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REVERSIBLE SHIFT BETWEEN TWO STATES OF Ca^{2+}-ATPase IN HUMAN ERYTHROCYTES MEDIATED BY Ca^{2+} AND A MEMBRANE-BOUND ACTIVATOR

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Summary

The (Ca^{2+} + Mg^{2+})-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) from human erythrocytes occurred in two different states, A-state and B-state, depending on the membrane preparation.

The A-state showed low maximum activity (V) and the Ca^{2+} activation was characterized by a Hill coefficient, n_H, of about 1 and a Michaelis constant, K_{Ca}, about 30 μM.

The B-state showed high V, a n_H above 1, which indicates positive cooperativity of Ca^{2+} activation, and a K_{Ca} of about 1 μM.

With varying ATP concentrations, both the A-state and the B-state showed negative cooperativity and slightly different values of K_m.

The B-state was shifted to the A-state when the membranes were exposed to low Ca^{2+} concentrations. The shift reached 50% at approx. 0.5 μM Ca^{2+}. At the low Ca^{2+} concentrations an activator was released from the membranes.

The A-state was shifted to the B-state when the membranes were exposed to Ca^{2+} in the presence of the activator. The shift reached 50% at about 30 μM Ca^{2+}. The recovery of high V was time dependent and lasted several minutes. Increasing concentrations of Ca^{2+} and activator accelerated the recovery.

It is suggested that the A-state and the B-state correspond to enzyme free of activator and enzyme associated with activator, respectively. Furthermore, the two states may represent a resting and an active state, respectively, of the calcium pump.

Introduction

The Ca^{2+}-stimulated ATPase of erythrocyte membranes may exist in at least two different states [1], one state, showing high Ca^{2+} affinity and high maximum activity, being obtained when the Ca^{2+} concentration exceeds 10^{-7}—10^{-6} M

during the hemolytic step of membrane preparation. At Ca^{2+} concentrations below 10^{-7} M the ATPase assumes another state showing reduced Ca^{2+} affinity and low maximum activity [1,2].

It has been demonstrated that the membrane-free hemolysate contains a protein activator which, under certain circumstances, increases the maximum activity of the Ca^{2+}-ATPase (Bond and Clough [3]. Luthra et al. [4]). However, only the ATPase state with reduced Ca^{2+} affinity and low maximum activity is activated by the membrane-free hemolysate, whereas the other state is unaffected [1].

In the present investigation it is shown that the membranes prepared at Ca^{2+} concentrations above 10^{-6} M contain an activator which is able to increase the Ca^{2+} affinity and the maximum activity of the Ca^{2+}-ATPase of membranes prepared at Ca^{2+} concentrations below 10^{-7} M. The effect of the activator is dependent on the presence of Ca^{2+}.

Methods

Preparation of erythrocyte membranes

Ghosts were prepared from recently outdated bank blood as described previously [1]. The centrifugation of the membrane suspensions was performed in a Sorvall continuous flow device which secures full accessibility of the Ca^{2+}-ATPase [2]. By varying the hemolyzing buffer, two different types of membranes were prepared.

A-membranes. The hemolyzing buffer contained 6.7 mM sodium phosphate, 1.0 mM ethyleneglycol bis(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), pH 7.4, leading to pH 7.7—7.9 and a *p*Ca (—log [Ca^{2+}]) below 10 during hemolysis. The hemolysis was succeeded by two washings of the membranes with 10 mM Tris · HCl, pH 7.6, at 22° C.

In experiments including preincubation of A-membranes in the presence of activator (cf. below), the frozen-thawed A-membranes were washed prior to preincubation, once with 1.2 mM EGTA/70 mM Tris · HCl, pH 7.4 at 37° C and once with 70 mM Tris · HCl to remove traces of activator.

B-membranes. The hemolyzing buffer contained 6.7 mM sodium phosphate, 1.0 mM nitrilotriacetic acid, and 0.7 mM $CaCl_2$ leading to *p*Ca 4.5 during hemolysis and pH as above. During the two succeeding washings, *p*Ca varied between 5 and 6.

Preparation of activator

Unless otherwise stated, the activator was prepared by extracting B-membranes with 1.2 mM EGTA in 70 mM Tris · HCl, pH 7.4, at 37° C for 5 min (10—15 g dry membrane per l). The suspension was centrifuged 15 min at 45 000 × *g* and the supernatant constituted the activator. The concentration of dissolved membrane material, given as mg dry matter per l, was used as a measure of activator concentration.

Determination of ATPase activity

P_i method. The activity was assayed [5] by measuring P_i liberated at 37° C in a medium of 3 mM Tris · ATP/4 mM $MgCl_2$/1 mM EGTA or nitrilotriacetic acid

[2]/various concentrations of $CaCl_2$/70 mM Tris · HCl/0.7—1.0 g dry membrane per l medium, pH 7.2.

