DETERMINATION OF THE STOICHIOMETRY OF THE CALCIUM PUMP IN HUMAN ERYTHROCYTES USING LANTHANUM AS A SELECTIVE INHIBITOR

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1. Introduction

Since the discovery of ATP dependent calcium transport in human erythrocyte ghosts by Schatzmann [1] and its association with a calcium stimulated, magnesium dependent ATPase [(Mg + Ca)–ATPase] [1–4], few attempts have been made to determine the stoichiometry of the calcium transport system. The lack of a specific inhibitor of transport (Mg + Ca)–ATPase, such as ouabain in the case of the Na,K–ATPase associated with the Na-pump [5], made the determination of the stoichiometry difficult, since ATPase activity related to calcium transport could not be readily separated from the total ATPase activity present. Ruthenium red appeared to be a promising tool for determining the stoichiometry, as Watson et al. [6] reported that this hexavalent dye specifically inhibited (Mg + Ca)–ATPase in human erythrocyte membrane fragments without affecting the Mg–ATPase or Na,K–ATPase activities. We found that ruthenium red inhibited calcium transport to a maximum of only 60% when applied to the external side of resealed ghosts [7]. Schatzmann and Tschabold [8] reported that the lanthanides praseodymium (Pr^{3+}) and holmium (Ho^{3+}) inhibited 100% of the active calcium transport in resealed ghosts at 1 mM concentration. Weiner and Lee [9] found that low concentrations of La^{3+} also specifically inhibited Ca-activated ATPase activity in membrane fragments without affecting either Mg–ATPase or Na, K–ATPase activities.

In this communication we show that whereas 0.1 mM La^{3+} completely blocked Ca efflux when applied on the external side of resealed human erythrocyte ghosts, La^{3+} inhibited only 50% of the total Mg- and Ca-dependent ATPase activities. A stoichiometry of 2 (two calcium ions transported per mole of ATP hydrolyzed) was obtained from these experiments. The stoichiometry was the same in resealed ghosts prepared in the presence or absence of 1 mM EDTA during the hemolysis procedure.

2. Materials and methods

2.1. Preparation of ghosts

Human blood, preserved in acid citrate dextrose solution, was obtained from the Red Cross Blood Bank and used within 20 days of collection. The cells were washed twice in isotonic saline solution. The buffy coat was removed by suction with a loss of the top third of the red cells. Ghosts were prepared by a modification of the method of stepwise hemolysis described by Schrier [10]. The washed cells were consecutively hemolyzed in 10 vol of 0.08, 0.06 and 0.04 M NaCl at 2–4°C. In some experiments (see figs.) the above solutions also contained 1 mM EDTA (disodium salt). The ghosts were finally hemolyzed in 0.015 M NaCl and 0.005 M Tris–maleate (pH 7.1) at 2–4°C. One ml of the ghost preparation containing 4.5 mg protein was suspended in 3 ml of the loading medium containing (in final concentration) 4 mM $MgCl_2$, 4 mM Na_2ATP, 10 mM Tris–maleate (pH 7.1) and 3 mM $CaCl_2$. The tubes were equilibrated for 5–10 min at 2–4°C.

Resealing of the ghosts was achieved by the addition of 0.2 ml of 2.876 M NaCl and incubation for 10 min at 25°C. The tubes were returned to the ice bath and washed twice at 2–4°C with a solution containing 2

mM $MgCl_2$, 125 mM NaCl and 20 mM Tris-maleate, pH 7.1. The washed ghosts were suspended to a final vol of 3 ml with the same solution which contained, in addition, 1 mM $CaCl_2$. In some experiments $LaCl_3$ was added in the external medium for 10 min prior to incubation. The tubes were then incubated at 37°C for the required time periods.

