPase assays were performed on the other.

As shown earlier, in the absence of La, Ca was rapidly extruded from low-Ca ghosts whilst in the presence of La active transport was halted (Fig. 6a).

As was expected, a considerable ATPase activity was obtained during incubation with no added La, amounting to about 3 μmol P_i/ml ghosts after 8 min incubation (Fig. 6a). In the presence of La, by contrast, the activity was only 0.5 μmol P_i/ml ghosts after the same period, thus indicating that La was not fully inhibitory.

In high-Ca ghosts, similarly, La fully inhibited Ca efflux but partially affected the enzymatic activity, which reached a value of about 0.4 μmol P_i/ml ghosts after 8 min incubation (Fig. 6b). On the other hand, when La was omitted, ghost Ca decreased linearly with time during 8 min incubation. Concomitantly, ATPase activity increased linearly from 0 to 1.5 μmol P_i/ml ghosts over the same period (Fig. 6b).

Either with low- or high-Ca ghosts, La-sensitive ATP hydrolysis followed a time course which was virtually identical to that of active Ca extrusion. Moreover, the magnitude of La-sensitive ATPase activity was closely correlated with the extent of active transport.

The results clearly demonstrate that there is a tight correspondence between Ca extrusion and ATP hydrolysis either at low or high internal free Ca.

Stoichiometry of active efflux

In view of the above results, it was of importance to determine if the stoichiometry of the pump remains unaltered when ghosts are exposed to high internal Ca. Ghosts containing either low or high free Ca concentrations were incubated as described in the preceding section. The ATPase activity of these ghosts is a mixture of ATP synthesis and hydrolysis since the cells were not thoroughly starved. However, for the La-inhibitable fraction the situation is not ambiguous. For this reason, the initial rates of both La-sensitive ATP hydrolysis and active extrusion were assessed on the same batch of ghosts at saturating Ca concentrations.

As was expected, the rate of extrusion (in μmol Ca/ml ghosts per min) from ghosts loaded with 22 μM free Ca was about 0.32 and was decreased to about 0.15 when ghosts were loaded with 2 mM total Ca (Table II). Concomitantly, te rate of ATP hydrolysis was reduced from 0.34 to 0.12 μmol P_i/ml ghosts per min under the same conditions.

The ratio of La-sensitive Ca efflux to La-sensitive ATP hydrolysis, was not different in low- or high-Ca ghosts, being about 0.94 and 1.18, respectively. These results demonstrate that the stoichiometry of the pump is not altered by exposure to high free Ca concentrations.

The effect of some cations on pumping rate

Early work has shown that the $(Ca^{2+} + Mg^{2+})$-ATPase activity of human red cells, measured in the presence of ouabain, is stimulated to an appreciable extent by alkaline cations [10].

As this activity is the enzymatic expression of the Ca pump of red cells, the question arises as to whether such cations have a similar effect on the pump activity.

TABLE II

THE STOICHIOMETRY OF Ca EXTRUSION FROM HIGH- AND LOW-Ca GHOSTS

Ghosts were prepared and incubated as described in legend to Fig. 6. Initial rates of Ca extrusion and ATPase activity are results from different paired experiments. Ca/P_i, initial rate of La-sensitive Ca transport and La-dependent ATPase activity

Cells loaded with.	Expt	Transport (μmol Ca/ml ghosts per min)	ATPase (μmol P_i/ml ghosts per min)	Ca/P_i
22 μM free Ca	1	0.33	0.32	1.03
	2	0.32	0.34	0.94
	3	0.30	0.35	0.86
	4	0.35	0.37	0.95
	5	0.31	0.34	0.91
		0.32 ± 0.019	0.34 ± 0.018	0.94 ± 0.062 *
2 mM total Ca	1	0.14	0.12	1.17
	2	0.15	0.12	1.25
	3	0.16	0.15	1.07
	4	0.13	0.11	1.18
	5	0.15	0.12	1.25
		0.15 ± 0 011	0.12 ± 0.015	1.18 ± 0.074 *

* $P > 0$ 10.

To test this possibility, cells were lysed in the presence of equimolar amounts of Mg and ATP, and different free Ca concentrations ranging from 5 to 22 μM. Isotonicity was restored with either Na, choline or Mg and ghosts were incubated with external Ca according to method b, in an isosmotic medium of the same cation used for resealing.

