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Association of (Ca+Mg)-ATPase Activity With ATP-Dependent Ca Uptake in Vesicles Prepared From Human Erythrocytes

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Ghost membranes prepared from human erythrocytes exhibit 2 distinct (Ca+Mg)-ATPase[1] activities (Quist and Roufogalis, Arch Biochem Biophys 168:240, 1975). (Ca+Mg)-ATPase activity dependent on a water soluble protein fraction is selectively lost from ghost membranes during preparation of vesicles under low ionic strength, slightly alkaline conditions. In this study, the Ca^{2+} dependence of the remaining membrane bound (Ca+Mg)-ATPase activity and ATP-dependent Ca uptake in vesicles were compared. The Ca^{2+} activation curves for (Ca+Mg)-ATPase activity and Ca uptake into vesicles were parallel over a Ca^{2+} range of 0.3–330 μM, and both curves have 2 apparent K_A values for Ca^{2+} of 0.45 and 100 μM. Addition of a concentrated soluble protein fraction containing predominantly spectrin to the vesicles increased (Ca+Mg)-ATPase activity over twofold but did not affect the rate of Ca uptake. These findings suggest that the (Ca+Mg)-ATPase activity remaining in vesicles after extraction of the water soluble proteins is associated with the Ca pump whereas (Ca+Mg)-ATPase activity dependent on the soluble protein fraction is associated with some other function.

Key words: human erythrocytes; ATP-dependent Ca uptake; (Ca+Mg)-ATPase; spectrin; inside-out vesicles

While it is generally accepted that (Ca+Mg)-ATPase activity in human erythrocyte membranes is associated with Ca transport (1, 2), others have suggested that at least part of the (Ca+Mg)-ATPase activity may be associated with contractile-like proteins in these membranes (3, 4, 5). Recently, it was demonstrated by using $LaCl_3$ as a selective inhibitor of transport (Ca+Mg)-ATPase that only 50% of the total (Ca+Mg)-ATPase activity in resealed ghosts (6) and intact erythrocytes (7) is associated with Ca transport.

Further evidence for the presence of at least 2 separate (Ca+Mg)-ATPase activities was recently obtained in erythrocyte ghost membranes (5). Incubation of ghosts under conditions producing formation of vesicles and a loss of water soluble membrane proteins (8) resulted in a selective loss of (Ca+Mg)-ATPase activity having a K_A for Ca^{2+} of 2 μM

[1] Abbreviations: (Ca+Mg)-ATPase – Ca^{2+}-activated, Mg^{2+}-dependent adenosine triphosphate; EGTA – ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, Tris – Tris (hydroxymethyl)-methylamine; SDS – sodium dodecyl sulfate.

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(5). This activity previously referred to as high affinity (Ca+Mg)-ATPase activity is maximally activated by 5–10 μM Ca^{2+} (5, 9, 10). Addition of the water soluble protein fraction (5) containing predominantly spectrin (11) to extracted vesicles under isotonic conditions restores this (Ca+Mg)-ATPase activity. This component of (Ca+Mg)-ATPase activity is thought to be involved in the regulation of erythrocyte membrane deformability (12).

The (Ca+Mg)-ATPase activity remaining in vesicles prepared from ghost membranes has 2 K_A values for Ca^{2+} of 0.5 and 100 μM (5). This so-called low-affinity (Ca+Mg)-ATPase activity is maximally activated by 200–300 μM Ca^{2+} (5, 9, 13). In this study, we show that (Ca+Mg)-ATPase activity and ATP-dependent Ca uptake in resealed erythrocyte vesicles have similar affinities for Ca^{2+}. We also show that addition of the soluble protein fraction to vesicles increases (Ca+Mg)-ATPase activity over twofold but does not affect the rate of Ca uptake.

