Biochimica et Biophysica Acta, 600 (1980) 467—479
© Elsevier/North-Holland Biomedical Press

BBA 78848

EFFECTS OF CALCIUM AND SOLUBLE CYTOPLASMIC ACTIVATOR PROTEIN (CALMODULIN) ON VARIOUS STATES OF (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY IN ISOLATED MEMBRANES OF HUMAN RED CELLS *

M.G. LUTHRA and H.D. KIM

Department of Physiology, College of Medicine, University of Arizona, Tucson, AZ 85724 (U.S.A.)

(Received December 18th, 1979)

Key words: (Ca^{2+} + Mg^{2+})-ATPase; Ca^{2+} concentration; Activator protein; Calmodulin; (Human red cell membrane)

Summary

(Ca^{2+} + Mg^{2+})-ATPase activity of red cells and their isolated membranes was investigated in the presence of various Ca^{2+} concentrations and cytoplasmic activator protein. Red cell ATPase activity was high at low Ca^{2+} concentrations, and low at moderate and high concentrations of Ca^{2+}. In the case of isolated membranes, both low and moderate Ca^{2+} concentrations produced higher (Ca^{2+} + Mg^{2+})-ATPase activity than high Ca^{2+} concentration. Membrane-free hemolysate containing soluble activator of (Ca^{2+} + Mg^{2+})-ATPase produced a significant increase in (Ca^{2+} + Mg^{2+})-ATPase activity only at low Ca^{2+} concentration. Regardless of Ca^{2+} and activator concentrations, the enzyme activity in the membrane was lower than lysed red cells. The low level of (Ca^{2+} + Mg^{2+})-ATPase activity seen at high Ca^{2+} concentration can be augmented by lowering the Ca^{2+} concentration with EGTA in the assay medium. However, once the membrane was exposed to a high Ca^{2+} concentration, the activator could no longer exert its maximum stimulation at the low Ca^{2+} concentration brought about by addition of EGTA. This loss of activation was not attributable to the Ca^{2+}-induced denaturation of activator protein but rather related to the alteration of (Ca^{2+} + Mg^{2+})-ATPase states in the membrane. On the basis of these data, it is suggested that only a small portion of (Ca^{2+} + Mg^{2+})-ATPase activity of isolated membranes can be stimulated by the soluble activator and that (Ca^{2+} + Mg^{2+})-ATPase most likely exists in various states depending upon Ca^{2+} concentration and the presence of activator. The enzyme state exhibiting the high degree of stimulation by activator may undergo irreversible damage in the presence of high Ca^{2+} concentrations.

* Preliminary data on this study were presented at 63rd Annual Meeting of Federation of American Societies for Experimental Biology, April 2—10, at Dallas.

Introduction

It has been well established that a steep Ca^{2+} concentration gradient of nearly 10 000-fold is maintained across the red cell membranes by means of $(Ca^{2+} + Mg^{2+})$-ATPase and a low permeability to Ca^{2+} [1—3]. An increase in intracellular Ca^{2+} is known to elicit deleterious effects including change in cell shape and deformability [4—7], shortened life span and a selective K^+ loss known as the 'Gárdos effect' [8,9]. Perhaps one of the most provocative observations in recent years on $(Ca^{2+} + Mg^{2+})$-ATPase has been the discovery in crude hemolysate of a soluble cytoplasmic protein which can stimulate this membrane-bound enzyme activity [10—12]. The activator protein has now been purified to its apparent homogeneity with a mol. wt. of 18 000 [13,14] and probably exists in polymer forms with mol. wt. 34 000—58 000 (Luthra, M., unpublished result). A possible physiological role of this protein was demonstrated in experiments by MacIntyre and Green [15] and Hinds et al. [16] on inside-out human red cell vesicles which can be made to actively transport Ca^{2+} in the presence of ATP under suitable conditions. It was found that inclusion of the activator protein resulted in an augmentation of Ca^{2+} flux into the vesicles.

Of particular interest is accumulating evidence that this and the activator-like proteins have been found not only in red cells of various mammals [12], chickens (Luthra, M., unpublished result) and the pig reticulocytes [12] but also in other biological tissues including the brain [17], heart [18], muscle [19] and corpus luteum (Luthra, M., unpublished result). Regardless of the source of the parental cells from which the activator is derived, all can stimulate human red cell membrane $(Ca^{2+} + Mg^{2+})$-ATPase and vice versa to a varying degree [12,20]. Moreover, troponin C from muscle [20] and cyclic-AMP phosphodiesterase modulator protein from the brain and heart [14,20,21] and cytoplasmic protein from corpus luteum of monkey can also enhance human red cell $(Ca^{2+} + Mg^{2+})$-ATPase activity. In point of fact, a suggestion has been made that the human red cell activator and cyclic-AMP phosphodiesterase modulator protein are very similar, if not identical, proteins [14,20, 21]. In keeping with this view, we have recently found that both the red cell and the brain protein can be rendered ineffective upon oxidation of methionine residues by treatment with such reagents as *N*-bromosuccinimide [22]. In this communication, results of our investigation on the relationship of Ca^{2+} and the activator concentration as a determinant for various Ca^{2+}-affinity ATPase activity will be presented. The companion paper will deal with the question of how the mechanism of $(Ca^{2+} + Mg^{2+})$-ATPase undergoes alterations in the course of cell aging.

Materials and Methods

L-Histidine, vanadium-contaminated * Na_2ATP, Trizma base and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO). Saponin was a

---

* Vanadium is known to inhibit red cell membrane ATPases to a varying degree depending upon assay conditions. Under assay conditions employed herein, vanadium does not significantly inhibit $(Ca^{2+} + Mg^{2+})$-ATPase activity.

product of Calbiochem (La Jolla, CA) and used without further purification. Chloroform and methanol were purchased from Mallinckrodt Chemical Works (St. Louis, MO).