2.2. Assay of ATPase and calcium

For determination of ATPase activity the reaction was stopped by the addition of 1 ml of 20% trichloracetic acid. Inorganic phosphate was determined by the method of Fiske and SubbaRow [11]. For determination of the velocity of Ca efflux the reaction was stopped by the addition of 6 ml of ice cold 119 mM NaCl and 6 mM $LaCl_3$, in order to displace superficially bound calcium. The pellet was washed once more in this solution. Calcium was extracted by the procedure of Sparrow and Johnstone [12] and measured by atomic absorption spectrophotometry (Techtron AA-5). The precision of triplicate determinations was found to be ±5–10% (standard error of the mean). Protein determinations were made by the method of Lowry et al. [13].

In order to determine the contribution of leaky ghosts to total ATPase activity, ghosts were resealed in the normal medium but in the absence of ATP and washed as above. The formation of P_i after the addition of 1 mM ATP to the incubation medium at 37°C was used to estimate the ATPase activity due to non-resealed or leaky ghosts. This was found to be 0.07 μmol P_i/hr/mg protein or 13% of the total ATPase activity of ghosts resealed in the presence of 3 mM calcium.

Mg–ATPase activity was determined in the absence of calcium by direct incubation of the hemolyzed cells with ATP at 37°C. These cells were leaky since calcium is required for resealing [14] (unpublished observations). The Mg–ATPase activity, which is an approximate estimate only, was found to be 0.125 μmol P_i/hr/mg protein or 24% of the total ATPase activity of ghosts resealed in the presence of 3 mM calcium.

2.3. Source of chemicals

Inorganic salts were of analytical reagent grade. Na_2ATP (Sigma) and $LaCl_3$ (Fisher) were used as received. Certified atomic absorption calcium reference solution (Fisher) was used as a standard for atomic absorption spectroscopy. Trichloracetic acid and $LaCl_3$ were analyzed for calcium contamination by the above method.

3. Results

Ouabain sensitive Na,K–ATPase was inactive in ghosts resealed with NaCl in the absence of added KCl [7] and therefore under these conditions does not contribute to total ATPase activity. Table 1 shows the effect of varying the concentration of $LaCl_3$ in the external medium on calcium efflux from resealed ghosts loaded in 3 mM $CaCl_2$. An I_{50} value of 6.0×10^{-5} M was obtained. $CaCl_2$ (1–5 mM) in the external medium did not inhibit calcium efflux or ATPase activity, in agreement with the findings of Schatzmann [14].

Fig. 1A shows the time course of calcium efflux in the presence or absence of 0.1 mM $LaCl_3$ in resealed ghosts prepared by stepwise hemolysis in 1 mM EDTA. Fig. 1B shows the same, except that the ghosts were prepared in the absence of EDTA. In the absence of $LaCl_3$, after a short lag time due to warming the tubes from 2 to 37°C, there is a rapid loss of calcium which levels off after about 14 min. The levelling off may be due to desaturation of the transport system or to endogenous membrane calcium. The rates of calcium efflux in fig. 1A and 1B, determined from the slopes of the curves between 4 and 10 min, were 0.474 and 0.440 μmol Ca/hr/mg protein, respectively. In the presence of 0.1 mM La^{3+} calcium efflux was completely abolished in both EDTA and non-EDTA treated ghosts.

Table 1
Inhibition of calcium efflux by La^{3+}. $LaCl_3$ was applied in the external medium (Materials and methods).

La^{3+} (mM)	Calcium Efflux (μmoles/hr/mg protein)	Inhibition %
0	0.474	0
0.01	0.474	0
0.03	0.368	22
0.06	0.236	50
0.10	0	100
0.50	0	100

