Journal of Supramolecular Structure 10:215–225 (1979)
Normal and Abnormal Red Cell Membranes 375–385

Properties of (Mg^{2+} + Ca^{2+})-ATPase of Erythrocyte Membranes Prepared by Different Procedures: Influence of Mg^{2+}, Ca^{2+}, ATP, and Protein Activator

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Erythrocyte membranes prepared by three different procedures showed (Mg^{2+} + Ca^{2+})-ATPase activities differing in specific activity and in affinity for Ca^{2+}. The (Mg^{2+} + Ca^{2+})-ATPase activity of the three preparations was stimulated to different extents by a Ca^{2+}-dependent protein activator isolated from hemolysates. The Ca^{2+} affinity of the two most active preparations was decreased as the ATP concentration in the assay medium was increased. Lowering the ATP concentration from 2 mM to 2-200 μM or lowering the Mg:ATP ratio to less than one shifted the (Mg^{2+} + Ca^{2+})-ATPase activity in stepwise hemolysis membranes from mixed "high" and "low" affinity to a single high Ca^{2+} affinity. Membranes from which soluble proteins were extracted by EDTA (0.1 mM) in low ionic strength, or membranes prepared by the EDTA (1–10 mM) procedure, did not undergo the shift in the Ca^{2+} affinity with changes in ATP and $MgCl_2$ concentrations. The EDTA-wash membranes were only weakly activated by the protein activator. It is suggested that the differences in properties of the (Mg^{2+} + Ca^{2+})-ATPase prepared by these three procedures reflect differences determined in part by the degree of association of the membrane with a soluble protein activator and changes in the state of the enzyme to a less activatable form.

Key words: (Mg^{2+} + Ca^{2+})-ATPase, erythrocyte membranes, endogenous protein activator

Controversy exists as to the properties, number and function of (Mg^{2+} + Ca^{2+})-ATPase(s) present in erythrocyte membranes. Quist and Roufogalis [1, 2], employing a stepwise hemolysis procedure for preparation of erythrocyte membranes, identified mixed "high" and "low" Ca^{2+} affinity (Mg^{2+} + Ca^{2+})-ATPase activity. A correlation of ATPase

Received March 5, 1978; accepted August 29, 1978.

0091-7419/79/1002-0215$02.30

activity in the low Ca^{2+}-affinity range and calcium transport has been found in intact erythrocytes as well as in preparations of resealed "ghosts" and vesicles [1, 3, 4]. Katz and Blostein [5] employing an EDTA-wash procedure for preparation of erythrocyte membranes and low ATP concentrations (2 μM) observed an association of (Mg^{2+} + Ca^{2+})-ATPase activity and related Ca^{2+}-dependent phosphoprotein formation in the high Ca^{2+} affinity range. The recent discovery of a soluble protein activator [6–8] of (Mg^{2+} + Ca^{2+})-ATPase further increases the difficulty in determining whether there are two distinct ATPases or two states of a single (Mg^{2+} + Ca^{2+})-ATPase regulated by other proteins in the membrane. In the present study, we have utilized three different erythrocyte membrane preparations, all of which are currently being used to study the properties of the (Mg^{2+} + Ca^{2+})-ATPase activity, to investigate a number of factors that affect the Ca^{2+} affinity and activity of this enzyme complex.

MATERIALS AND METHODS

Human erythrocyte membranes were prepared by three different procedures Essentially they were the following: i) a 1:10 hemolysis of packed cells in distilled water followed by washing in EDTA (1–10 mM), then further washes and storage in Tris-HCl, pH 7.4 [9, 10]; ii) a stepwise 1:10 hemolysis in decreasing concentrations of NaCl (0.08–0.015 M) followed by washing and storage in 0.015 M NaCl and 0.005 M Tris-maleate, pH 7.2 [2]; and, iii) a 1:14 hemolysis in 20 imOsm imidazole, pH 7.4, followed by washing and storage in imidazole medium [11]. Complete details of these procedures are given in the references cited.

Extracted erythrocyte membranes containing low affinity (Mg^{2+} + Ca^{2+})-ATPase activity were prepared by the procedure of Fairbanks et al [12] as previously described [2]. Essentially, membranes prepared by the stepwise hemolysis procedure were incubated in 5 vol of 1 mM Tris- 0.1 mM EDTA, pH 8, and agitated at 37° for 30–45 minutes. Membranes separated by centrifugation at 20,000 × g for 10 minutes were resuspended in 5 mM Tris-15 mM NaCl, pH 7.2, to the original volume of packed cells from which the membranes were derived. Since this procedure removes up to 25% of membrane protein [12, 13], activities of membranes and extracted membranes were compared per ml of packed cells.