Methods

Preparation of red cells and membranes. Blood was drawn from healthy donors and collected in heparinized tubes. The blood was centrifuged, and plasma and buffy coat were removed. The packed red cells were washed three times with 0.172 M Tris-HCl buffer, pH 7.6, and resuspended in the same buffer to give 50% hematocrit. An aliquot was stored on ice and another was used for isolation of membranes in 20 imosM Tris-HCl, pH 7.6, as described previously [10]. A membrane suspension of approx. 20% packed red cells was prepared by appropriate dilution of membranes in 20 imosM Tris-HCl, pH 7.6, while the intact red cells were suspended in 0.172 M Tris-HCl, pH 7.6, to give a hematocrit of 10%. Both membranes and red cells were exposed to saponin at a final concentration of 0.1 mg saponin/ml of suspension. Red cells were kept at room temperature for 15 min, and then transferred to 4°C, while membranes were kept all the time at 4°C. Both membranes and saponin-lysed cells were used within 20 min.

Preparation of membrane-free hemolysate. Unless otherwise indicated, the supernatant from the first step of hypotonic lysis was used as a source of $(Ca^{2+} + Mg^{2+})$-ATPase activator without further concentration.

Measurement of $(Ca^{2+} + Mg^{2+})$-ATPase activity. A total of 0.7 ml assay mixture was composed of the following: 3.1 mM $MgCl_2$, 2.1 mM Na_2ATP, 68.5 mM NaCl, 28 mM KCl, 1.5 mM Tris and 69 mM histidine, pH 7.5, 0.1 ml hemolysate and either 0.1 ml of saponin-lysed cells or 0.1 ml of membranes. The concentration of Ca^{2+} in the medium was varied by the addition of varying amounts of either EGTA or Ca^{2+} as indicated. Whenever EGTA was used, no attempt was made to calculate the concentration of free Ca^{2+} in the incubation medium or in the membrane, since formation on the dissociation constants, K_d, of EGTA and membrane-bound Ca^{2+} is lacking. Whenever Ca^{2+} was added to the medium, the added Ca^{2+} concentrations were indicated. The reaction was allowed to proceed at 37°C for 2 h by adding ATP and then terminated by the addition of chloroform/methanol (2 : 1, v/v) and the inorganic phosphorous was determined essentially by the method of Fiske and Subbarow [23] as described previously [11]. For the purpose of simplicity, the abscissa was divided arbitrarily into four parts of added EGTA or Ca^{2+}, representing between 1.8 and 0.21 mM EGTA, between 0.21 mM EGTA and 0.08 mM Ca^{2+}, between 0.08 and 0.18 mM Ca^{2+}, and between 0.18 to 0.71 mM Ca^{2+} and were designated as no calcium, low calcium, moderate calcium and high calcium concentrations, respectively. It was found that between 1.8 and 0.21 mM EGTA concentrations, ATPase activities was quite small and that almost no activation by the activator took place. Thus, levels of ATPase activities obtained at 1.8 mM EGTA were referred to as basal activities due to $(Na^+ + K^+)$- and Mg^{2+}-stimulated ATPases. This value was subtracted from the total activity and the residual activity was referred as $(Ca^{2+} + Mg^{2+})$-ATPase. The activities were expressed as μmol P_i released/ml packed cell per 2 h at 37°C.

Results

*Effect of varying Ca^{2+} concentration * on (Ca^{2+} + Mg^{2+})-ATPase activity of red cells and isolated membranes*

Fig. 1 shows the effect of Ca^{2+} on (Ca^{2+} + Mg^{2+})-ATPase activity of saponin-hemolysed red cells and membranes. Low calcium concentrations (0.21 mM EGTA to 0.08 mM Ca^{2+}) produced a marked stimulation of Ca^{2+}-ATPase activity of lysed red cells, while moderate (0.08—0.18 mM Ca^{2+}) and high (0.18—0.71 mM Ca^{2+}) calcium concentrations produced a progressive inhibition of (Ca^{2+} + Mg^{2+})-ATPase activity. Like red cells, isolated membranes exhibited increased (Ca^{2+} + Mg^{2+})-ATPase activity at low calcium concentrations. However, unlike red cells, the membrane (Ca^{2+} + Mg^{2+})-ATPase activity continued to increase at moderate Ca^{2+} and only at high calcium concentration was slight inhibition of the enzyme apparent. At 2.5 mM calcium no ATPase activity was detectable in the membranes (data not shown). These data imply that upon isolation of membranes not only the reduction in (Ca^{2+} + Mg^{2+})-ATPase took place but the calcium-dependent characteristics were also altered.

Effect of cytoplasmic activator on (Ca^{2+} + Mg^{2+})-ATPase activity of isolated membranes at various concentrations of calcium

Addition of membrane-free hemolysate containing activator ** of (Ca^{2+} + Mg^{2+})-ATPase [10] produced a marked stimulation of ATPase activity at Ca^{2+} concentrations where membranes normally display very low (Ca^{2+} + Mg^{2+})-ATPase (Fig. 3a). In contrast to membranes alone, but similar to red cells (Fig. 1), the enzyme activities were high at low calcium concentrations and low at both moderate and high calcium concentrations when the activator was present. However, at all calcium concentrations tested the activities in the presence of hemolysate were much higher than the membrane alone but still lower than the lysed red cells (Table I). To state it another way, even under optimum stimulatory conditions, the recovery of the membrane enzyme activity to the level of red cells was incomplete. The lower panel in Fig. 3 illustrates percent increase in membrane (Ca^{2+} + Mg^{2+})-ATPase activity by the activator