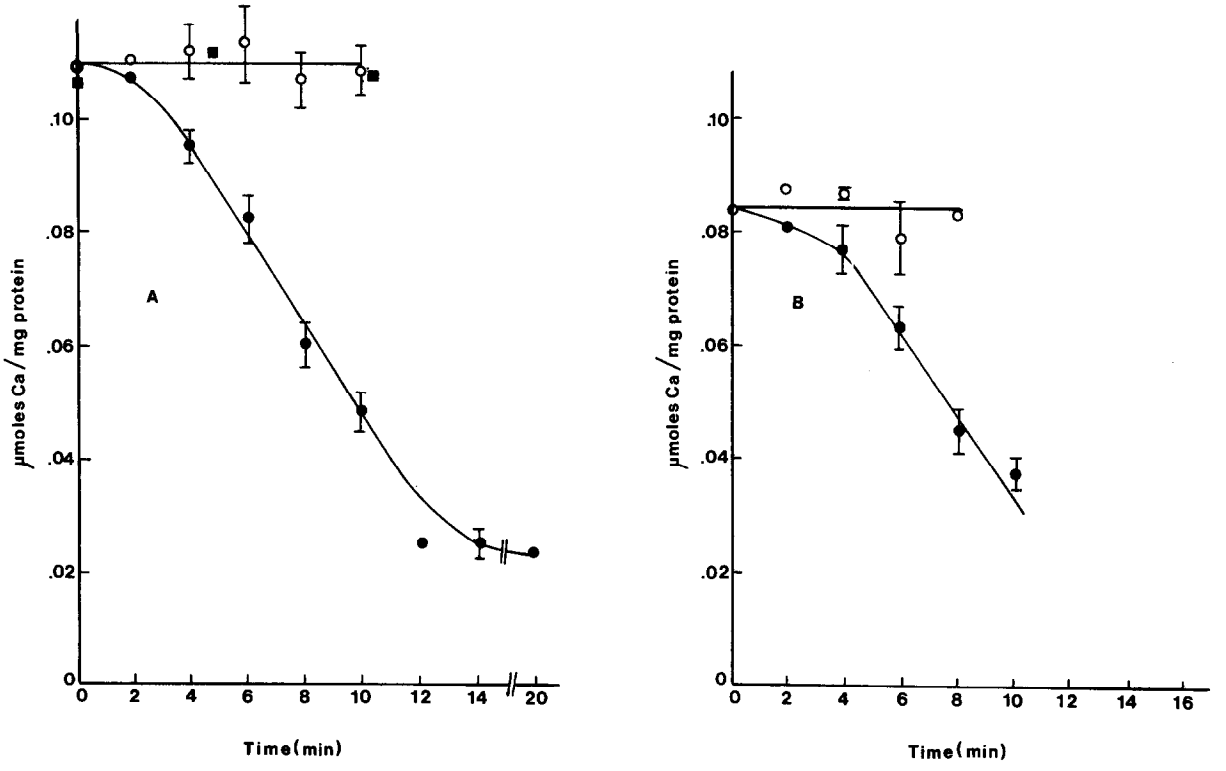

Fig. 1. Effect of La^{3+} on calcium efflux from resealed ghosts. A, 1 mM EDTA was used during the stepwise hemolysis procedure. B, in the absence of EDTA. Ghosts were loaded in 3 mM $CaCl_2$ medium as described in Materials and methods. $LaCl_3$ was applied to the external medium during incubation. The data represent means of three separate experiments, and the bars represent the standard errot of the determinations. (●–●–●), control; (○–○–○), in the presence of 0.1 mM $LaCl_3$; (■–■–■), in the absence of ATP in the loading medium.

Fig. 2A shows the time course of ATP hydrolysis in resealed ghosts prepared with EDTA, in the presence and absence of 0.1 mM $LaCl_3$ in the external medium. Fig. 2B shows the same, except that the ghosts were prepared in the absence of EDTA. After a similar brief lag time, the rate of ATPase activity increased exponentially and began to level off after approx. 14 min, similarly to the calcium efflux curves (fig. 1). The rates of ATP hydrolysis were likewise estimated by determining the slopes of the curves between 4 and 10 min. In the absence of $LaCl_3$, the rates of ATP hydrolysis in fig. 2A and 2B were 0.520 and 0.480 µmol P_i/hr/mg protein, respectively. The addition of 0.1 mM $LaCl_3$ in the external medium reduced total ATPase activity to 0.285 and 0.250 µmol P_i/hr/mg protein, respectively.

