Biochimica et Biophysica Acta, 942 (1988) 65–72
Elsevier

BBA 74026

Comparison of the active calcium extrusion, calcium buffering capacity and ATPase activity in rabbit reticulocytes and mature red cells

Miguel Lucas, Rosario Mata and Antonio Romero

Departamento de Bioquímica, Hospital Universitario, Facultad de Medicina, Sevilla (Spain)

(Received 11 January 1988)

Key words: Calcium ion extrusion; Calcium buffering; ATPase; Reticulocyte; Erythrocyte; (Rabbit blood)

The purpose of the present work was to study the changes in the pattern of calcium homeostasis following the loss of intracellular organella during red cell maturation. Reticulocytes and mature red cells were prepared from anaemic rabbits blood after daily bleeding. Experimental protocols were designed to study the calcium buffering capacity in intact and digitonin-disrupted cells, the calcium pumping rate and, the Ca^{2+}-translocating ATPase activity in the aforementioned red cells subpopulations. In digitonin-disrupted cells, a vesicular calcium pool, sharing the properties of mitochondria, could be detected in reticulocytes but no in mature red cells. Calcium content and calcium buffering capacity were significantly lower in reticulocytes than in mature red cells. The pattern of active calcium extrusion was quite similar in the two cell subpopulations, although reticulocytes had somewhat higher calcium affinity. Besides, an estimation of the calcium pumping rate gave higher values in reticulocytes than in mature erythrocytes. These values were 21 and 9 mmol/l cells per h, respectively. Maximal activities of the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase and basal Mg^{2+}-ATPase were significantly higher in reticulocytes than in mature red cells, but no differences were observed regarding calcium affinity. The results show that changes in the properties of the Ca^{2+}-translocating ATPase and intracellular calcium buffering systems are mechanisms involved in the process of red cell maturation.

Introduction

During the maturation, reticulocytes lose their intracellular organella leading to mature erythrocytes which are maintained virtually free of calcium due to the low calcium permeability and the powerful calcium pumping ATPase. Changes in membrane transport properties occur during this maturation process affecting active sodium and potassium transport and passive calcium permeability [1,2,3].

There are suggestive reports pointing out that the ($Ca^{2+} + Mg^{2+}$)-ATPase of plasma membrane decreases in the course of reticulocyte maturation [4,5]. A remarkably low maximal ($Ca^{2+} + Mg^{2+}$)-ATPase activity in the presence of calmodulin described in reticulocytes [6] seems inconsistent with this. However, neither the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase nor the active extrusion of calcium by the pumping system have been properly studied in reticulocytes.

Lew and Garcia-Sancho [7] have reported heterogeneous Ca^{2+} pumping among red cells per-

Abbreviations: EGTA, ethyelene glycol bis(β-aminoethyl ether)*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: M. Lucas, Departamento de Bioquímica, Hospital Universitario, Facultad de Medicina, 41009 Sevilla, Spain.

meabilized to Ca^{2+} by the divalent cation ionophore A23187. Striking differences in the calcium and ATP content of red cell subpopulations at steady state have been described which may be due to factors affecting active Ca^{2+} extrusion from the cells (Garcia Sancho and Lew, personal communication).

The purpose of the present work was to study the possible differences between reticulocytes and mature red cells regarding the patterns of calcium buffering and calcium translocating ATPase, undertaken to get further insight into the mechanism of maturation of red blood cells. With this in mind, we applied the A23187 permeabilization procedure [8] in combination with the use of cobalt [9] to study the active extrusion of calcium by subpopulations of red blood cells from rabbits with reticulocytosis provoked by daily bleeding. Here, we describe different patterns of calcium homeostasis, ATP concentration and active calcium-extrusion between reticulocytes and mature erythrocytes. Besides, concerning the high-affinity Ca^{2+}-ATPase, we observed striking differences among the ghosts prepared from the studied red cell subpopulations of anaemic rabbits.

Materials and Methods

Reticulocytosis was induced in rabbits of 3–4 kg by daily bleeding, about 50 ml each day, from the ear vein. Fractionation of red cells was according to density by the procedure of Rapoport et al. [10]. In brief, 50 ml of blood with 20–40% reticulocytosis were washed three times with 5 volumes of 0.9% NaCl. The red cells were resuspended at 50% haematocrit in 0.9% NaCl and sedimented at about $2000 \times g$ for 30 min. The buffy coat was carefully removed during the washing steps and the last centrifugation. The top 15% (F1) and the bottom 10% (F4) of the cell column were collected and washed again with the adequate buffer for the purpose of separate experiments. F1 contained 60–100% reticulocytes, whereas in F4 the reticulocyte content was below 4%.