The preparations of (γ-^{32}P)ATP and the procedures for measuring ATPase activity as $^{32}P_i$ released from (γ-^{32}P)ATP have been described previously [9, 10] and are summarized in the legend to Fig. 1. Incubation times at the various ATP concentrations were chosen so that the assays were linear with respect to time and membrane concentration. Free Ca^{2+} was buffered in the incubation medium, with ethyleneglycolbis (β-aminoethyl)-N,N-tetraacetate (EGTA) in a final concentration of 0.1 mM. The free Ca^{2+} concentration was determined by the association constants for the interaction of EGTA and ATP with Ca^{2+} for the pH and ATP concentrations at which the experiment was conducted [14].

Protein activator was prepared from erythrocyte hemolysates by the method of Luthra et al [6] using a carboxy methyl Sephadex column (CM 50, Pharmacia) and concentrating the desired fraction by ultrafiltration. An endogenous soluble activator was also isolated from erythrocyte membranes: 12 ml of membranes prepared by the stepwise hemolysis procedure were incubated in 5 vol of 1 mM Tris-HCl-0.1 mM EDTA, pH 6.5 for 35 minutes at 37°. The 100,000 × g supernatant was concentrated 20-fold by ultrafiltration (Amicon XM-100 A filter), layered on a 5–20% isokinetic sucrose gradient and centrifuged at 38,000 × g for 16–18 h. Activator was assayed by incubating 300 μl of various fractions with extracted membranes under standard conditions (see Fig. 3) at 300 μM Ca^{2+}.

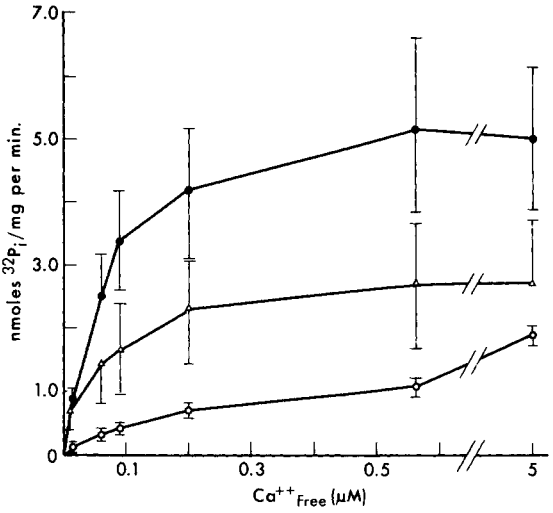

Fig. 1. $(Mg^{2+} + Ca^{2+})$-ATPase activity of erythrocyte membranes prepared by different procedures. Erythrocyte membranes prepared by stepwise hemolysis (●——●), imidazole-wash (△——△) or EDTA-wash (○——○) procedures were first incubated at 37° for 10 min in the presence of 0.1 mM Tris-EGTA, pH 7.4. The membranes were assayed at 37° for 10 min in a final volume of 0.125 ml containing 0.075 ml membranes (2–3 mg protein/ml), 40 mM Tris-HCl, pH 7.4, 0.2 mM [γ-^{32}P] ATP (50×10^3 cpm per 0.125 ml final volume), 1.0 mM $MgCl_2$ in the presence of various amounts of added $CaCl_2$ (0.02–5.0 μM Ca^{2+} free). The reaction was terminated by the addition of 0.75 ml cold trichloroacetic acid (5%) containing 5 mM Na_2ATP and 2 mM KH_2PO_4. 0.45 ml of charcoal (0.15 g/ml in 5% trichloroacetic acid) was added and following centrifugation for 2 min (Eppendorf microcentrifuge) aliquots (0.4 ml) were counted in 10 ml Aquasol scintillation fluid. The results shown are the mean ± SE of at least 3 different membrane preparations in each case. The ATPase activity refers to Ca^{2+}-stimulated components after subtraction of values measured without Ca^{2+} added.

Materials: All chemicals were purchased from Sigma Chemical. $^{32}P_i$ (carrier free) and Aquasol were purchased from New England Nuclear. All inorganic chemicals used were Reagent grade. Charcoal (Norit A) was purchased from Fisher Scientific.