4. Discussion

The addition of 0.1 mM $LaCl_3$ to the external medium completely abolished ATP dependent calcium efflux from resealed ghosts (fig. 1). However, 0.1 mM $LaCl_3$ only inhibited total ATPase activity by 50% (fig. 2), indicating that a significant proportion of the ATPase activity is not associated with calcium transport. 50% of this remaining ATPase activity was estimated to be Mg–ATPase activity, which is not sensitive to La^{3+} at the concentrations used in this study (see also Weiner and Lee [9]). The remaining 25% of total ATPase may be associated with a (Mg + Ca)–ATPase activity not associated with calcium transport.

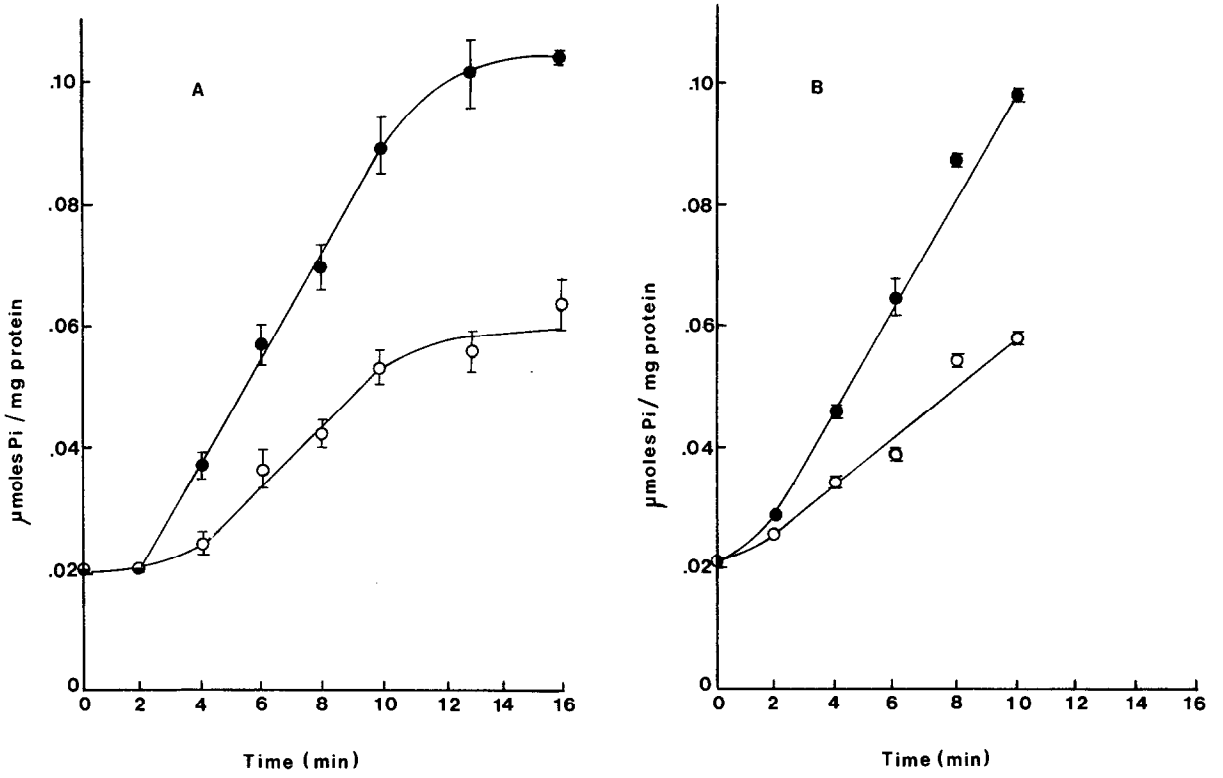

Fig. 2. Effect of La^{3+} on ATPase activity in resealed ghosts. A, 1 mM EDTA was used during stepwise hemolysis. B, in the absence of EDTA. Ghosts were loaded in 3 mM $CaCl_2$ medium as described in Materials and methods. $LaCl_3$ was applied to the external medium during incubation. The data are means of three separate experiments, ± the standard error of the determinations. (●–●–●), control; (○–○–○), in the presence of 0.1 mM $LaCl_3$.