Calcium flux was assayed with $^{45}CaCl_2$ by either centrifugation or filtration procedures. For the centrifugation method, aliquots of the cell suspension were transferred to Eppendorf tubes containing 10 volumes of incubation medium, without the radioactive tracer, and centrifuged at approximately $10\,000 \times g$ for 30 s. The supernatant was discarded and the top of the pellets dried by carefully touching with a filter paper stick. The cells were lysed in water, the proteins precipitated with 0.3 M perchloric acid and aliquots of the clear supernatant used for counting in a β spectrometer. The uptake of radioactive calcium was also measured by filtration through filters previously soaked with non-radioactive incubation medium. At specified time intervals, aliquots were removed and filtered under vacuum conditions. The filters were washed once with 10 ml of non-radioactive medium, dried and afterwards dissolved in the scintillation coktail. The type of filters were glass fiber for intact red cells experiments and MA Millipore 0.45 μm pore size for disrupted cells. Since there were substantial differences among the protocols of different experiments, the specific incubation medium and protocols are given in the figure legends for each experiment.

ATPase activity was assayed by measurement of the inorganic phosphate released from ATP under the conditions indicated in each experiment. Incubations were stopped with 0.6 M perchloric acid and inorganic phosphate was determined in aliquots of the perchloric extract with 1 ml of a mixture consisting 0.6 M H_2SO_4, 0.5% (w/v) ammonium molybdate and 2% ascorbic acid.

Total water content of the cells was determined by gravimetry of the cell pellets before and after drying to constant weight. Extracellular volume of the cell pellets was determined with [^{14}C]saccharose as previously described [11]. The fraction of intracellular water, obtained after subtraction of extracellular volume, averaged 0.74 ± 0.06 and 0.63 ± 0.05 for reticulocytes and mature erythrocytes, respectively. ATP was measured in neutralyzed cell perchloric extracts wit the coupled hexokinase–glucose-6-phosphate dehydrogenase reactions [12].

Chemicals: ATP and Hepes were from Boehringer Mannheim; Ruthenium red and azide from Merck; digitonin from Sigma; [^{14}C]saccharose and $^{45}CaCl_2$ from Amersham.

Results

The disruption of plasma membrane by digitonin allowed to show the existence in reticulocytes of a vesicular calcium pool sharing the properties of mitochondria which could not be detected in the mature erythrocyte fraction. Under the assay conditions described in Fig. 1, the mitochondrial pool accumulated calcium up to nearly 2.5 mmol/l of cells. It may be calculated that the calcium buffering capacity of mitochondria, in digitonin-disrupted reticulocytes, shifted free calcium from 35 μM to nearly 0.3 μM. A residual calcium pool, insensitive to Ruthenium red but released by A23187, could be detected. The size of this pool was equivalent to 0.16 ± 0.003 and 0.09 ± 0.001 mmol/l of cells in reticulocytes and mature erythrocytes, respectively.

In a set of experiments, calcium uptake was assayed in intact red cells (Fig. 2). The distribution ratio of the radioactive tracer (cpm per ml cells/cpm per ml of medium) averaged 0.075 ± 0.009 and 0.079 ± 0.008 μl/μl cells for reticulocytes and mature eytrocytes, respectively, and did not change with time between the 1st and 60th min of incubation. The most striking feature regarding Fig. 2 is the significant difference in the distribution ratio of calcium after permeabilization with A23187. Intracellular calcium at equilibrium was nearly 6-times lower in reticulocytes than in mature erythrocytes. It should be pointed out that the distribution ratio did not change at higher A23187 concentrations, suggesting the existence of equilibrium conditions [6].