In some experiments the concentrations of Tris · ATP and $MgCl_2$ were varied in order to determine the Michaelis constant for the substrate. The ATP concentration was kept constant during the assay by addition of 2.5 mM phosphoenolpyruvate and 30 mg per l medium of pyruvate kinase. These ATPase determinations were performed in the presence of optimum Ca^{2+} concentrations and 1.0 mM Mg^{2+}. The concentrations of ATP, Mg · ATP, and free magnesium ion were calculated on the basis of stability constants [6].

ADP method. ADP production at 37°C was linked to NADH oxidation and monitored continuously at 366 nm. The medium of the two methods was identical except for ATP (1.7 mM), $MgCl_2$ (3 mM), dry membrane (0.4—1.0 g per l medium) and the addition of 0.42 mM NADH, 2.5 mM phosphoenolpyruvate and 40 mg per l medium of pyruvate kinase/lactate dehydrogenase (Boehringer Mannheim).

The specific activity was not dependent on the membrane concentrations used during ATPase assay.

The Ca^{2+}-stimulated ATPase activity was determined as the difference between (Ca^{2+} + Mg^{2+})-dependent and Mg^{2+}-dependent activity and expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$ dry membrane exclusive of hemoglobin.

Methods of analysis

The determinations of pH, dry matter, protein, hemoglobin, ATP, calcium (atomic absorption) and Ca^{2+} (ion selectrode) were performed as previously described [2,5].

Results

Kinetics of Ca^{2+}-ATPase in A-membranes and B-membranes

Ca^{2+} activation. It appears from Fig. 1 and Table I that the Ca^{2+}-ATPase of non-preincubated A-membranes showed much higher Michaelis constant for Ca^{2+}, K_{Ca}, lower Hill coefficient, n_H, and lower maximum activity, V, than non-preincubated B-membranes in accordance with previous results [1,2].

Effect of ATP concentration. Contrary to the kinetics of Ca^{2+} activation, the kinetics of A-membranes and B-membranes, obtained by varying the concentration of ATP, were not very dissimilar (Table II). The Michaelis constant, K_m, of the A-membranes, whether calculated on the basis of Mg · ATP or uncomplexed ATP (cf. Rega and Garrahan, ref. 7), was a little lower than the K_m of B-membranes. However, the negative cooperativity, demonstrated by Wolf et al. [8], was found in both A-membranes and B-membranes, as indicated by the low Hill coefficients ($n_H < 1$, Table II). The K_m of B-membranes based on Mg · ATP agrees with the value reported for membrane-bound Ca^{2+}-ATPase by Wolf et al. [8]. The K_m of A-membranes was not changed when the Ca^{2+} concentration during ATPase assay was 30 μM instead of 150 μM (not shown).

Preincubation of B-membranes

The different properties of A-membranes and B-membranes were caused by exposure of the membranes to different Ca^{2+} concentrations during membrane

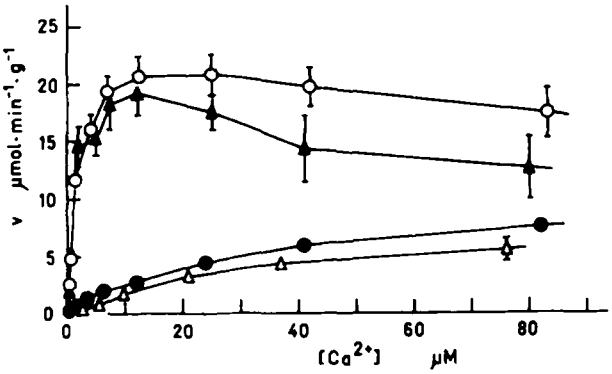

Fig. 1. Ca^{2+}-stimulated ATPase activities (v) in different membrane preparations dependent on Ca^{2+} concentration during ATPase assay. The results are of three experiments with different blood specimens. ATPase medium: 3 mM Tris · ATP/4 mM $MgCl_2$/1 mM EGTA or nitrilotriacetic acid/various concentrations of $CaCl_2$/70 mM Tris · HCl/0.7—1.0 g dry membrane per l medium, pH 7.2. P_i production measured. ●, A-membranes; ▲, A-membranes (2—3 g dry matter per l medium) preincubated for 1 h at 37°C with 140 μM Ca^{2+} and activator (cf. Methods, 400 mg dry matter per l medium) in 70 mM Tris · HCl, pH 7.4, succeeded by two washings in 10 μM Ca^{2+}/70 mM Tris · HCl to remove excess of activator. ○, B-membranes; △, B-membranes preincubated for 0.5 h at 37°C with 1 mM EGTA in 70 mM Tris · HCl, pH 7.4, two washings in 70 mM Tris · HCl. Vertical bars, ±S.E.