Raising free Ca from about 1 to 6 μM in the presence of Na or choline, brought about a progressive increase in transport rate up to saturating levels, reaching a maximum value of 0.13 μmol Ca/ml ghosts per min, which is about one third of that obtained in a high-K medium (Fig. 5b). The apparent K_{Ca}, estimated from the graph, was about 2 μM.

When Mg was the main cation, by contrast, activation of the pump occurred at higher Ca concentrations (4 μM), saturating at about 10 μM with a rate not much different from that found in the presence of Na or choline. The apparent K_{Ca} was slightly increased to nearly 5.4 μM.

These findings taken together suggest that active Ca transport is markedly stimulated by K but apparently not affected by Na. They also show that a high Mg concentration has a marked inhibitory effect at low internal Ca.

Sideness of action of alkaline cations

Contrary to expectation, the preceding experiment showed that Ca extrusion is unaltered when Na is replaced by choline. The lack of effect of Na appears conflictive since both Na and K activate the (Ca^{2+} + Mg^{2+})-ATPase of human erythrocytes [10]. The possibility then rises that Na^+ ions may have different effects on Ca transport depending on which side of the membrane they are present.

In order to investigate any possible asymmetric activation by the above ions, high-K ghosts were resealed to alkaline cations, as described in Methods. The ghosts were then incubated with K^+, Na^+, Mg^+ or choline$^+$ as main external cations. After this, they were lysed and analysed for Na, K and Ca. The ghost concentration of these ions was referred to the initial ghost volume in order to correct for volume changes occurring during incubation. The Na and K concentration was used to assess the degree of resealing to alkaline cations.

Virtually no Na entry was obtained after 6 min incubation in a high-Na medium. By contrast, K ions

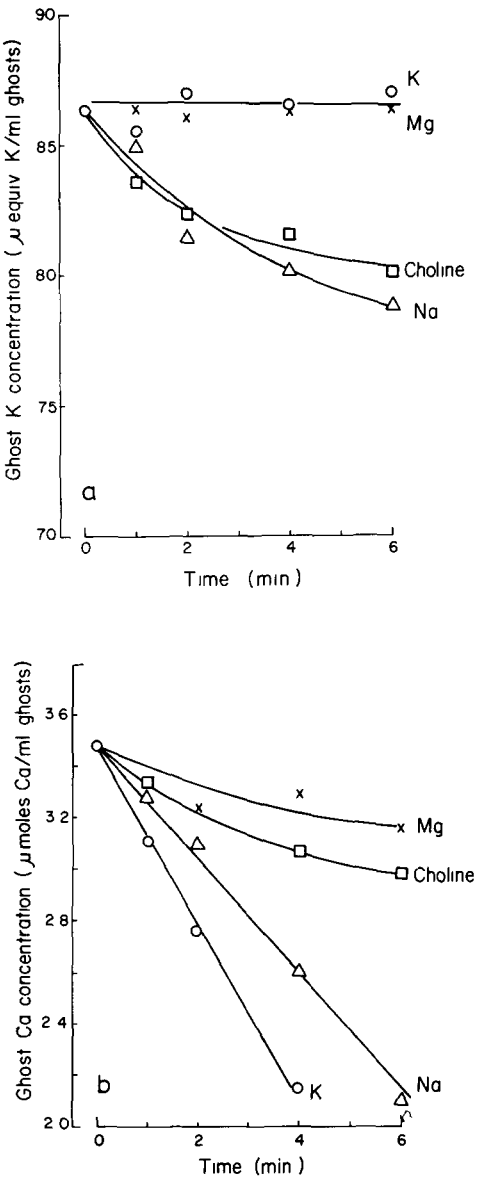


Fig 7 Alteration of K and Ca contents of ghosts resealed to alkaline cations. High-Ca ghosts, which had been loaded with both ATP and an excess of Ca over EGTA, were resealed to alkaline cations by pre-incubating for 30 min at 37°C in a high-K medium, containing 4 mM inorganic phosphate; 5 mM adenine and 10 mM inosine. After this, they were washed and incubated in fresh media of the different cations indicated in the graph, in the presence of 5 mM Ca, 5 mM adenine and 10 mM inosine Samples were taken at the times indicated above to determine the concentration of K (part a) and Ca (part b) in the ghosts. Results from a single experiment are presented. The free Ca concentration of ghosts before incubation was 18 μM.

leaked out from the ghosts at rates which depended on the composition of the external medium. Thus, a moderate K loss of about 12 μequiv./ml ghosts in 6 min occurred after incubation with Na^+ or choline$^+$, whilst practically no K was lost if these ions were replaced by Mg^{2+} (Fig. 7a). In addition, K loss was not linear with time, thus suggesting the presence of ghosts fractions with different permeabilities towards K.