MATERIALS AND METHODS

Preparation of Vesicles

Whole human blood preserved in acid-citrate-dextrose was obtained from the Red Cross blood bank and was used within 20 days of collection. Ghosts were prepared from washed erythrocytes by stepwise hemolysis as previously described (6). Vesicles were prepared from only freshly prepared ghosts by a modification of the method of Steck et al. (8). One volume of ghosts (3.5–4.0 mg protein/ml) was diluted with 5 volumes of 0.5 mM NaCl or 0.1 mM EGTA and 1.0 mM Tris-maleate (pH 8.0) and incubated for 30 min at 37°C. Both procedures result in a complete disruption of ghosts to vesicles (diameter < 1 μm) as determined by phase contrast microscopy. The suspension was centrifuged for 15 min at 20,000 × g at 4°C. The supernatant containing predominantly bands I, II, and V polypeptides as determined by SDS-polyacrylamide gel electrophoresis (5, 11) was collected and concentrated sixfold by ultrafiltration and is referred to as the soluble protein fraction. The pellet consisting of vesicles was suspended to the original volume of ghosts used in 15 mM NaCl and 5 mM Tris-maleate, pH 7.1. To facilitate resealing, 1 volume of the vesicle preparation was suspended in 3 volumes of medium containing 10 mM Tris-maleate, pH 7.1, 4 mM $MgCl_2$, and 0.5 mM $CaCl_2$. After equilibration for 5 min at 4°C, isotonicity was restored with 2.9 M NaCl (4 ml to 80 ml of suspension). The suspension was incubated for 10 min at 25°C and centrifuged at 7,000 × g for 10 min at 4°C. The vesicles were washed twice with 30 volumes of 55 mM Tris-maleate, pH 7.2, 6.4 mM $MgCl_2$, and 66 mM NaCl to remove extravesicular Ca^{2+} at 7,000 × g at 4°C and suspended with the same solution to the original volume of ghosts used. One volume of this vesicle preparation was suspended in 2 volumes of 55 mM Tris-maleate, pH 7.2, at 37°C, 6.4 mM $MgCl_2$, 66 mM NaCl, and 0.15 mM EGTA. The concentration of $CaCl_2$ was varied in this mediium. In some experiments the soluble protein fraction was included to a final concentration of 0.5 mg of protein/ml.

Determination of Ca Uptake and ATPase Activity

To study Ca uptake and ATPase activity, the vesicle preparation was preincubated for 10 min at 37°C and the reaction was started by the addition of Na_2ATP (2 mM final concentration). At appropriate time intervals, 2 ml and 1 ml aliquots were removed for either inorganic phosphate (P_i) or Ca analysis, respectively. The concentration of Ca^{2+}

was calculated by equations given by Katz et al. (14) using a Ca-EGTA stability constant of of $10^{10.65}$ (15).

The Ca content of the vesicles was determined by suspending 1 ml of vesicle suspension in 8 ml of cold 110 mM NaCl and 0.5 mM $LaCl_3$. $LaCl_3$ was included to block Ca uptake (6) and to displace loosely bound extravesicular Ca (16). The tubes were centrifuged at 15,000 × g for 15 min and the supernatant was completely removed by aspiration. Further washing of the vesicles with the $LaCl_3$ solution did not lower the Ca content of the vesicles. Ca was extracted from the pelleted vesicles by the method of Sparrow and Johnstone (17) as previously described for resealed ghosts (6). Ca was determined by atomic absorption spectrophotometry (Techtron AA-5) in the presence of 30 mM $LaCl_3$.

ATPase activity in the vesicles was stopped by mixing 2 ml of suspension with 1 ml of 20% trichloroacetic acid. Inorganic phosphate (P_i) was determined by the method of Fiske and SubbaRow (18). (Ca+Mg)-ATPase activity was corrected for Mg-ATPase activity obtained in the absence of added Ca by subtraction.

Characterization of Vesicles

The proportion of resealed inside-out vesicles present was determined by measuring the fraction of latent acetylcholinesterase activity (19) in the presence and absence of 0.1% Triton X-100 as described by Steck and Kant (20). Similarly, the proportion of resealed right-side-out vesicles was found by determining the amount of latent glyceraldehyde 3-phosphate dehydrogenase activity (21). Protein concentration was determined by the method of Lowry et al. (22).