Since the calcium-equilibrium patterns induced by A23187 under the conditions of Fig. 2 may be conditioned by changes in cell volume, or pH, the ionic composition of the medium described by Ferreira and Lew [8], was adopted in further experiments. The experiment in Fig. 3 shows the calcium content of red cells ($[Ca_i^T]$), permeabilized with A23187, as a function of external calcium ($[Ca_o]$), calculated as described in the legend to

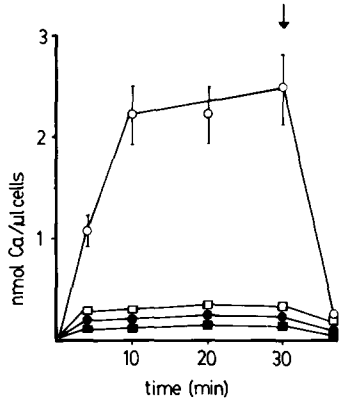

Fig. 1. Calcium transport in digitonin disrupted red cells. The washed red cell fractions were resuspended at 20% haematocrit in Na-Hepes buffer, consisting of (mmol/l): 120 NaCl, 1.2 MgCl, 5 KCl, 40 Hepes (pH 7.4). Calcium transport was assayed in red cells suspensions at a final haematocrit of 7% in a medium containing (in mmol/l) 44 NaCl, 4.9 MgCl, 1.8 KCl, 15 Hepes (pH 7.4), 0.37 EGTA, 0.37 $^{45}CaCl_2$, 7 succinate, 3.7 ATP. Circles refer to reticulocytes and squares to mature red cells, F1 and F4 fractions, respectively. Filled symbols refer to the presence of 7 μM Ruthenium red. The cells were preincubated for 5 min, and then, the tubes were supplemented with 0.14 mg/ml digitonin (zero time). At the indicated times, aliquots were removed and filtered through 0.45 μm pore size Millipore filters (see Methods). The arrow indicates the addition of 3.7 μM A23187. Results are given in nmol calcium per μl of cells. Mean $\pm$ S.E. of four separate experiments. Similar results were obtained in K-Hepes buffer, i.e. when NaCl was replaced by KCl.

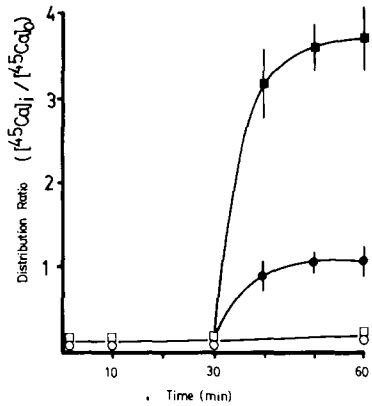

Fig. 2. Time course of calcium uptake in intact red cells. Effect of A23187. The uptake of calcium was assayed in Na-Hepes buffer (see legend to Fig. 1) supplemented with 2 mM KH_2PO_4, 5 mM glucose and 0.9 mM $^{45}CaCl_2$. At the indicated times, 100-μl aliquots were removed and processed by the centrifugation procedure, as described in Materials and Methods. At the 30th min the assay medium was supplemented with 2.1 μM A23187 (closed symbols) except in control tubes (open symbols). Circles refer to reticulocytes and squares to mature erythrocytes. Haematocrit was 16.8%. Results are given as the distribution ratio (cpm per ml cells/cpm per ml of medium). Mean $\pm$ S.E. of three separate experiments. With 1.6 mM $CaCl_2$, the results were quite similar (single experiment not shown).

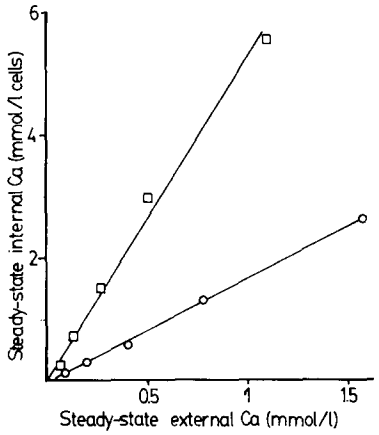

Fig. 3. Calcium content of red cells as a function of external calcium. Red cells were incubated in Tris-buffer which consisted of (in mmol/l): 75 NaCl, 75 KCl, 0.2 $MgCl_2$, 10 inosine, 10 Tris-HCl (pH 7.5). Haematocrit, 12.5%. Total $^{45}CaCl_2$ added was in the range 0.1–1.7 mmol/l suspension. A23187 was added to give a concentration of 100 μmol/l cells. After 15 min of incubation at 37 °C, 100-μl aliquots were removed and processed by the centrifugation procedure (see Materials and Methods). Total calcium content of red cells ($[Ca_i^T]$) was calculated from the distribution ratio of the radioactive tracer and the molarity of calcium in the suspension. Steady-state external calcium (Ca_o) concentration was obtained from the difference between total calcium in the suspension ($[Ca_s]$) and total calcium in the cells taking in account the haematocrit (Ht):

$$[Ca_o] = [Ca_s] - Ht \cdot [Ca_i^T]/(1 - Ht).$$

The values were adjusted by the least-squares method and gave slope values of 5 and 1.68 for mature red cells and reticulocytes, respectively. Correlation coefficients were close to 0.99 in both cases. ○, reticulocytes; □, mature erythrocytes.