---

* Calcium content in both the assay buffer and the membrane was measured by atomic absorption spectroscopy. Total calcium concentration in the assay medium containing membrane was 7.5 μM, out of which 4.0 μM was due to the calcium contamination in the buffer alone. To achieve lower than 7.5 μM calcium in the assay medium, varying amounts of EGTA were added. To calculate the free calcium concentration in the assay medium, the equation of Katz et al. [24] was used with the following assumptions. (i) Membrane-bound calcium behaves similar to calcium in the buffer in terms of its interaction with EGTA. (ii) The binding constant of the calcium-EGTA complex at pH 6.8 in histidine buffer is the same as that at pH 7.5, and this was taken to be $4.7 \cdot 10^5$ [25]. The data presented in Fig. 2 show that there are at least two Ca^{2+} activation peaks of (Ca^{2+} + Mg^{2+})-ATPase and an inhibition as a function of calculated free Ca^{2+} concentration. The same data are presented in Fig. 1 in which calcium concentrations are denoted by added EGTA or calcium concentrations in the assay media. Even though in Fig. 1 calcium concentration is represented somewhat on an alinear scale, still the pattern of (Ca^{2+} + Mg^{2+})-ATPase activity is similar to that presented in Fig. 2. However, due to the uncertainty of the binding constant of the membrane-bound calcium and EGTA complex as well as the lack of accurate information on the effect of pH on the binding constant of the calcium-EGTA complex in histidine, the data in the following figures are represented as added EGTA or calcium instead of calculated concentration on the abscissa.

** Similar data are also obtained with the use of purified activator protein (calmodulin) [22].

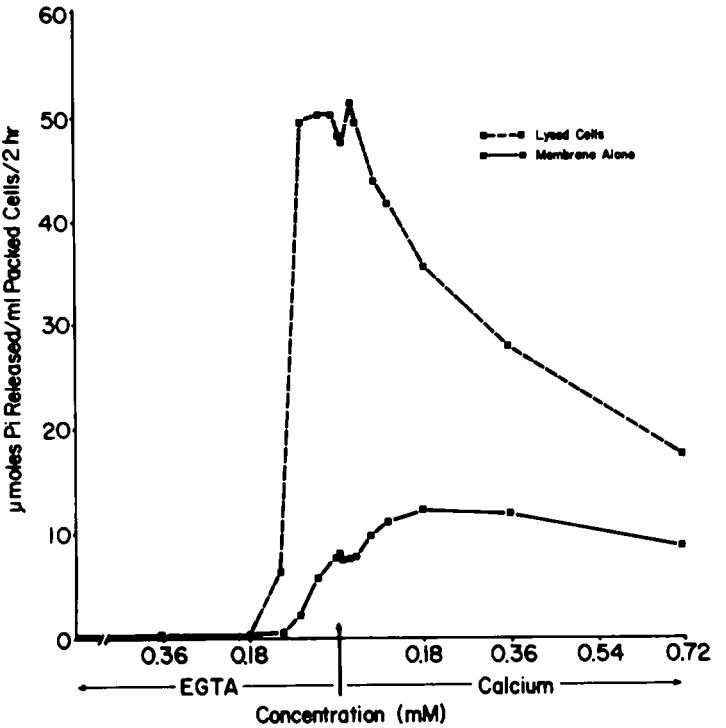


Fig. 1. Saponin-exposed (Ca^{2+} + Mg^{2+})-ATPase activity of human red cells and membranes at various concentrations of calcium.

TABLE I

A COMPARISON OF (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY BETWEEN LYSED CELLS AND ISOLATED MEMBRANES EITHER WITH OR WITHOUT HEMOLYSATE AT VARIOUS CONCENTRATIONS OF EGTA OR CALCIUM

9 μl of lysed red cells or their isolated membranes either with or without hemolysate were tested for ATPase activity. Hemolysate obtained from 3.4 and 9 μl cells was added to isolated membranes. Results are expressed as μmol P_i released/ml packed cells per 2 h.

μM	Membranes alone	+ Hemolysate		Lysed cells
		3.4 μl	9.0 μl	
EGTA				
18.0	0	0.4	0.7	0.2
14.4	0.2	1.0	1.5	2.5
7.2	0.5	2.9	5.9	10.6
0	0.8	11.0	16.5	26.9
Ca^{2+}				
7.2	1.6	16.3	21.6	31.7
14.4	2.1	19.3	24.2	32.5
28.8	3.1	23.6	25.0	32.7
43.2	3.9	25.1	24.3	35.1
108.0	6.6	23.2	23.6	30.0
180.0	7.9	19.1	20.0	27.2
252.0	8.0	18.4	17.8	23.2
360.0	7.7	16.0	14.7	20.8
720.0	7.9	10.5	11.0	13.4
1800.0	2.2	4.3	2.9	5.3