Although a low Na permeability was recovered by the ghosts, the results demonstrate that K permeability remains altered after resealing. This may be a consequence of Ca binding to the K channel. As the haematocrit is 5%, the maximal rise in external K would be about 0.6 mM under conditions were the maximal K loss occurs. Therefore, it can be assumed that a large K gradient is retained.

Active Ca efflux from these ghosts was markedly altered by the composition of the external medium. A large extrusion occurred in the presence of Na or K, whereas their replacement considerably reduced Ca efflux. Thus, ghost Ca (in μmol/ml ghosts) was decreased from about 3.5 to 3.1 after 6 min incubation with Mg or choline and further diminished to nearly 2.1 by incubating in the presence of Na over the same period (Fig. 7b). In the presence of K, ghost Ca was also decreased to about 2.1 but within a shorter incubation time.

The maximal rate of extrusion (±1. S.D.) obtained in 5 experiments with external Na or K was 0.19 ± 0.017 and 0.36 ± 0.036 μmol Ca/ml ghosts per min, respectively.

The above results clearly show that in high-K ghosts, the Ca pump is stimulated by external Na or K, the latter ion being more effective. In addition, the results suggest that Na only activates from outside, since no effect above that of choline is found in ghosts which had not been resealed to alkaline cations.

Discussion

By lysing and resealing in the presence of dextran, the original content of small solutes and ions within human erythrocytes can be altered to a greater extent than that of proteins. Dextran presumably prevents the formation of large haemolytic holes by adhering to the membrane surface, thus reducing the rate of protein loss during osmotic lysis. The ghosts obtained in this way are nearly as heavy as unlysed cells and they retain some of the important enzymes for ATP synthesis. These characteristics have facilitated some studies on the Ca pump, which are discussed below.

Influence of metabolism on active extrusion

Ca extrusion was stimulated to different extents by the various substrates tested. The degree of stimulation was markedly dependent on the capacity of ghosts of synthetising ATP from the particular substrate and hence, on the actual ATP concentration. In keeping with this view, two different maximal rates of transport can be suggested from the linear decrease with time of ghost Ca found during the first 5 min incubation with inosine or adenine plus inosine (Fig. 3). Such a result is in accordance with the biphasic ATP activation curve of Ca efflux described for human red cells [11].

On the other hand, when Ca efflux was supported by the above substrates, the extent of activation varied inversely with intracellular Ca concentration. This behaviour is expected from the well known inhibitory effect of Ca on glycolysis [8]. The above finding stresses the importance of ATP synthesis as rate limitant of active transport in ATP-deficient cells when substrates other than ATP are metabolized.

Activation of Ca efflux by ATP

The greatest stimulation of the Ca pump was obtained when ATP was incorporated into low-Ca ghosts. The maximal rate was 0.340 μmol Ca/ml ghosts per min, which is about 1.7-times the maximal value reported in the literature [4].

It is very unlikely that the active efflux was overestimated in the present work, for the following reasons: first, ghosts were not washed after incubation and besides, they were tight to Ca. Secondly, the incubation was ended by adding the ghost suspension to an ice-cold medium, which contained 2 mM $LaCl_3$. Under these conditions, the pump was rapidly inactivated and the efflux time reduced to the experimental incubation period.

The reasons for an increased pump activity in our experimental conditions may perhaps, be attributed to the use of dextran.

It is known that dextran markedly reduces the loss of Hb that occurs during osmotic lysis [3] and it is

likely that the loss of other proteins and smaller polypeptides is similarly affected. The activation of Ca extrusion by inosine or adenine plus inosine supports this idea.

In the above context, an activator [12,13] and an inhibitor of the Ca pump [14,15] have been both described in the human erythrocyte cytoplasm. Interplay of these effector molecules seems to regulate pump activity in a complex way [16]. It seems possible that the increased pumping rate found in the presence of dextran can be due to a greater retention of calmodulin, a selective elution of the inhibitor or both.

On the other hand, it has been shown that Ca uptake by inside-out red cell membrane vesicles is markedly diminished as the ratio of soluble cytoplasmic proteins to erythrocyte membrane proteins is increased beyond an optimum value [15]. It is thus expected that by diluting the original cell content upon lysis, a higher Ca transport rate may be obtained with ghosts, as suggested by Sarkadi et al. [15].