the figure. Intracellular calcium and equilibrium in reticulocytes was much lower than in mature erythrocytes, over the entire range of calcium concentrations. The fraction of ionized calcium, α, could be calculated from the slopes of the curves according to the equation $\alpha = r^z\ [Ca_o]/[Ca_i^T]$, where $r^2 = 2$ (see Ref. 8), assuming that calcium distribution was at equilibrium under the present experimental conditions. The last assumption seems to be valid because of: (i) the linearity of the plots at the wide range of calcium concentrations and: (ii) the high ionophore concentration (nearly 100 μmol/l cells). The critical point is that the calculated values of α were: $\alpha = 2/5.5 = 0.36$ in mature erythrocytes and, $\alpha = 2/1.68 = 1.2$ in reticulocytes. Assuming a maximum value of 1 for α, the calcium buffering capacity, B/K_b, can be calculated from the equation (see Ref. 8):

$$[Ca_i^T] = [Ca^{2+}][(V_w/V_c) + (B/K_b)],$$

and

$$\alpha = [(V_w/V_c) + (B/K_b)]^{-1}$$

referring to: internal free calcium, $[Ca^{2+}]$; cell water volume, V_w; cell volume, V_c; the concentration of intracellular buffer of calcium, B and; the dissociation constant between Ca^{2+} and this buffer, K_b. The values obtained were: (i) for mature erythrocytes $0.36 = 1/(0.64 + B/K_b)$, and $B/K_b = 2.1$; (ii) for reticulocytes $1 = 1/(0.74 + B/K_b)$, and $B/K_b = 0.26$. These considerations support the idea of a possible defective calcium buffering capacity in reticulocytes as compared to mature erythrocytes. Alternatively, reticulocytes display a higher calcium pumping rate which in turn could hinder the accesibility of calcium to the low-affinity calcium buffering system.

In a set of experiments, the active calcium extrusion through the calcium pump was studied after the addition of either cobalt or albumin to A23187 permeabilized cells, as described by Tiffert et al. [9]. Under the assay conditions described in Fig. 4, the distribution ratio of calcium at equilibrium in mature erythrocytes was nearly two times higher than in reticulocytes. Total internal calcium, Ca_i^T, approached to 81 ± 0.7 and 140 ± 4 μmol/l cells in reticulocyte and mature erythrocyte fractions, respectively. Results in Fig. 4 also show the curves of active calcium extrusion when the leak induced by A23187 was interrupted with either cobalt or albumin.

Calcium efflux patterns of Fig. 4 were further studied by linear regresion analysis of the calcium pumping rate versus internal calcium concentrations. In a first approach, a logarithmic adjustement (not shown) revealed apparent second-order kinetic. The exponents of the computed equations were 2.61 and 1.88 for reticulocytes and mature erythrocytes, respectively, indicating a quite different degreee of activation by internal calcium at the sites of the calcium pump.