RESULTS

$(Mg^{2+} + Ca^{2+})$-ATPase Activities of Different Erythrocyte Membrane Preparations

The $(Mg^{2+} + Ca^{2+})$-ATPase activities of the three erythrocyte membrane preparations in the presence of various free Ca^{2+} concentrations (0.02–5 μM) is illustrated in Fig. 1. The ATP concentration in these experiments was 200 μM. The imidazole-wash preparation and the stepwise hemolysis preparation produced $(Mg^{2+} + Ca^{2+})$-ATPase activity curves of a similar shape, although in our hands, the $(Mg^{2+} + Ca^{2+})$-ATPase activity was higher in membranes prepared by the stepwise hemolysis procedure. The EDTA-wash preparation produced a $(Mg^{2+} + Ca^{2+})$-ATPase activity curve of low activity, particularly at low free Ca^{2+} concentrations. Figure 2 is a reciprocal plot of the same data illustrating the K_{diss} for Ca^{2+} and the maximum velocity at saturating Ca^{2+} concentrations ($V_{Ca^{2+}}$) of the $(Mg^{2+} + Ca^{2+})$-ATPase of the three erythrocyte membrane preparations. The plots were constructed using the weighted statistical analysis of Wilkinson [15]. At this ATP concentration, membranes prepared by the step-wise hemolysis procedure and the imidazole-wash

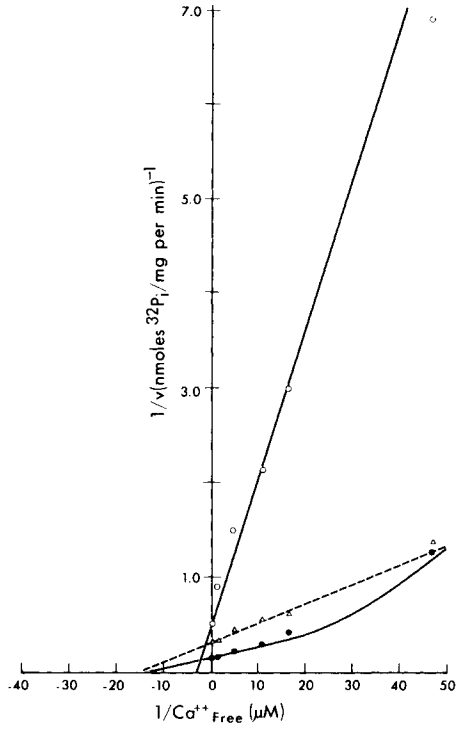

Fig. 2. K_{diss} and $V_{Ca^{2+}}$ of the (Mg^{2+} + Ca^{2+})-ATPase activity of erythrocyte membranes prepared by different procedures. Reciprocal plots of data illustrated in Fig. 1 at 200 μM ATP in the (●——●) stepwise hemolysis preparation, (△– – –△) imidazole-wash preparation and (○——○) EDTA-wash preparation. Plots were constructed according to the weighted statistical analysis of Wilkinson [15].

procedure have a similar K_{diss} for Ca^{2+}, but the EDTA-wash preparation has a significantly lower Ca^{2+} affinity than either of the other two preparations ($P < 0.001$, student t-test). The EDTA-wash preparation also has a significantly lower $V_{Ca^{2+}}$ than either of the other two preparations ($P < 0.001$).

The effect of ATP concentration on the K_{diss} for Ca^{2+} and the $V_{Ca^{2+}}$ for the (Mg^{2+} + Ca^{2+})-ATPase activities of the three erythrocyte membrane preparations is illustrated in Table I. The K_{diss} and the $V_{Ca^{2+}}$ values were determined by the statistical analysis of Wilkinson [15]. At ATP concentrations of 2-200 μM the K_{diss} for Ca^{2+} increased as the ATP concentration was increased. The K_{diss} values were not statistically different between the stepwise and imidazole-wash preparations. The EDTA-wash preparation, on the other hand, showed a significantly higher K_{diss} for Ca^{2+} at all three ATP concentrations ($P < 0.001$). At 2,000 μM ATP the differences among the three preparations in K_{diss} for Ca^{2+} were eliminated. As expected, $V_{Ca^{2+}}$ values in the three preparations increased progressively as the ATP concentration increased. At ATP concentrations of 20–2,000 μM the $V_{Ca^{2+}}$ of the stepwise preparation was significantly higher than that of the EDTA-wash preparation ($P < 0.001$) and the imidazole-wash preparation ($P < 0.01$ at 20 μM ATP and $P < 0.001$ at 200 and 2,000 μM ATP).