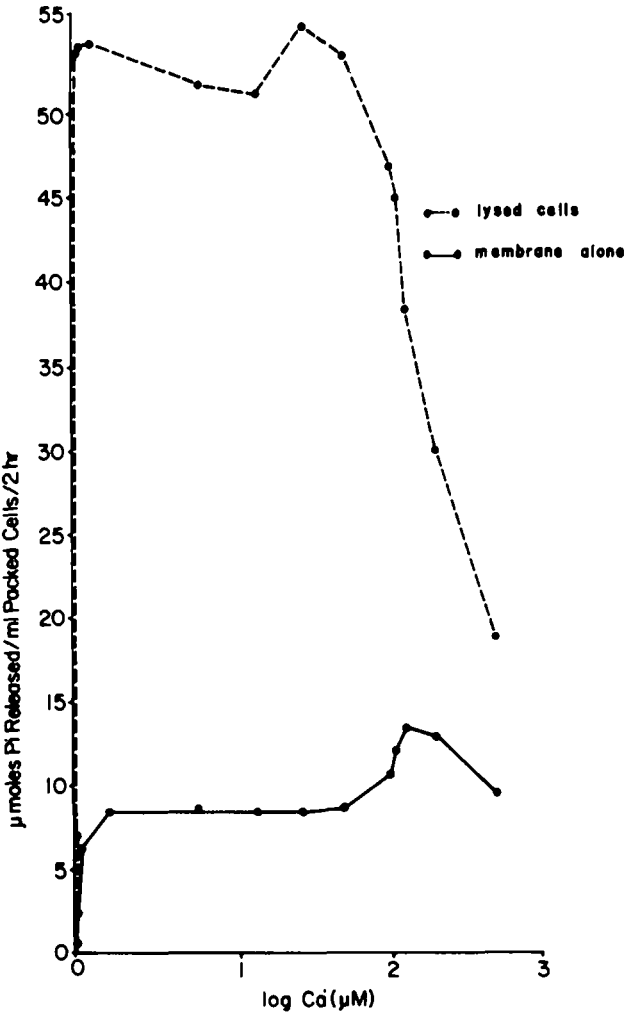


Fig. 2. Saponin-exposed (Ca^{2+} + Mg^{2+})-ATPase activity of human red cells and membranes at various concentrations of calculated free calcium.

at various concentrations of calcium. Apparently, as much as 500% (sometimes even up to 10-fold) increase in (Ca^{2+} + Mg^{2+})-ATPase activity could be produced at very low calcium concentrations (i.e., at 0.054 mM EGTA). Although further elevation in calcium concentration (between 0.040 mM EGTA and 0.08 mM calcium) resulted in an increase in (Ca^{2+} + Mg^{2+})-ATPase, the percent stimulation by the activator was actually decreased substantially (Fig. 3b). At calcium concentrations between 0.005 and 0.05 mM another small peak of activation was consistently observed as shown in Fig. 3b. Furthermore, in response to increasing Ca^{2+} concentration, the enzyme activities in the membrane alone and stimulation by the activator protein follow opposite directions. Contrary to progressively increasing enzyme activity of the membrane alone, the stimulation of the enzyme activity by the activator gradually falls. Thus,

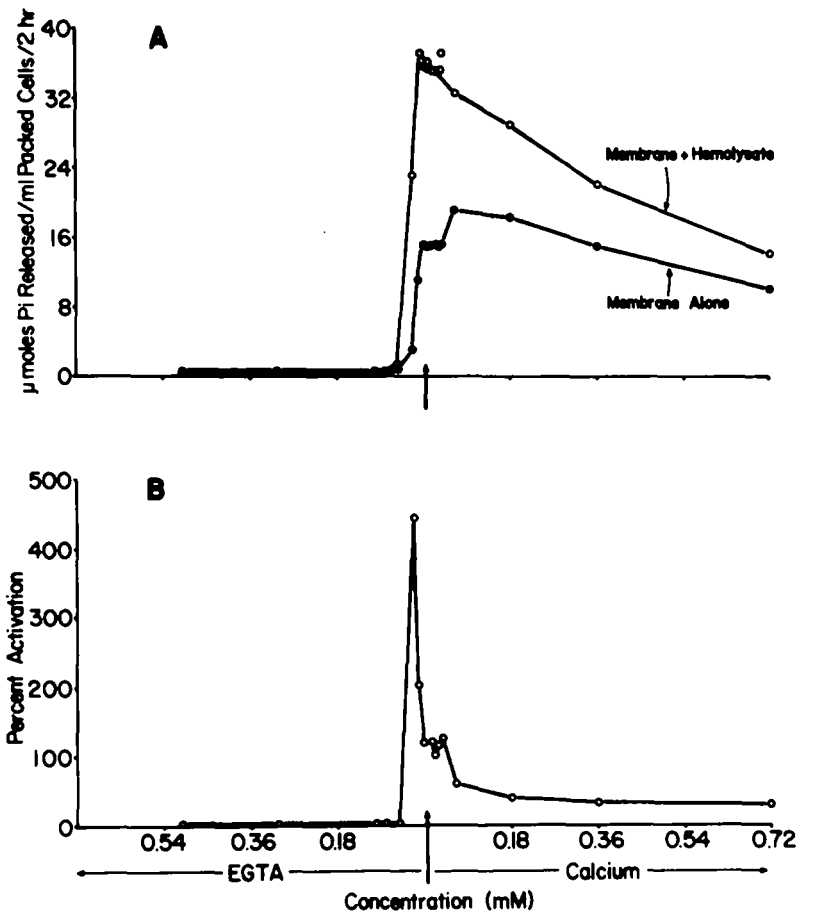


Fig. 3. Effect of calcium and membrane-free hemolysate on $(Ca^{2+} + Mg^{2+})$-ATPase activity of isolated membranes from human red cells. (A) $(Ca^{2+} + Mg^{2+})$-ATPase activity of isolated membranes in the presence (○———○) or absence (●———●) of hemolysate in response to varying amounts of EGTA or calcium. (B) Percent increase in membrane $(Ca^{2+} + Mg^{2+})$-ATPase activity by hemolysate at varying concentration of EGTA or calcium.

a maximum stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase by the activator protein occurs within a narrow range of Ca^{2+} concentrations at which the membrane alone exhibited a fraction of its maximal $(Ca^{2+} + Mg^{2+})$-ATPase activity. These findings could be interpreted to mean that as the critical levels of Ca^{2+} concentrations are raised, a highly activator-dependent enzyme state may diminish until a transformation into a relatively activator-insensitive state takes place.