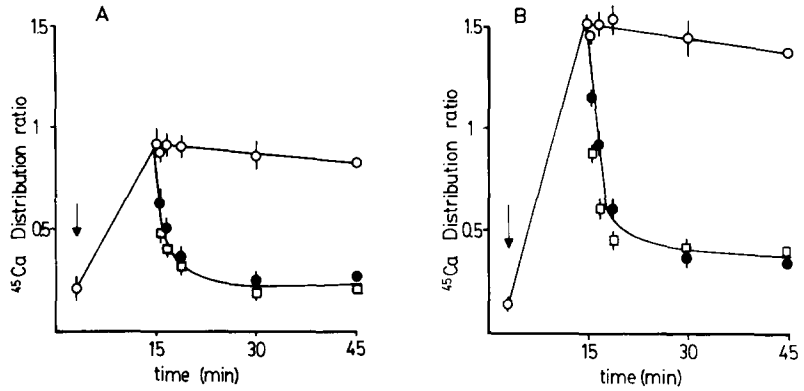

Fig. 4. Time course of the calcium-pump-mediated calcium efflux. Red cell fractions were incubated in Tris-buffer (see legend to Fig. 3) containing 89.7 μM $^{45}CaCl_2$. Haematocrit, 12%. A23187 was added (see arrow) to give a concentration of 100 μmol/l cells. At the 15th min, the incubation medium was supplemented with either 0.2 mM $CoCl_2$ (●) or 16 mg/ml albumin (■), except in control tubes (open circles). At the indicated times, 50-μl aliquots were removed and processed by filtration through glass fiber filters (see Methods). Fig. 4A refers to reticulocytes and 4B to mature erythrocytes. The results, given as the distribution ration, are the mean ± S.E. of seven separate experiments, except for the 60th min aliquots (two experiments). The filtration procedure was chosen to allow the frequency of sampling after the addition of cobalt and albumin.

The calcium pumping rate, ϕ, fitted also rather well with the exponential of internal calcium, and can be expressed by an equation of the form: $\phi = A \exp(K [Ca_i^T])$, where A and K refer to constants of the linear regression given by the derived equation: $\ln \phi = \ln A + K[Ca_i^T]$. From the slopes of the curves it can be deduced that the calcium pumping rate in mature red cells was significantly lower than in reticulocytes by nearly 60%, on the basis of internal calcium concentrations. Besides, an approximation to the calcium pumping rate at equilibrium, may be obtained by introducing the values of internal calcium before the addition of cobalt (15th min in Figs. 4A and 4B) in the corresponding equations of Fig. 5. The calculated values were 21 and 9 mmol/l cells h for reticulocytes and mature erythrocytes, respectively.

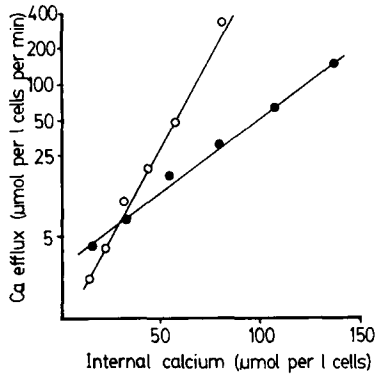

Fig. 5. Calcium pumping rate as a function of internal calcium. The slopes of the net calcium-efflux curves of Fig. 4 after addition of cobalt were plotted against total internal calcium. The equations which fit the experimental points are: $\phi = 0.7 \exp(0.077 [Ca_i^T])$, in reticulocytes (open circles); $\phi = 2.79 \exp(0.029 [Ca_i^T])$, in mature erythrocytes (filled circles). The parameters of the equations were computed by linear regression analysis of $\ln \phi$ against $[Ca_i^T]$. Correlation coefficients were > 0.99.

TABLE I

ATP LEVELS IN RETICULOCYTES AND MATURE ERYTHROCYTES: EFFECT OF A23187, COBALT AND ALBUMIN

Red blood cells were incubated at 37 °C at a haematocrit of 18% in a series of tubes with or without 12 μM A23187. After 15 min, the tubes containing A23187, but one, were supplemented with either 0.2 mM $CoCl_2$ or 16 mg/ml albumin. Incubations were stopped 30 min later with 0.6 M perchloric acid. ATP was determined as described in Methods. F1 refers to reticulocytes and F4 to mature erythrocytes. Results, in μmol ATP/ml cells, are the mean ± S.E. of four separate experiments.

Additions	F1	F4
None	1.55 ± 0.15	1.48 ± 0.14
A23187	0.53 ± 0.08	0.18 ± 0.05
A23187 and cobalt	0.94 ± 0.08	0.39 ± 0.05
A23187 and albumin	0.81 ± 0.03	0.43 ± 0.02

ATP levels fell in the presence of the Ca^{2+}-ionophore to nearly 35% and 12% of the initial values in reticulocytes and mature erythrocytes, respectively. The drop was from quite normal values (1.5 mmol/l cells). The addition of either cobalt or albumin led, after 30 min incubation, to a partial recovery of ATP levels (see Table I).