Effect of ATP Concentration on Ca^{2+} Affinity

Since ATP affected the affinity of the $(Mg^{2+} + Ca^{2+})$-ATPase in the membrane preparations, the effect of ATP on the shape of the Ca^{2+}-activation curve was examined in detail in membranes prepared by the stepwise hemolysis procedure (Fig. 3). In these experiments, $MgCl_2$ was kept constant (6.4 mM). At 2 mM ATP (Fig. 3A) Ca^{2+} activation of the $(Mg^{2+} + Ca^{2+})$-ATPase activity occurred over a wide range of Ca^{2+} concentrations (0.3–300 μM), demonstrating mixed high and low Ca^{2+}-affinities [2, 16]. By contrast, at 2 or 20 μM ATP, $(Mg^{2+} + Ca^{2+})$-ATPase activity was saturated at around 10 μM Ca^{2+}, the ATPase activity reaching a plateau at higher Ca^{2+} concentrations (Fig. 3A). At 200 μM ATP the Ca^{2+}-activation curve was intermediate but tended to exhibit mainly high Ca^{2+}-affinity ATPase activity (Fig. 3A). To determine whether these ATP-dependent changes in the shape of the Ca^{2+}-activation curve were associated with soluble proteins in the erythrocyte membrane, the effect of ATP on the shape of the Ca^{2+}-activation curve was again examined following extraction of soluble proteins in 1.0 mM Tris-0.1 mM EDTA, pH 8 for 35 min at 37°. As shown previously [2], loss of soluble proteins resulted in $(Mg^{2+} + Ca^{2+})$-ATPase activity of predominantly low Ca^{2+}-affinity. The ATP dependence of the

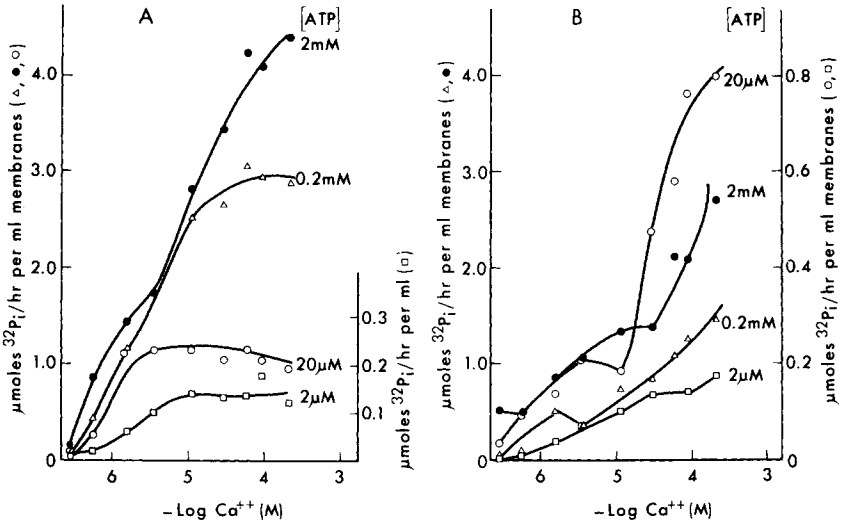

Fig. 3. Effect of ATP on the Ca^{2+} affinity of $(Mg^{2+} + Ca^{2+})$ -ATPase in erythrocyte membranes (A) and pH 8-extracted membranes (B). Erythrocyte membranes were prepared by the stepwise hemolysis procedure. Extracted membranes were obtained as described in Methods. $(Mg^{2+} + Ca^{2+})$-ATPase activity was assayed in a final volume of 0.6 ml containing 0.05 ml of membranes or 0.10 ml of extracted membranes (approximately 4 and 3 mg protein/ml), 55 mM Tris-maleate, pH 7.2, 6.4 mM $MgCl_2$, 66 mM NaCl, 0.1 mM EGTA, 0.1 mM ouabain, 0.002-2.0 mM Na_2ATP containing [γ-^{32}P]ATP (15–30 × 10^3 cpm) and $CaCl_2$ (0.3-300 μM Ca^{2+} free). Membranes were incubated at 37° for 2 min (2–20 μM ATP), 20 min (200 μM ATP) and 60 min (2000 μM ATP). The reaction was terminated with 0.2 ml of 5% cold trichloroacetic acid. 0.4 ml of charcoal (0.15 g/ml in 5% trichloroacetic acid) was added and following centrifugation for 2 min (Eppendorf microcentrifuge) aliquots (0.4 ml) were counted in 10 ml Aquasol scintillation fluid. The influence of 2–2,000 μM ATP on free Ca^{2+} was calculated (14) and found to be negligible. The curves shown represent a typical experiment from the same batch of erythrocyte membranes.