Reversal of inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase activity induced at high calcium concentrations

To determine whether the inhibition caused by high calcium concentration could be reversible, experiments were designed in which isolated membranes were incubated at 37°C in assay medium containing 0.7 mM $CaCl_2$ with and without activator for a total of 80 min. Aliquots were taken for ATPase activity measurements during 80 min of incubation. As expected, the stimulation

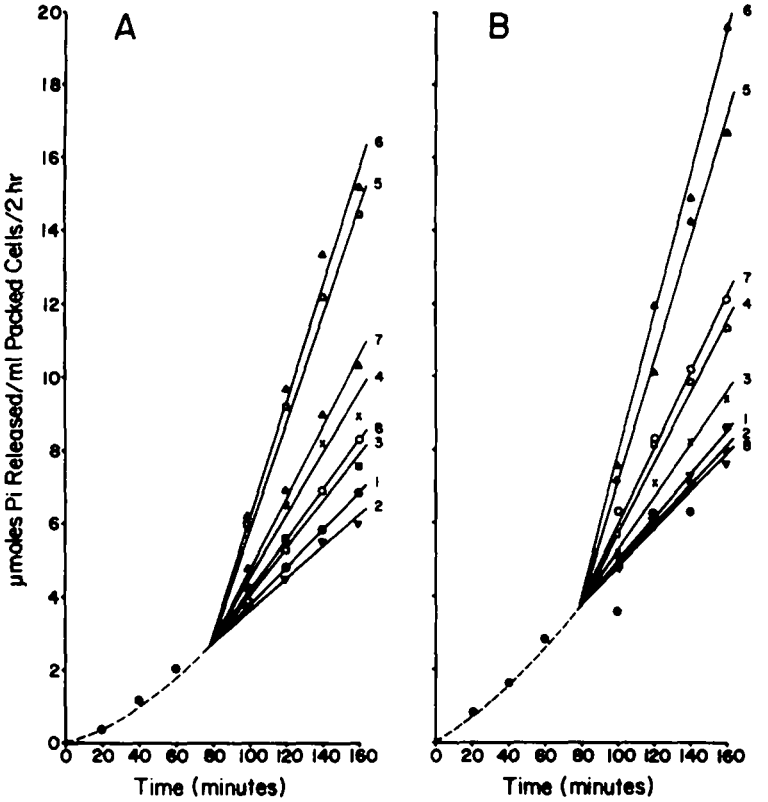


Fig. 4. Effect of EGTA on calcium-induced inhibition of (Ca^{2+} + Mg^{2+})-ATPase activity of human red cell membranes in the absence or presence of hemolysate. Isolated membranes were incubated in the ATPase assay medium containing 0.7 mM $CaCl_2$ without (A) or with (B) hemolysate for 80 min. Aliquots were taken and mixed with varying amounts of EGTA at various time points. The enzyme activity was monitored thereafter for additional 80 min. Concentrations of EGTA used were (1 and 2) 0 mM; (3) 1.8 mM; (4) 0.35 mM; (5) 0.70 mM; (6) 0.88 mM; (7) 1.02 mM; (8) 1.2 mM.

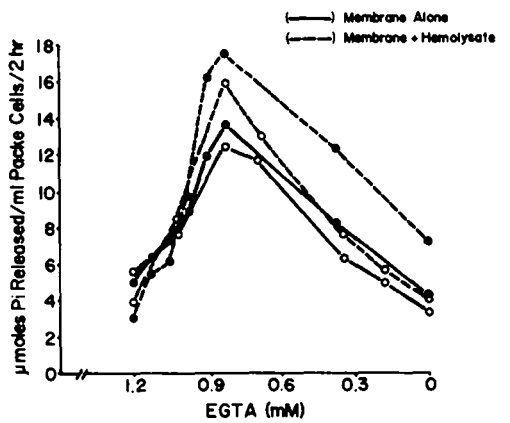


Fig. 5. Effect of EGTA on calcium-induced inhibition of (Ca^{2+} + Mg^{2+})-ATPase activity of human red cell membranes in the absence or presence of hemolysate. The data on reversal of calcium inhibition of (Ca^{2+} + Mg^{2+})-ATPase by EGTA were taken from Fig. 3 (●) and from another similar experiments (○) at 80 min incubation in EGTA and plotted against the concentration of EGTA in the assay medium.

by activator in this time period was small due to a high inhibitory concentration of Ca^{2+}. At the end of this period, incubated samples were mixed with varying amounts of EGTA and ATPase activity was followed for a total of 80 min. For the purpose of comparison, the reversal of the enzyme activity by EGTA at the end of an 80 min EGTA exposure period in Fig. 4 was plotted against EGTA concentration in the medium as shown in Fig. 5. It is evident that upon reduction of calcium in the assay medium, the bulk of inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase activity due to exposure of membranes to high calcium could be reversed. However, the maximum percent activation produced