The properties of the Ca^{2+}-pump were further examined by testing ATPase activity in red cell ghosts. Results in Fig. 6 show the calcium dependence. Half-maximal activity was achieved at 0.26 and 0.4 μM calcium in reticulocytes and mature erythrocytes ghost, respectively. Maximal activity was reached at about 2 μM calcium and gave values of 132 ± 10 and 40 ± 5.8 mmol P_i released in 60 min per litre of the original cell volume, in reticulocytes and mature erythrocytes, respectively. The lower activity in mature red cells was not overcome when the incubation medium was supplemented with either the cytosolic fraction obtained from the red cells lysate or pure calmodulin (not shown). The lysate of mature red cells slightly inhibited ATPase activity of reticulocytes ghosts, but the degree of inhibition was not significant. Table II summarizes the results of separate experiments designed to seek the possible contribution of ouabain- and azide-sensitive components to ATPase activity. Under the described assay conditions, a minor decrease of ATPase activity was detected in the presence of the inhibitors. It should be pointed out that the above given ATPase activities did not differ from those obtained in ghosts prepared by freezing/thawing lysis. Besides, an exhaustive washing to remove endogenous calmodulin (see Ref. 21 for method) rendered ghosts that required exogenous calmodulin for maximal stimulation by calcium ions of ATPase activity (not shown).

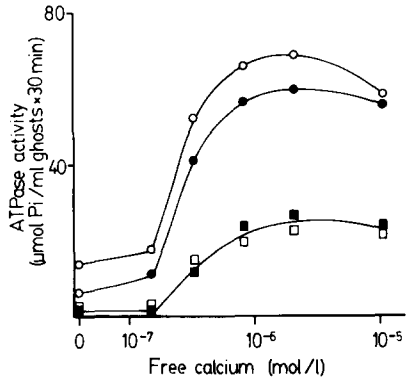

Fig. 6. Calcium dependence of ATPase activity in red cell ghosts. The red cell fractions were diluted in Na-Hepes buffer at a final haematocrit of 15% and then digitonin was added to the cell suspension to give a final concentration of 0.5 mg/ml. After 20 s the cell suspension was diluted by adding 9 volumes of a medium consisiting of 30 mM imidazole-histidine (pH 7) and 100 mM KCl. The permeabilized cells were spun down at nearly $3000 \times g$ and resuspended in the same buffer. The final volume of ghosts was brought to the original volume of cell suspension. The splitting of ATP was determined by the release of P_i (see Methods) in a medium conssisting of (in mmol/l): 100 KCl, 9 sodium azide, 0.5 ouabain, 6 $MgCl_2$, 5 ATP, 0.5 EGTA and 0.2–0.5 $CaCl_2$. Besides, te medium contained 0.05 mg/ml digitonin. The ghost concentration was equivalent to a haematocrit of 3%, on the basis of the original cell volume. Incubation was at 37 C for 30 min. Free calcium concentrations were calculated from the calcium/EGTA ratio according to Portzehl [20]. Circles refer to F1, the reticulocyte fraction, and squares to F4, the mature erythrocyte fraction. In other series of tubes, 20 μl of the supernatant obtained from digitonin-disrupted cells (H1 from F1 and H4 from F4) replaced an equal volume of water in a final incubation volume of 100 μl: ●, F1 plus H4; ■, F4 plus H1. No differences were observed amidst a number of different conditions, i.e: F1 plus H1, F4 plus H4, or the addition of saturating amounts of calmodulin (not shown).

TABLE II

ATPase ACTIVITY IN RETICULOCYTES AND MATURE ERYTHROCYTES: EFFECT OF MAGNESIUM, CALCIUM AND INHIBITORS

Red cell ghosts were prepared from reticulocyte and mature erythrocyte fractions as described in the legend of Fig. 6. Total ($Ca^{2+} + Mg^{2+}$)-ATPase activity was assayed in the presence of 6 mM $MgCl_2$, 0.5 mM EGTA and 0.4 mM $CaCl_2$; other conditions as described in Fig. 6. Mg^{2+}-dependent ATPase refers to basal activity in the absence of added calcium. Ca^{2+}-stimulated ATPase refers to the activity obtained by subtraction of basal activity from total ($Ca^{2+} + Mg^{2+}$)-ATPase activity. The calculated free calcium concentration was 1.4 μM. Where indicated as 'inhibitors', the incubation medium contained in addition 9 mM sodium azide and 0.5 mM ouabain. Results are the mean ± S.E. of 4–6 separate experiments, given as nmol P_i released in 30 min per μl of cells.