TABLE I

EFFECT OF PREINCUBATION ON K_{Ca}, n_H, AND V OF Ca^{2+}-STIMULATED ATPase IN A-MEMBRANES AND B-MEMBRANES

The three experiments from Fig. 1. Michaelis constant and Hill coefficient for Ca^{2+}-activation (K_{Ca} and n_H), and maximum activity (V) were determined as previously described [2]. Mean ± S.E.

	A-membranes		B-membranes	
	Non-preincubated	Preincubated	Non-preincubated	Preincubated
K_{Ca} (μM)	32.7 ± 2.0	1.09 ± 0.08	1.43 ± 0.19	33.4 ± 5.1
n_H	0.87 ± 0.12	2.07 ± 0.12	1.49 ± 0.13	1.14 ± 0.04
V (μmol · min^{-1} · g^{-1})	10.1 ± 0.5	19.2 ± 1.8	22.6 ± 2.3	8.0 ± 1.7

TABLE II

DEPENDENCE ON ATP CONCENTRATION OF Ca^{2+}-ATPase IN A-MEMBRANES AND B-MEMBRANES

Two experiments with different blood specimens. ATPase assay as in Fig. 1, except for addition of 2.5 mM phosphoenolpyruvate and 30 mg per l medium of pyruvate kinase, in order to keep the ATP level constant during assay. Ca^{2+} concentrations during assay were 150 μM (A-membranes) and 30 μM (B-membranes). Michaelis constant (K_m), on the basis of ATP or Mg · ATP (cf. text), and Hill coefficient (n_H) were determined as previously described [2]. Mean ± S.E., tested by Student's t-test.

	A-membranes	B-membranes	Significance
K_m ATP (μM)	1.45 ± 0.26	3.23 ± 0.22	$P < 0.05$
K_m Mg · ATP (μM)	16.5 ± 2.9	36.3 ± 2.3	$P < 0.05$
n_H	0.73 ± 0.01	0.74 ± 0.05	$P > 0.5$

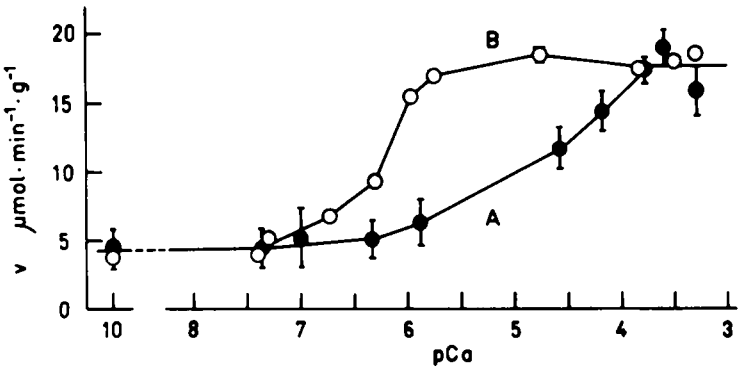

Fig. 2. Ca^{2+}-stimulated ATPase activity (v) of preincubated A-membranes and B-membranes vs. pCa during preincubation. The results are of two experiments with different blood specimens. ATPase assay as in Fig. 1, 40 μM Ca^{2+} during assay. A-membranes (●): preincubation, 1 h at 37°C, 2—3 g dry membrane per 1 medium/activator (670 mg dry matter per 1 medium)/1 mM EGTA/various concentrations of $CaCl_2$/70 mM Tris · HCl, pH 7.4. B-membranes (○): preincubation as A-membranes, except for omission of activator. The preincubated membranes were washed as in Fig. 1. Vertical bars, ±S.E.

preparation [2]. Preincubating B-membranes with EGTA at 37°C and an ionic strength of 0.075 M, K_{Ca} of the Ca^{2+}-ATPase increased and V was reduced to the level of non-preincubated A-membranes (Fig. 1, Table I). Fig. 2 shows that v decreased when the membranes were preincubated with decreasing Ca^{2+} concentrations, especially in the region of 10^{-6}—10^{-7} M. This is similar to the change of kinetics obtained by varying the Ca^{2+} concentration during the hemolytic step of membrane preparation at 8°C and an ionic strength of 0.025 M [2].

Centrifuging the preincubated B-membranes, an activating factor was detectable in the supernatant. In order to investigate the effect of this factor an EGTA extract was prepared from B-membranes (cf. Methods). In the following this extract is called activator, unless otherwise stated.

Preincubation of A-membranes with activator

Preincubating A-membranes in the presence of activator and 140 μM Ca^{2+}, K_{Ca} of the Ca^{2+}-ATPase decreased and V increased to the level of non-preincubated B-membranes (Fig. 1, Table I). The n_H increased above the level of B-membranes. However, n_H in B-membranes was previously found to be 1.8 [2].