RESULTS

In the final vesicle preparation, 44–48% of the total membranes consisted of resealed inside-out vesicles (i.e., 46% of the total acetylcholinesterase activity was latent) and 39% resealed right-side-out vesicles (39% of the total glyceraldehyde-3-phosphate dehydrogenase activity was latent). The remaining activity suggests that approximately 15% of the membranes were unsealed. Inside-out vesicles were not routinely separated from right-side-out vesicles as (Ca+Mg)-ATPase activity of this vesicle preparation was lost after separation on a dextran T-110 gradient according to Steck and Kant (20).

The presence of latent acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase activities indicates that the vesicles are relatively impermeable to their respective substrates. Other observations indicate that the vesicles are impermeable to Ca and ATP. In the absence of extravesicular $CaCl_2$, there was no change in vesicular Ca content of vesicles incubated for 25 min at 37°C. EGTA (0.1 mM) in the extravesicular medium would presumably extract Ca from leaky vesicles. In the absence of added $CaCl_2$ and in the presence of 2 mM ATP in the extravesicular medium, the Ca content also does not change under the above conditions. Therefore, the right-side-out vesicles are impermeable to ATP as Ca is rapidly lost from vesicles resealed in the presence of AFP when incubated at 37°C (not shown). In agreement with Steck and Kant (20), the vesicles retain their relative impermeability as determined by latent marker enzyme activities for several days when stored at 4°C although (Ca+Mg)-ATPase activity is greatly reduced.

The Ca^{2+} dependence of ATP dependent Ca uptake and (Ca+Mg)-ATPase activity in the vesicles is compared on Fig. 1. The Ca^{2+} activation curves for Ca uptake and (Ca+Mg)-ATPase activity are essentially parallel over a Ca^{2+} concentration range of 0.1–330 μM. A plot of this data according to Eadie (23) yields curves with 2 distinct slopes with K_A

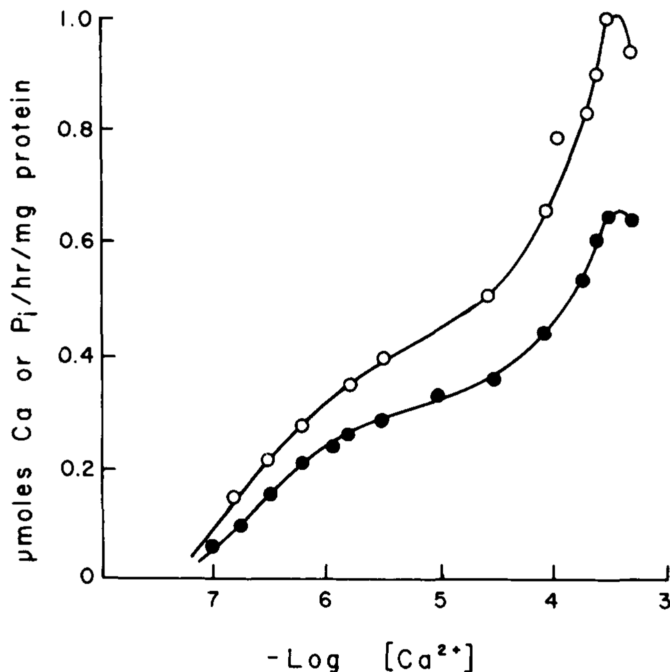


Fig. 1. Ca^{2+} dependence of (Ca+Mg)-ATPase activity (•) and ATP-dependent Ca uptake (○) in vesicles prepared from human erythrocyte ghosts.