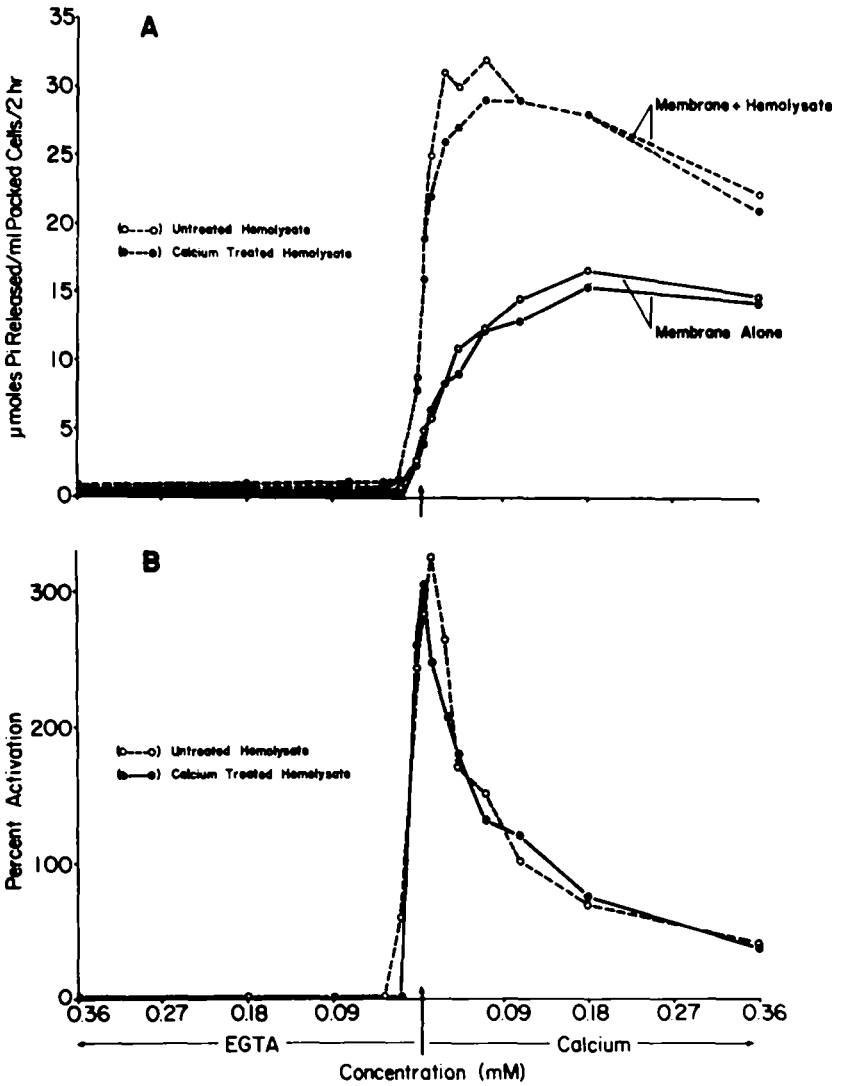


Fig. 6. Stimulatory effect of hemolysate upon high Ca^{2+} exposure. Hemolysate was incubated in 0.7 mM calcium for 30 min at 25°C and then excess calcium was removed by dialysis against 3 l of 20 imosM Tris-HCl buffer, pH 7.6, with frequent changes. Hemolysate without calcium was treated under identical conditions. Activation of membrane $(Ca^{2+} + Mg^{2+})$-ATPase (A) was carried out. Percent activation (B) with calcium-treated and untreated hemolysate at various concentrations of calcium is represented.

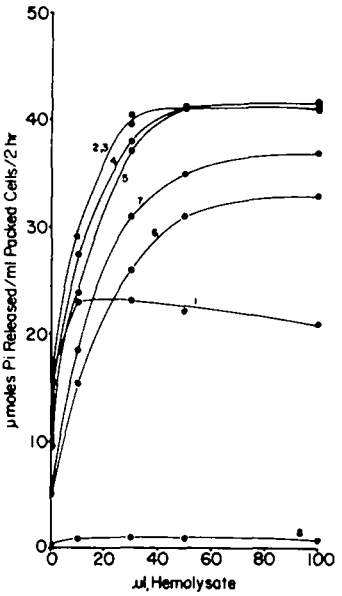


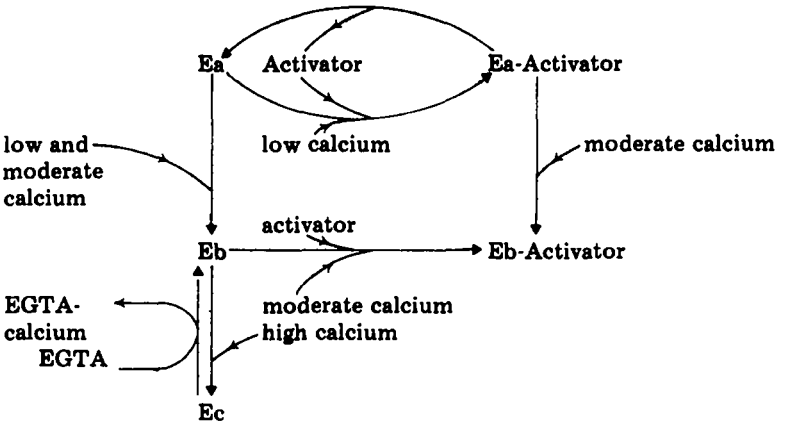
Fig. 7. Effect of increasing amount of hemolysate on $(Ca^{2+} + Mg^{2+})$-ATPase activity at various calcium concentrations. Concentration of membrane-free hemolysate was varied from 10 to 100 µl in 0.7 ml of ATPase assay medium containing the following Ca^{2+} or EGTA. (1) 0.35 mM Ca^{2+}, (2) 0.0012 mM Ca^{2+}, (3) 0 mM Ca^{2+} and 0 mM EGTA, (4) 0.018 mM EGTA, (5) 0.036 mM EGTA, (6) 0.076 mM EGTA, (7) 0.054 mM EGTA and (8) 0.108 mM EGTA.