The same authors have found that Hb shifts to higher protein ratios the whole activation-deactivation curve of Ca uptake by inside-out vesicles. Concentrations of cytoplasmic proteins otherwise inhibitory of Ca transport become stimulatory in the presence of Hb. The greater amounts of residual Hb retained by dextran-resealed ghosts, may perhaps explain the differences in maximal transport rates encountered with other ghosts preparations [5,17, 18].

The magnitude of active transport

The maximal efflux rate found in the present work is equivalent to 20.4 mmol Ca/l ghosts per h, which is between 2000–20 000 times greater than the passive entry [19]. The question arises what the reasons of a powerful extrusion mechanism might be when the influx of the transported ions is virtually negligible.

Red cells aged in vitro are more permeable to Ca than fresh cells [20] and it seems possible that cells aged in vivo may be also more permeable to Ca. The presence of a powerful extrusion mechanism would guarantee the survival of the mature cell by maintaining very low levels of free Ca and preventing the opening of the K channel [21].

As the red cell ages, however, the increasing activation of the pump would finally lead to ATP depletion since the pump V would exceed the glycolytic ATP synthesis rate. This situation would lead to a raised internal Ca, which in turn would markedly reduce cell viability and facilitate sequestering and destruction of senescent cells. The Ca pump thus seems to have a paradoxical existence in red cells.

Activation of the pump by internal Ca

As reported for other ghosts preparations [16], both the maximal extrusion rate and apparent K_{Ca} depend on internal free Ca. Thus, in low-Ca ghosts the apparent K_{Ca} is about 2.7 μM. This value is nearly similar to that reported by Schatzmann [5] for red cell ghosts or Ferreira and Lew [4] for intact erythrocytes, thus indicating that the pump affinity for Ca is not altered by dextran.

On the other hand, in high-Ca ghosts the maximal efflux rate is virtually a half of that found in low-Ca ghosts and the apparent K_{Ca} is increased 100-fold, thus reaching the estimate of Sarkadi et al. [22] in intact red cells. Although allowances for imprecision of the calculation of internal free Ca in high-Ca ghosts have to be made, the kinetic parameters of the pump in these ghosts are still very different from those obtained in low-Ca ghosts.

The reasons for such differences are not known. Perhaps, an interaction of calmodulin with the Ca pump is involved. Calmodulin binding to human red cell membranes increases both the affinity for Ca and the maximal activity of the $(Ca^{2+} + Mg^{2+})$-ATPase [23,24]. Under such conditions, the apparent K_{Ca} is shifted by a factor of nearly 10 and the Hill coefficient of the activation curve is increased from 1 to about 2 [24].

In the present work, the K_{Ca} values found between low- and high-Ca ghosts differ by a factor of 100 and a very small change in the Hill coefficient was observed. Similar qualitative results were obtained by Niggli et al. [25] upon adding calmodulin, when the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocytes was reconstituted into phosphatidylcholine liposomes. In this case, an increase in free Ca concentration to about 500 μM nearly completely abolished activation by calmodulin. These effects, however, were absent when the enzyme was reconstituted into phosphatidylserine liposomes.

The above observations suggest that dissociation

and association of calmodulin to the pump does not entirely account for by the difference in the kinetic parameters encountered in the present work.

The morphological changes associated with a raised internal Ca may be also related. Intact cells or ghosts reduce their volume when loaded with low Ca concentrations and indentations (spicles) appear in their membrane, thus giving a crenated aspect to the whole cell [26,27]. A reduction in cell volume will increase the concentration of cytoplasmic soluble proteins. This would alter the soluble to membrane proteins ratio, which in turn may reduce the pumping capacity of crenated ghosts, as suggested previously.

At higher Ca concentrations, the crenation process becomes more acute and spicles may be lost from the membrane. If these membrane fragments contain Ca pumps, such a process may lead to a reduction in the cell pumping capacity. The apparent K_{Ca}, however, will not be altered.

An increase in K_{Ca} may be obtained if the access of Ca to the pumping sites is limited by a diffusion barrier. Thus, the permeability of the barrier would determine the rate of entry to the compartment from where Ca would be actively expelled.