TABLE I. K_{diss} for Ca^{2+} and $V_{Ca^{2+}}$ of $(Mg^{2+} + Ca^{2+})$-ATPase of Erythrocyte Membranes Prepared by Different Procedures: Effect of ATP Concentration.

	ATP (μM)	Preparations		
		Stepwise	EDTA-wash	Imidazole-wash
K_{diss} (μM)	2	0.018 ± 0.006	0.185 ± 0.021[a]	0.018 ± 0.004
	20	0.020 ± 0.005	0.192 ± 0.031[a]	0.030 ± 0.007
	200	0.062 ± 0.012	0.398 ± 0.039[a]	0.060 ± 0.006
	2000	0.118 ± 0.013	0.149 ± 0.012[f]	0.165 ± 0.023
$V_{Ca^{2+}}$ (nmoles $mg^{-1}min^{-1}$)	2	0.398 ± 0.026	0.303 ± 0.011[f]	0.322 ± 0.013[c]
	20	1.781 ± 0.080	1.155 ± 0.061[b]	1.036 ± 0.049[d]
	200	5.419 ± 0.235	2.042 ± 0.078[a]	2.896 ± 0.077[e]
	2000	11.340 ± 0.377	4.250 ± 0.104[b]	7.474 ± 0.331[e]

Erythrocyte membranes prepared by different procedures were assayed for $(Mg^{2+} + Ca^{2+})$-ATPase activity as described in Fig. 1, in the presence of the following ATP and $MgCl_2$ concentrations: i) 2 μM ATP (5×10^3 cpm [γ-^{32}P] ATP per 0.125 ml final volume) and 25 μM $MgCl_2$, ii) 20 μM ATP (5×10^3 cpm [γ-^{32}P] ATP per 0.125 ml final volume) and 100 μM $MgCl_2$, iii) 200 μM ATP (50×10^3 cpm [γ-^{32}P] ATP per 0.125 ml final volume) and 1,000 μM $MgCl_2$ and, iv) 2,000 μM ATP (500×10^3 cpm [γ-^{32}P] ATP per 0.125 ml final volume) and 6,000 μM $MgCl_2$. Membranes were incubated at 37° for 2 min (2 μM ATP), 3.5 min (20 μM ATP), 10 min (200 μM ATP) and 25 min (2,000 μM ATP). The results are expressed as the K_{diss} ± SE and $V_{Ca^{2+}}$ ± SE of 3 pooled experiments in each case.

[a] Indicates a significant difference between the EDTA-wash preparation and the other two preparations ($P < 0.001$).

[b] Indicates a significant difference between the EDTA-wash preparation and the stepwise hemolysis preparation ($P < 0.001$).

[c] Indicates a significant difference between the stepwise hemolysis preparation and the imidazole-wash preparation ($P < 0.05$).

[d] Indicates a significant difference between the stepwise hemolysis preparation and the imidazole-wash preparation ($P < 0.01$).

[e] Indicates a significant difference between the stepwise hemolysis preparation and the imidazole-wash preparation ($P < 0.001$).

[f] Not significantly different.

Ca^{2+}-affinities seen in the erythrocyte membranes (Fig. 3), the prominantly low Ca^{2+}-affinity of the $(Mg^{2+} + Ca^{2+})$-ATPase activity was maintained at all ATP concentrations examined (2–2,000 μM).