by activator at low calcium concentration was largely abolished (compare Figs. 3 and 5). The loss of high activation at low calcium concentration could not be attributed to the denaturation of $(Ca^{2+} + Mg^{2+})$-ATPase activator protein in the hemolysate by high Ca^{2+} or EGTA. As shown in Fig. 6, hemolysate upon exposure to 0.7 mM calcium did not significantly alter its pattern of stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase. Furthermore, varying amounts of hemolysate at all calcium concentrations tested showed a maximum activation at 50 µl of hemolysate (Fig. 7) suggesting that increasing amounts of activator could not overcome the calcium-induced inhibitory effect on $(Ca^{2+} + Mg^{2+})$-ATPase activation. Similarly, experiments performed in hemolysed red cells revealed that once the hemolysed red cells were exposed to 0.7 mM calcium, they too lose a part of $(Ca^{2+} + Mg^{2+})$-ATPase activity which could not be recovered even after the sequestration of calcium by EGTA (data not presented). Thus, these data imply that a portion or form of the enzyme which is maximally stimulated by the activator protein was irreversibly damaged upon exposure of membranes to a high calcium, even in the presence of an activator.

Discussion

The main classes of ATPase activity found in human red cells are ouabain-insensitive, Mg^{2+}-stimulated, $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase types [26]. To expose the ATPase activity in red cells it is necessary to disrupt

the cells so as to permit the accessibility of external substrate ATP to the membrane-bound enzyme. Thus, in most of the previous studies on (Ca^{2+} + Mg^{2+})-ATPase, isolated red cell membranes were used. As expected, the activities obtained by various investigators on isolated red cell membranes were different depending upon the procedure of isolation of red cell membranes [27]. In general, under condition, where hemoglobin-free membranes were isolated by hypotonic lysis showed two affinity Ca^{2+}-ATPase activities [28—30] and a stimulation by monovalent cations [31]. Hemoglobin-free membranes in hypotonic media are usually much lower than the isotonic red cell lysates obtained by the use of either the freeze-thaw technique [11,27] or by the application of saponin-induced hemolysis [32]. Only a partial recovery of this activity could be achieved by the addition of membrane-free hemolysate which contains activator of (Ca^{2+} + Mg^{2+})-ATPase of red cell membranes [10—12]. The present study was undertaken to determine the effect of a soluble activator on various calcium affinity ATPase activities in red cell membranes. The data presented in this report show that red cell lysate prepared in isotonic buffer solution had only one optimum activation peak of Ca^{2+}-ATPase as a function of calcium concentration in the assay medium containing monovalent cations and magnesium. In contrast to red cell lysates, isolated membranes under similar conditions of assay showed two optimal activation peaks in response to calcium concentration in the medium. The highest degree of stimulation by the soluble cytoplasmic activator was observed only in low calcium where membranes alone exhibit only a fraction of the maximum Ca^{2+}-ATPase. It is worth pointing out that a calcium concentration which resulted in a decline of activator stimulation was still sufficient to produce an enhancement of Ca^{2+}-ATPase activity in membranes alone (Fig. 3). Thus, these observations may imply that in the membranes, the form of ATPase activity which could be stimulated at low calcium concentration was being converted into another form which is relatively insensitive to the activator with increasing calcium concentration. Further increase in calcium concentrations was found to produce another smaller optimum activity followed by a progressive inhibition. This inactivation can be reversed by sequestration of high calcium with EGTA. The data presented in Fig. 1 and Table I reveal that the cytoplasmic activator at low calcium concentration is capable of lowering the calcium requirement and increasing the V of various active states of the (Ca^{2+} + Mg^{2+})-ATPase. These results corroborate the findings recently reported by Gopinath and Vincenzi [20], Scharff and Fonder [33] and Hanahan et al. [34]. These authors suggested that high-affinity (Ca^{2+} + Mg^{2+})-ATPase is probably an activator-associated enzyme form, while low-affinity (Ca^{2+} + Mg^{2+})-ATPase is a free enzyme form and these forms are interconvertible, depending upon the absence or presence of an activator. This proposal alone, however, does not explain why the (Ca^{2+} + Mg^{2+})-ATPase activity of the reconstituted systems containing hypotonic membrane-free hemolysate as high as found in intact red cells is still lower than the saponin-lysed red cells (Fig. 1 and Table I and Ref. 32). Furthermore, the results on irreversibility of activator stimulation in membranes exposed to high calcium concentrations and on incomplete recovery of ATPase activity in lysed red cells when exposed to high calcium (data not presented) cannot be explained by the above proposal. These



SCHEME 1.

observations suggest that in addition to the effect of an activator, there are certainly other factors including calcium, and the concentration and hypotonicity of the hemolysing medium used for membrane preparation which play a critical role in the maintenance of the various forms of red cell membrane ($Ca^{2+} + Mg^{2+}$)-ATPase. To account for these effects, the following tenative working hypothesis is proposed in scheme 1.

Ea represents a form of Ca^{2+}-ATPase activity which is highly activated by the soluble activator at low calcium and is termed as Ea-activator. This state is reversible only at low calcium concentrations. This part of the scheme is similar to that presented by Scharff and Fonder [33]. Eb is the transformed form of Ea. This transformation takes place due to exposure of red cells to hypotonic medium which accompanies the release of a soluble activator. Similarly, this transformation occurs on storage of membranes and can be blocked partially if membranes are stored at 4°C in the presence of a soluble activator (Luthra, M., unpublished results), suggesting that a soluble activator probably favors the maintenance of the Ea form of the enzyme in isolated membranes. More importantly, if Ea is converted to Eb due to moderate calcium concentration in the assay, such a transformation probably cannot be reversed by a soluble activator. On the other hand, with further increase in calcium concentration, the Eb form is converted into another state which is most probably an inactive form of the enzyme. Data not presented here show that 2.5 mM added calcium concentration completely inhibited the Ca^{2+}-ATPase activity of isolated membranes. However, the reversal of Ec to the Eb form can be achieved by the addition of EGTA in the assay (Fig. 4). In the above scheme, both Ea and Eb forms of the enzyme are shown to form Ea-activator and Eb-activator, while Ea-activator is the only highly active form of the enzyme. Eb-activator form can be produced from Ea-activator if calcium concentration in the assay is increased (Fig. 1 and 2). Whether or not all these forms exist in intact functional cells is a matter of pure speculation. Furthermore, the role of membrane phospholipids which are known to affect Ca^{2+}-ATPase activity of isolated membranes [35] and partially purified Ca^{2+}-ATPase [36] are still to be ascertained in terms of their influence on various states of the enzyme.