	F1	F4
Mg^{2+}-dependent	18.3 ± 2.6	4.8 ± 1.9
Mg^{2+}-dependent, inhibitors	16.8 ± 1.1	3.5 ± 0.8
Ca^{2+}-stimulated	52.1 ± 5.7	10.2 ± 0.4
Ca^{2+}-stimulated, inhibitors	43.1 ± 4.7	13.7 ± 1.8

Discussion

Maturation of the human reticulocyte produces changes in cation permeability and in particular a selective loss of calcium permeability, as studied in ATP depleted cells, although calcium content of reticulocytes does not differ from mature red cells [3]. Since calcium uptake in ATP-depleted cells measures the passive calcium permeability of energy-replete cells [3], the higher calcium permeability of reticulocytes should be counteracted by the active calcium extrusion which is achieved by the plasma membrane ATPase [13].

The results of the present work suggest a quite different behaviour between reticulocytes and mature erythrocytes regarding calcium handling. In this comparative study, three different experimental approaches were employed: (i) the measurement of intracellular calcium levels under equilibrium conditions induced by permeabilization with A23187 [8]; (ii) the measurement of Ca^{2+}-pump mediated ^{45}Ca extrusion from preloaded cells and; (iii) the measurement of plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase activity in red cell ghosts.

Concerning the cell calcium content at equilibrium, as it was determined by permeabilization with A23187 (Fig. 3), the most striking feature was the low intracellular calcium levels reached in reticulocytes as compared to mature erythrocytes. It seemed as if rabbit reticulocytes lacked the low-affinity large-capacity calcium buffer described in human red cells [8,16] and present in rabbit erythrocytes (Fig. 3, this work). The calcium buffering capacity in intact reticulocytes, as estimated in the present work, was nearly 7-times lower than in mature erythrocytes. These results contrast with the buffering capacity of digitonin-disrupted reticulocytes where, as should be expected, a high capacity calcium buffering system, sensitive to mitochondrial inhibitors and A23187, was clearly demonstrated (Fig. 1).

The uphill Ca extrusion rates at equilibrium, 21 and 9 mmol/l cells h, computed from the exponential equations are in the range of previous estimated values [7,8]. The critical point is that the calcium extrusion was through calcium-saturated pumps as can be deduced from two experiments: (i) a positive correlation was obtained (not shown) when the pumping rate, in the time course of calcium efflux, was plotted against the quotient pumping rate/[Ca] and; (ii) the calcium pumping rate was linear in time at intracellular calcium concentrations which are saturating for calmodulin [17,18] and the Ca^{2+}-translocating ATPase (Fig. 6, see also Ref. 18). It should be pointed out that higher calcium concentrations (not shown) were not inhibitory for calcium efflux neither in reticulocytes nor in mature erythrocytes. The possibility that calcium could replace magnesium at an activating site [19], under the assay conditions induced by A23187, emerges as an explanation for the lack of inhibition by relatively high calcium concentrations of the uphill Ca^{2+} efflux through Ca^{2+}-saturated pumps, should be further analyzed.

Concerning ATPase activity, the results of the present work contrast with the previously reported [6] reduction of maximal ($Ca^{2+} + Mg^{2+}$)-ATPase activity in rabbit reticulocytes. The differences could be due to the procedure by which membranes were prepared and the high calcium concentration in the assay conditions described by Cameron and Green [6]. Our results agree with the described decrease of Ca^{2+}-ATPase in the course of reticulocytes maturation [4]. Moreover, in preliminary experiments (not shown) we could obtain, after exhaustive washing, reticulocyte ghosts which were highly sensitive to exogenously added calmodulin. The salient point in the present work is that reticulocyte ghosts display a high-calcium affinity ($Ca^{2+} + Mg^{2+}$)-ATPase with maximal activity higher than in mature erythrocyte ghosts, in the presence of saturating calmodulin concentrations. The described changes in lipid composition on maturation of reticulocytes [14] could be the reason for the different pattern of ATP hydrolysis, as reported for the purified ATPase of red cell membranes [15]. However, this possibility remains to be investigated.

Whether the proposal of Garcia-Sancho and Lew (personal communication, see also Ref. 7), on the existence of red cell subpopulations with uneven calcium distribution, pumping rate and ATP content, is or is not involved in the different behaviour of reticulocytes and mature erythrocytes, is a matter of work in progress, undertaken to get further insight into the mechanism of maturation and ageing of red blood cells.

Acknowledgements

We wish to thank Dr. J. García-Sancho for helpful discussions and criticism of the manuscript. This work was supported by Grant No. 1042-84 from the Comision Asesora de Investigacion Cientifica y Tecnica.

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