Effect of $MgCl_2$ Concentration on Ca^{2+}-Affinity

The relationship of Mg:ATP ratio to the Ca^{2+}-affinity of the $(Mg^{2+} + Ca^{2+})$-ATPase was examined in both erythrocyte membranes and extracted membranes prepared by the stepwise hemolysis procedure. In these experiments, ATP was kept constant at 2 mM. In the erythrocyte membranes, lowering the $MgCl_2$ concentration from 6.4 mM (Mg:ATP = 3.2:1) to 1.0 mM (Mg:ATP = 0.5:1) resulted in a change in the Ca^{2+} activation curve from a mixed high and low affinity to predominantly high Ca^{2+}-affinity activity saturating at 10 μM free Ca^{2+} or less (Fig. 4). Lowering the Mg:ATP ratio from 1 to 0.5 resulted in an abrupt decrease in maximal velocity. The extent to which these effects were related to soluble membrane proteins were examined in the extracted membranes. In contrast to the erythrocyte membranes, lowering the $MgCl_2$ from 6.4 mM to 1 mM progressively reduced the activity of the $(Mg^{2+} + Ca^{2+})$-ATPase without any apparent effect on Ca^{2+}-affinity, the enzyme requiring higher than 10 μM Ca^{2+} for saturation throughout (Fig. 5).

Characterization of Soluble Protein Activator

We confirmed a previous finding [2] that addition of the soluble fraction (extracted from membranes with 1 mM Tris-0.1 mM EDTA, pH 8) to the extracted membranes restored the original high Ca^{2+}-affinity and velocity of $(Mg^{2+} + Ca^{2+})$-ATPase activity (not shown). A similar result was obtained with soluble fraction extracted under the same conditions at pH 6.5 for 35 min at 37° (not shown). When the soluble protein fraction was layered on a 5–20% isokinetic sucrose gradient, an activator of the $(Mg^{2+} + Ca^{2+})$-ATPase activity of the extracted membranes was found in the fraction above the hemoglobin band. The failure of the activator protein to penetrate the sucrose gradient suggests that it may be similar to the low molecular weight $(Mg^{2+} + Ca^{2+})$-ATPase activator isolated from hemolysates of erythrocytes [6–8]. Work is in progress to further investigate this aspect.

Endogenous protein activator isolated from hemolysates by the method of Luthra et al [6] stimulated the $(Mg^{2+} + Ca^{2+})$-ATPase activity of all three preparations studied but to varying degrees (Table II); erythrocyte membranes prepared by the imidazole-wash procedure were stimulated to a greater degree than membranes prepared by the two other procedures.

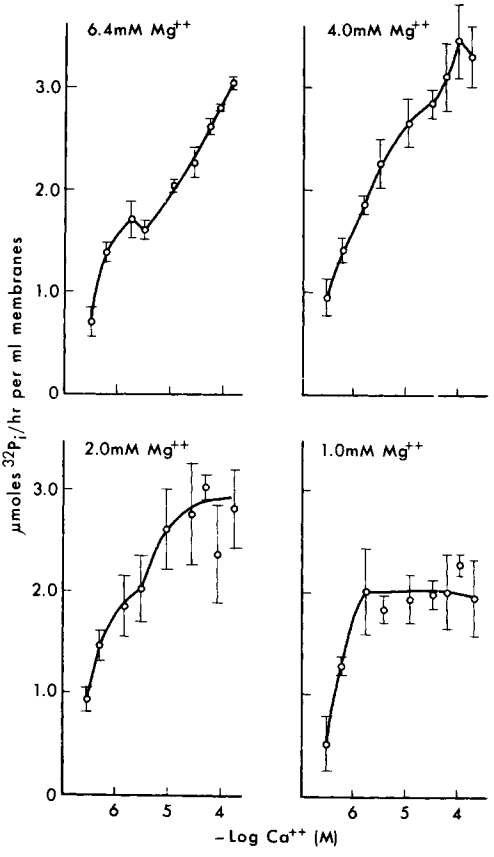

Fig. 4. Effect of $MgCl_2$ on the Ca^{2+} affinity of $(Mg^{2+} + Ca^{2+})$-ATPase in erythrocyte membranes. Erythrocyte membranes were prepared by the stepwise hemolysis procedure. $(Mg^{2+} + Ca^{2+})$-ATPase was assayed as described in Fig. 3, in the presence of 2 mM ATP and various $MgCl_2$ concentrations (1–6.4 mM). The results are the mean ± SE of three membrane preparations.