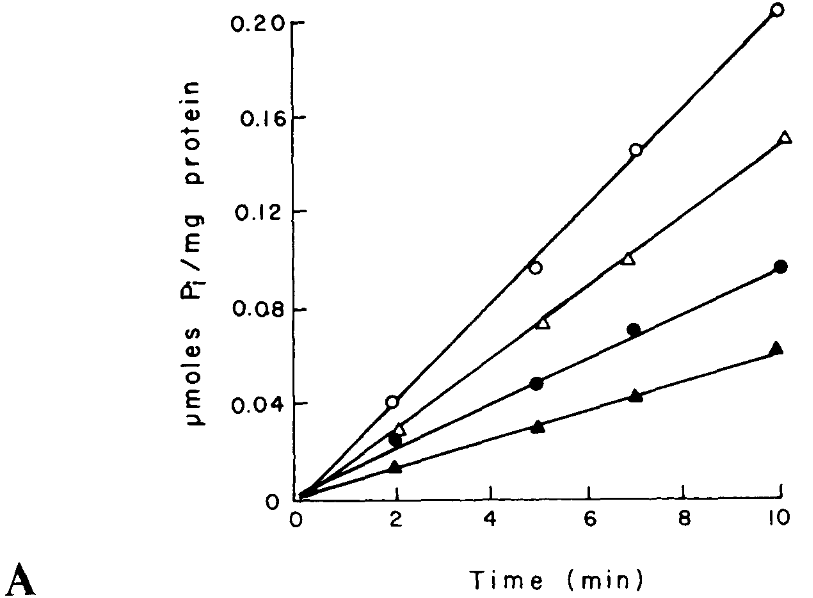
values for Ca^{2+} of 0.45 and 100 μM. Both Ca uptake and (Ca+Mg)-ATPase activity are saturated at 330 μM Ca^{2+}. In this study, Ca uptake was dependent on ATP at all Ca^{2+} concentrations studied, and Ca uptake and (Ca+Mg)-ATPase activity were linear for at least 10 min (see Fig. 2A and 2B).

The rate of Ca uptake is approximately 1.5-fold greater than (Ca+Mg)-ATPase activity over the Ca^{2+} concentration range studied (Fig. 1). The presence of unsealed membranes (15% of total membranes) may yield an apparent lower stoichiometry of 1.5 (μmoles Ca taken up/μmoles ATP hydrolyzed). Unsealed membranes can be readily calculated to contribute to 25% of the total (Ca+Mg)-ATPase activity in this vesicle preparation based on the following 2 assumptions. First, only inside-out vesicles (46%) and unsealed membranes (15%) would contribute to (Ca+Mg)-ATPase activity due to the inaccessibility of ATP to the cytoplasmic side of right-side-out vesicles (39%). Second, unsealed membranes would not likely contribute to Ca uptake as the membranes are washed with 0.5 mM $LaCl_3$ solution prior to Ca analysis (see Methods). By subtracting (Ca+Mg)-ATPase activity due to unsealed membranes from total (Ca+Mg)-ATPase activity in Fig. 1, the stoichiometry of Ca uptake becomes 2.0.

Addition of the soluble protein fraction to the vesicles increased (Ca+Mg)-ATPase activity 2.5- and 2.1-fold at Ca^{2+} concentrations of 29 and 230 μM, respectively (Fig. 2A). Under the same conditions, addition of the soluble protein fraction to the vesicles was found not to have any effect on the rate of ATP-dependent Ca uptake.

DISCUSSION

In order to determine which component of total (Ca+Mg)-ATPase activity in erythrocyte membranes is associated with Ca transport, ATP-dependent Ca uptake and