Acknowledgements

The authors wish to acknowledge Barbara Kingsolver and Karen Scherer for their excellent technical assistance and Dr. R.P. Watts for his valuable suggestions. This work was supported in part by grants from the American Heart Association, Arizona affiliate to M.G. Luthra and the National Institute of Health Grant (No. AM 17723) to H.D.K. One of us (H.D.K.) is the recipient of a Research Career Development Award of the NIH AM 00316.

References

1 Passow, H. (1963) in Cell Surface Reactions (Brown, H.D., ed.) p. 57 Scholars Library, New York
2 Olson, E.J. and Cazort, R.J. (1969) J. Gen. Physiol. 53, 311—315
3 Schatzman, H.J. and Vincenzi, F.F. (1969) J. Physiol. 201, 369—395
4 Lacelle, P.L., Kirkpatrick, F.H., Udkow, M.P. and Adkin, B. (1973) in Red Cell Shape: Physiology-Pathology-Ultrastructure (Besis, M., Weed, R.E. and Leblond, P.F., eds.), pp. 68—78, Springer Verlag, New York
5 Eaton, J.W., Skelton, T.D., Swofford, H.S., Koplin, C.E. and Jacob, H.S. (1973) Nature 246, 105—106
6 Palek, J. (1973) Blood 42, 988
7 Kirkpatrick, F.H., Hillman, D.G. and La Celle, P.L. (1975) Experientia 31, 653—654
8 Gárdos, G. (1958) Acta Physiol. Hung. 14, 1—5
9 Szász, I. and Gárdos, G. (1974) FEBS Lett. 44, 213—216
10 Bond, G.H. and Clough, D.L. (1973) Biochim. Biophys. Acta 323, 592—599
11 Luthra, M.G., Hildenbrandt, G.R. and Hanahan, D.J. (1976) Biochim. Biophys. Acta 419, 164—179
12 Luthra, M.G., Hildenbrandt, G.R., Kim, H.D. and Hanahan, D.J. (1976) Biochim. Biophys. Acta 419, 180—186
13 Luthra, M.G., Au, K.S. and Hanahan, D.J. (1977) Biochem. Biophys. Res. Commun. 77, 678—687
14 Jarret, H.W. and Penniston, J.T. (1978) J. Biol. Chem. 253, 4676—4682
15 Macintyre, J.D. and Green, J.W. (1977) Fed. Proc. 36, 271
16 Hinds, T.R., Larson, F.L. and Vincenzi, F.F. (1978) Biochem. Biophys. Res. Commun. 81, 455—461
17 Cheug, W.Y. (1971) J. Biol. Chem. 246, 2859—2869
18 Kakiuchi, S., Yamazaki, R. and Nakajima, H. (1970) Proc. Jap. Acad. Sci. 46, 587—592
19 Dabrowska, R., Sherry, J.W.F., Aromatorio, D.K. and Hartshorne, D.J. (1978) Biochemistry 17, 253—258
20 Gopinath, R.M. and Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203—1209
21 Jarret, H.W. and Penniston, J.T. (1977) Biochem. Biophys. Res. Commun. 77, 1210—1216
22 Luthra, M.G. and Kim, H.D. (1979) Life Sci. 24, 2441—2448
23 Fiske, H. and Subbarow, J. (1975) J. Biol. Chem. 66, 375—400
24 Katz, A.M., Repke, D.I., Upshaw, J.E. and Polaseik, M.A. (1970) Biochim. Biophys. Acta 205, 473—490
25 Ogawa, Y. (1968) J. Biochem. Tokyo 64, 255—258
26 Schrier, S. (1977) Blood 50, 227—237
27 Hanahan, D.J., Ekholm, J. and Hildenbrandt, G.R. (1973) Biochemistry 12, 1374—1387
28 Wolf, H.V. (1972) Biochem. J. 130, 311—314
29 Quist, E.E. and Roufogalis, B.D. (1975) Arch. Biochem. Biophys. 168, 240—251
30 Scharff, O. and Fonder, B. (1977) Biochim. Biophys. Acta 483, 416—424
31 Bond, G.H. and Green, J.W. (1971) Biochim. Biophys. Acta 241, 393—398
32 Hanahan, D.J. and Ekholm, J.E. (1978) Arch. Biochem. Biophys. 187, 170—179
33 Scharff, D. and Fonder, B. (1978) Biochim. Biophys. Acta 509, 67—77
34 Hanahan, D.J., Taverna, R.D., Flynn, D.D. and Ekholm, J.E. (1978) Biochem. Biophys. Res. Commun. 84, 1009—1015
35 Ronner, P., Gazzottis, P. and Carafoli, E. (1977) Arch. Biochem. Biophys. 179, 578—583
36 Peterson, S.W., Ronner, P. and Carafoli, E. (1978) Arch. Biochem. Biophys. 186, 202—210