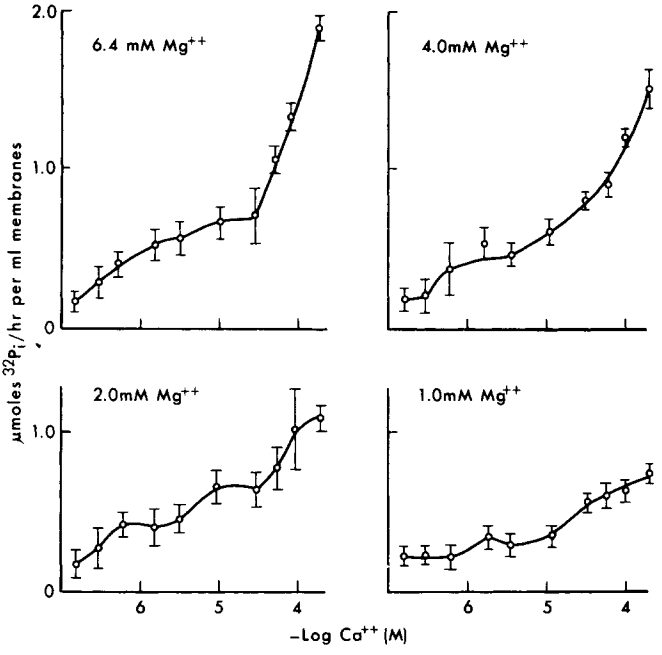

Fig. 5. Effect of $MgCl_2$ on the Ca^{2+} affinity of $(Mg^{2+} + Ca^{2+})$-ATPase in extracted membranes. Extracted membranes were obtained from erythrocyte membranes prepared by stepwise hemolysis, as described in Methods, and assayed as described in Fig. 3 for 60 min at 2 mM ATP. Results are the mean ± SE of three extracted membrane preparations.

TABLE II. Effect of Endogenous Protein Activator on $(Mg^{2+} + Ca^{2+})$-ATPase Activity of Erythrocyte Membranes Prepared by Different Procedures.

Preparation		% Stimulation
Stepwise	[3][a]	48.0 ± 13.6
EDTA-wash	[7]	111.1 ± 17.4
Imidazole-wash	[3]	311.0 ± 104.1[b]

Erythrocyte membranes prepared by three different procedures were assayed for $(Mg^{2+} + Ca^{2+})$-ATPase activity as described in Fig. 1 (200 μM ATP, 1,000 μM $MgCl_2$, and 0.1 μM free Ca^{2+}) in the presence and absence of protein activator (35 μg/0.25 ml final incubation volume) prepared by the method of Luthra et al [6]. Results are expressed as the mean % increase ± SE over activity observed in the absence of activator in at least three preparations in each case.

[a]indicates number of preparations in each group.

[b]indicates a significant difference between the imidazole-wash preparation and the EDTA-wash preparation ($P < 0.02$).

DISCUSSION

In this study we examined the factors responsible for differences in $(Mg^{2+} + Ca^{2+})$-ATPase activity and Ca^{2+}-affinity of three different erythrocyte membrane preparations commonly used to investigate the properties of this enzyme. The differences in K_{diss} for Ca^{2+} activation of $(Mg^{2+} + Ca^{2+})$-ATPase in the three preparations were dependent on the

ATP concentration. Membranes prepared by the stepwise and imidazole-wash procedure had a significantly higher Ca^{2+}-affinity than the EDTA-wash preparation at low ATP concentrations (2–200 μM). However, the Ca^{2+}-affinity of the former preparations progressively decreased as the ATP concentration in the assay medium was increased; at 2 mM ATP the K_{diss} of the three preparations was not significantly different. The EDTA-wash preparation exhibited a low Ca^{2+}-affinity at all of the ATP concentrations investigated. The EDTA-wash preparation also had the least active $(Mg^{2+} + Ca^{2+})$-ATPase activity. It is possible that the differences in Ca^{2+}-affinity and $V_{Ca^{2+}}$ noted in the three preparations were due to the different extent of association of the protein activator of $(Mg^{2+} + Ca^{2+})$-ATPase [11, 17] with the membranes. The protein activator has been shown to affect both these parameters [7] and is removed from membranes by EGTA [17, 18] or EDTA. However, the loss of activator from the EDTA-wash preparation may not be the only factor accounting for the low $(Mg^{2+} + Ca^{2+})$-ATPase activity and Ca^{2+}-affinity in these membranes, since this preparation was less activated by the addition of purified protein activator than the imidazole-wash membranes, a preparation with significantly higher $(Mg^{2+} + Ca^{2+})$-ATPase activity.