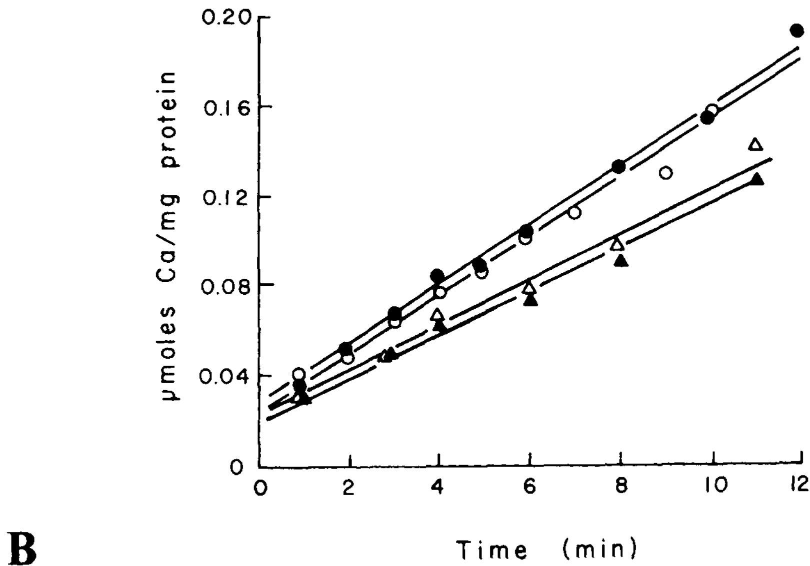


Fig. 2. Effect of the addition of the soluble protein fraction to vesicles on (Ca+Mg)-ATPase activity and ATP-dependent Ca uptake. A) Time course of (Ca+Mg)-ATPase activity in vesicles in the presence of 29 μM Ca^{2+} (▲) and 230 μM Ca^{2+} (●). Time course of (Ca+Mg)-ATPase activity after the addition of the soluble protein fraction to the vesicles in the presence of 29 μM Ca^{2+} (△) and 230 μM Ca^{2+} (○). B) Time course of Ca uptake in vesicles in the presence of 29 μM Ca^{2+} (▲) and 230 μM Ca^{2+} (●). Time course of Ca uptake after the addition of the soluble protein fraction to the vesicles in the presence of 29 μM Ca^{2+} (△) and 230 μM Ca^{2+} (○).

(Ca+Mg)-ATPase activity were compared in inside-out vesicles prepared from erythrocyte ghosts. Vesiculation requires incubation of ghosts in low ionic strength, slightly alkaline medium (8) in which the (Ca+Mg)-ATPase activity previously referred to as high-affinity activity is lost (5). The parallel nature of the Ca^{2+} activation curves for Ca uptake and (Ca+Mg)-ATPase activity and similarity of their Ca^{2+} affinities suggests that Ca transport is supported by (Ca+Mg)-ATPase activity remaining bound to the membrane after low ionic strength extraction of soluble proteins (Fig. 1). The apparent stoichiometry of 1.5 for Ca uptake in vesicles is lower than 2.0 obtained in resealed ghost (6) or intact erythrocytes (7, 24). However, correction for (Ca+Mg)-ATPase activity contributed by unsealed membranes to total (Ca+Mg)-ATPase activity raises the stoichiometry to 2.0 (see Results). The previously determined stoichiometry of less than 1 for ATP-dependent ^{45}Ca uptake into inside-out vesicles (26) might be due to leakage of Ca out of the vesicles, as the membranes were frozen prior to the ^{45}Ca-uptake studies. In our study, only freshly prepared vesicles were used and were resealed in the presence of $CaCl_2$ under conditions optimal for resealing of ghosts (6).

To determine if the soluble proteins extracted from ghosts under low ionic strength conditions contribute to ATP-dependent Ca uptake in vesicles, the concentrated soluble protein fraction was reconstituted with the vesicles. Recombination of the soluble protein fraction with the vesicles increased (Ca+Mg)-ATPase activity over twofold but did not alter the rate of Ca uptake into these vesicles (Fig. 2A and 2B). Therefore, (Ca+Mg)-ATPase activity dependent on the soluble protein fraction (5) or highly purified spectrin (25), may not be associated with the Ca pump. This finding is further evidence that high-affinity or more appropriately spectrin-dependent (Ca+Mg)-ATPase actitivy is involved in some other function such as regulation of erythrocyte membrane deformability (12).

In human erythrocyte vesicles, low-affinity or spectrin-independent (Ca+Mg)-ATPase activity is characterized by 2 apparent dissociation constants of 0.45 and 100 μM (Fig. 1). The existence of a Ca^{2+} binding site with a dissociation constant of 0.45 μM seems compatible for a Ca transport system which functions to maintain the intracellular concentration of Ca^{2+} below 1 μM (15). The physiological significance of the low Ca^{2+} affinity site (K_A of 100 μM) is less clear. This site is probably not an artifact of membrane preparation as Ca transport does not saturate until an intracellular Ca^{2+} concentration of 200 μM in intact human erythrocytes loaded with Ca^{2+} using the ionophore A-23187 (27). However, Scharff has found that the low affinity site is not apparent in ghost membranes prepared in the presence of 1.5 mM $CaCl_2$ (13). Scharff has suggested that Ca^{2+} may control the properties of membrane bound (Ca+Mg)-ATPase and that the different Ca^{2+} affinities for the pump (Ca+Mg)-ATPase may correspond to different states of the Ca pump (13).

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