Assuming that only the ATPase activity inhibited by La^{3+} is associated with calcium transport, the stoichiometry of the Ca transport system may be estimated. Subtraction of the rate of ATP hydrolysis in the presence of 0.1 mM $LaCl_3$ from the total ATPase activity gave rates of 0.235 and 0.230 µmol P_i/hr/mg protein in the absence or presence of 1 mM EDTA during the hemolysis procedure, respectively. These values give a stoichiometry of 2.02 and 1.91. These estimates are based on the assumption that La^{3+} selectively and completely blocks both calcium efflux and the ATPase activity associated with the calcium pump by binding on the external aspect of the cell membrane. These estimates would be low if ATPase activity other than that associated with calcium transport was inhibited by La^{3+}. Another possibility is that La^{3+} partially uncoupled calcium transport from ATPase activity, inhibiting the calcium efflux to a larger extent than the ATPase activity, thus giving a higher value for the stoichiometry. However, this possibility is less likely since La^{3+} does not completely block total calcium dependent ATPase activity even in membrane fragments (Weiner and Lee [9]), even though in this preparation La^{3+} is readily accessible to the ATPases. We have confirmed this result in membrane fragments prepared by osmotic shock (unpublished observations). Thus it appears that only a part of the total calcium dependent ATPase activity in resealed ghosts and fragments is sensitive to La^{3+}.

Recently, Schatzmann [14] concluded that the stoichiometry of the Ca pump in resealed erythrocytes was more likely 1 than 2. The reasons for the differences between the two estimates are not entirely clear. However, our estimate results from the use of La^{3+}

as a tool for inhibiting only that ATPase activity associated with calcium transport, while Schatzmann [14] corrected for Mg–ATPase activity only. Differences in the degree of functional reconstitution of leaky cells may also contribute to the discrepancy, since we have found that resealing cells to calcium is very dependent on both temperature and time (unpublished observations). Differences between the reconstitution of ghosts after one step hemolysis used by Schatzmann [3,14] and the stepwise hemolysis procedure [10] used in this study may also be important.

Recently there has been some controversy concerning the possibility that the red cell may have two Ca-dependent ATPase activities, namely, high and low calcium affinity (Mg + Ca)–ATPase(s) [15–18]. Scharf [19] and more recently Schatzmann [14] suggested that the low affinity (Mg + Ca)–ATPase in membrane fragments may be an artifact resulting from the conversion or denaturation of a high calcium affinity (Mg + Ca)–ATPase by EDTA treatment to an enzyme with lower affinity for calcium. In this study the rates of both ATPase activity and calcium efflux were comparable and similar stoichiometries were obtained in both EDTA and non-EDTA hemolyzed cells. Thus under the experimental conditions used here EDTA had no effect on either calcium transport or ATPase activity. The stoichiometry obtained here corresponds to that found for the calcium pump in sarcoplasmic reticulum [20]. Studies are now in progress to obtain further evidence for the presence and function of a magnesium and calcium-dependent ATPase activity unrelated to calcium transport. Preliminary results indicate that a high calcium affinity component of the total calcium-dependent ATPase activity can be removed by relatively mild conditions from human erythrocyte membrane fragments. After removal of this component La^{3+} is found to block calcium-dependent ATPase activity completely. Supporting the presence of two calcium-dependent ATPases, Knauff et al. [21] very recently demonstrated the possibility that two calcium-dependent phosphoprotein intermediates are formed in erythrocyte ghosts.

Acknowledgements

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