The influence of soluble membrane proteins on the properties of $(Mg^{2+} + Ca^{2+})$-ATPase was examined further in membranes prepared by the step-wise hemolysis procedure. This preparation, like the imidazole-wash preparation [11] contained a low molecular weight activator of $(Mg^{2+} + Ca^{2+})$-ATPase, extractable from the membranes by incubation in low ionic strength medium (1mM Tris) containing 0.1 mM EDTA. Whereas mixed high (saturation at 10 μM Ca^{2+}) and low Ca^{2+} affinity (not saturated up to 300 μM Ca^{2+}), $(Mg^{2+} + Ca^{2+})$-ATPase activities were apparent at 2 mM ATP, only high Ca^{2+} affinity activity was expressed at low ATP concentrations (2–20 μM). A similar shift to higher Ca^{2+} affinity activity was obtained when the $MgCl_2$ concentration was decreased, an abrupt change occurring when the Mg/ATP ratio was decreased below one. This shift in Ca^{2+}-affinity was also noted in the imidazole-wash preparation but not in the EDTA-wash preparation (Table I). Thus ATP and $MgCl_2$, or possibly Mg-ATP, appear to regulate the Ca^{2+}-affinity of the more active membranes.

Changes in Ca^{2+}-affinity of $(Mg^{2+} + Ca^{2+})$-ATPase with variation in ATP and $MgCl_2$ were not observed following extraction of soluble proteins from the membranes. Extraction resulted in the loss of approximately 25% of the membrane protein, including spectrin and actin (band 5) [13] and protein activator, producing a preparation of predominantly low Ca^{2+}-affinity $(Mg^{2+} + Ca^{2+})$-ATPase activity. Whether the change in Ca^{2+}-affinity in the extracted membranes resulted from the loss of a high affinity $(Mg^{2+} + Ca^{2+})$-ATPase from the membranes [2, 17], loss of a protein activator [18] or a change in state of the enzyme protein [20] is unclear. The addition of soluble protein extracts [2, 17], or purified $(Mg^{2+} + Ca^{2+})$-ATPase activator [7, Roufogalis and Mauldin, unpublished observations] to the extracted membranes restored their original high Ca^{2+}-affinity. The failure of ATP and Mg^{2+} to modulate the Ca^{2+}-affinity in extracted membranes suggests that their modulation is mediated through the soluble protein(s). Failure of ATP to alter the Ca^{2+}-affinity of the EDTA-wash preparation (Table I) suggests that this preparation was deficient in soluble proteins and perhaps protein activator compared to the other two preparations.*

*Predominantly high Ca^{2+}-affinity $(Mg^{2+} + Ca^{2+})$-ATPase has been reported in some membrane preparations at a Mg:ATP ratio equal to or less than one [21–23] indicating that other factors, such as membrane Ca^{2+} content [23–25] or the abundance of protein activator [18] can counteract the influence of these modulators.

However, failure of the partially purified activator to restore the activity of the EDTA-wash preparation to that of the other preparations (Table II) suggests that additional changes in membrane-bound (Mg^{2+} + Ca^{2+})-ATPase can occur as a result of the preparation conditions. This could result from the loss of other soluble proteins (spectrin and actin) from the membranes, a mechanism for which there is no direct experimental evidence at present, or an irreversible change in the state of the (Mg^{2+} + Ca^{2+})-ATPase [20] to a non-activatable form. An irreversible change in state does not appear to occur following the limited treatment of membranes with 1 mM Tris- 0.1 mM EDTA at 37° [2], but may have occurred on prolonged washing in EDTA (1-10 mM) during the preparation of the EDTA-wash membranes.

This work suggests that the differences in properties of the (Mg^{2+} + Ca^{2+})-ATPase prepared by different procedures may be due to the state of the enzyme determined at least in part by the degree of association of a protein activator and the Mg^{2+} and ATP concentrations used. This work also emphasizes that for the study and the comparison of (Mg^{2+} + Ca^{2+})-ATPase a careful definition be made of the method of erythrocyte membrane preparation, the subsequent treatment of the membranes at various ionic strengths and the conditions for assay with respect to Mg^{2+}, Ca^{2+} and ATP.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council of Canada, the British Columbia Heart Foundation and the Canadian Cystic Fibrosis Foundation.

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