$(Ca^{2+} + Mg^{2+})$-ATPase activity and pump stoichiometry

Ca extrusion corresponded very closely with $(Ca^{2+} + Mg^{2+})$-ATPase in low- and high-Ca ghosts. Moreover, La did not inhibit fully ATPase activity, although it completely stopped active extrusion, thus showing the presence of a La-resistent (Ca + Mg)-ATPase, as reported by others [16]. The extent of inhibition did not depend on internal Ca and was about 70–80% at 250 μM La, which is a value slightly higher than that obtained with intact cells [22].

The above findings support the view that the ghosts are tight to La, as must certainly be the case as they are tight to Ca. By relating the initial rate of La-sensitive ATP hydrolysis to the corresponding rate of extrusion, a valid estimate of pump stoichiometry can be made. The value obtained was close to 1, regardless of whether it was estimated in the presence or absence of EGTA. This value confirms other reports [5,11,18].

Cooperativity in the transport of Ca

As expected from previous work (see Ref. 16), the activation of the pump by Ca followed a sigmoid curve, with a Hill coefficient [28] of about 2. This result indicates cooperativity and suggests the involvement of two Ca^{2+} ions, in accordance with the findings of Ferreira and Lew [4].

The above results, taken together with those on pump stoichiometry, seem to indicate that the pump possesses two different sites for Ca one which activates the transport mechanism when it becomes occupied and an other which is directly involved in the translocation of the ion, as suggested by Schatzmann and Roelofsen [29].

Effect of the ionic composition on Ca extrusion

The stimulation by alkaline cations of active Ca transport and/or $(Ca^{2+} + Mg^{2+})$-ATPase has been reported earlier for membrane vesicles from rabbit skeletal muscle [30], dog heart [31] and human red cells [15,32]. We have confirmed and extended such findings to ghosts resealed in dextran solutions.

It was found that active extrusion was markedly affected by the ionic composition of both internal and external medium. Thus, the greatest efflux rate was observed when a high-K concentration was bathing both sides of the membrane. Full replacement of K by Na, Mg or choline considerably decreased the efflux rate. However, external Na was effective in enhancing Ca efflux from ghosts which had been resealed to alkaline cations, whilst choline or Mg showed virtually no effect.

These results show first, that K is required on both sides of the membrane for maximal stimulation of the Ca pump. Secondly, Na must be added from outside to activate Ca transport above the level obtained with Mg or choline.

The magnitude of the Na-stimulation was lower than that observed with external K. This is the same order of activation of the $(Ca^{2+} + Mg^{2+})$-ATPase of human red cells by Na and K [10,33]. Such observation suggests that the (Na, K)-dependent enzymatic activity is directly involved in active Ca efflux from human erythrocytes.

The above findings may explain the earlier observation that K activates the $(Ca^{2+} + Mg^{2+})$-dependent *p*-nitrophenyl phosphatase of human red cells [34–36]. This activity is regarded as the last step in a sequence of reactions and seems to point a role for K in the dephosphorylating step.

In such a context, it is of interest to mention that the activating effect of K and Na or the lack of action of Li on the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte membranes, is closely corresponded by an identical action of these ions on the inhibition by vanadate of the same enzyme [37,38]. As has been suggested that vanadate binds to the dephosphorylated E_2 form of the enzyme, thus blocking its conversion to E_1 [38], the above observation seems to support the idea that Na^+ and K^+ stimulate the overall ATPase reaction at the E_2-P_i to $E_2 + P_i$ step.

The effect of alkaline cations on both $Ca^{2+} + Mg^{2+}$-ATPase and Ca transport was studied recently in human red cell ghosts [39]. Unfortunately, the differences found between transport rates with Na or K were rather small and it seemed indifferent on which side of the membrane these ions were present.

The experiments reported in this work, by contrast, have clearly shown an asymmetrical effect of Na, whereby it becomes uneffective from inside the ghosts. Such finding is also conflicting with those reported by Sarkadi et al. [32]. The authors showed that Ca uptake by inside-out vesicles is activated to the same extent by Na or K. It is uncertain, however, whether these vesicles were tight to alkaline cations. Therefore, an activating effect of Na from inside inside-out vesicles cannot be ruled out under these conditions.

Similar studies made earlier on non-inverted membrane vesicles from skeletal muscle [30] or dog heart [31] have also shown differences between the effects of Na or K. Thus, with the latter preparation, K^+ ions caused a 3–5-fold increase in the initial velocity of Ca uptake whilst Na^+ ions were less effective than K^+, but more effective than Mg^{2+}, which is practically the same activation order obtained with ghosts.

More work is needed to fully characterize the sideness of action of alkaline cations on the Ca pump of human erythrocytes.

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