Reducing the Ca^{2+} concentration during preincubation, the ATPase activity of A-membranes was only partly reversed to the level of B-membranes, and below 10^{-6} M Ca^{2+} there was no effect of preincubation in the presence of activator (Fig. 2).

The results above indicate that the shift between the A-state and the B-state of Ca^{2+}-ATPase is reversible. The shift from A to B occurred in the presence of activator and Ca^{2+} and, according to Fig. 2, the shift reached 50% at 26 μM Ca^{2+}. However, the shift from B to A reached 50% at 0.5 μM Ca^{2+} (Fig. 2). These effective Ca^{2+} concentrations are similar to the values of K_{Ca} for non-preincubated A-membranes (33 μM) and B-membranes (1.4 μM), respectively (Table I), suggesting that Ca^{2+} affects the shift of state through binding to the

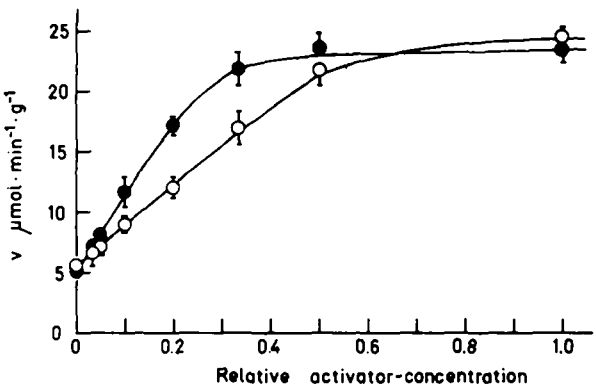

Fig. 3. Ca^{2+}-stimulated ATPase activity (v) of preincubated A-membranes vs. relative activator concentration (Ap_t) during preincubation. The results are of two or three experiments. ATPase medium as in Fig. 1, except for 1.7 mM ATP/3 mM $MgCl_2$/0.42 mM NADH/2.5 mM phosphoenolpyruvate/40 mg per l medium of pyruvate kinase/lactate dehydrogenase. Ca^{2+} concentration during assay was 40 μM. ADP production is measured. Preincubation, 1 h at 37°C, activator (relative concentration 1.0 corresponds to 340 mg dry matter per l medium)/1 mM EGTA/$CaCl_2$ yielding 100—140 μM Ca^{2+}/70 mM Tris · HCl, pH 7.4, succeeded by washings as in Fig. 1. The curves were calculated as described in the text assuming one activator site per enzyme molecule. •, 1.35 g dry membrane per l medium of preincubation; ○, 2.70 g dry membrane per l. Vertical bars, ±S.E.

Ca^{2+} sites which are involved in the Ca^{2+} activation of the ATPase.

Further experiments (not shown) demonstrated that the activating factor could be released by EGTA treatment of preincubated and washed A-membranes.

Effect of activator concentration

Fig. 3 shows that with the preincubation of A-membranes in the presence of activator and 10^{-4} M Ca^{2+}, the effect increased proportionally to low activator concentrations up to saturation. In the absence of activator the ATPase activity was about 5 $\mu mol \cdot min^{-1} \cdot g^{-1}$, as in non-preincubated A-membranes (Fig. 1), i.e., Ca^{2+} alone had no effect during preincubation. Lowering the membrane concentration during preincubation to half of the usual, the saturating effect of the activator was increased, half maximum activation being achieved at relative activator concentrations of 0.15 and 0.29 at the low and high membrane concentrations, respectively.

These results are consistent with the assumption that the activator preparation contains an activating factor which binds to the enzyme during the preincubation in the presence of Ca^{2+}. The nearly linear dependence on low activator concentrations in Fig. 3 and the dependence on membrane concentration may be explained by assuming that the Ca^{2+}-ATPase, in the presence of 10^{-4} M Ca^{2+}, binds nearly all the activating factor added up to saturation, i.e., the activating factor is bound to the enzyme with very high affinity. This view is supported by the observed stability of the B-state of the Ca^{2+}-ATPase even during prolonged preparation procedures in the presence of Ca^{2+} but in the absence of added activator [5,8].

Applying an enzyme model with one binding site for the proposed activator, it was possible to calculate saturation curves as those shown in Fig. 3. However,

models with more than one activator site per mol enzyme fitted the observations as well. The calculations (not shown) were based on the assumption that the saturating concentration of the activator was of the same order of magnitude as the concentration of enzyme and that the apparent Michaelis constant for the activator stimulation was much lower than the enzyme concentration.

Various activator-preparations added during ATPase assay

Fig. 4 shows that other activator preparations, viz. dialyzed membrane-free hemolysate prepared accroding to Bond and Clough [3] and activator prepared according to Luthra et al. [4], increased the activity of A-membranes in a way similar to the activator prepared from B-membranes. In analogy with the activator prepared by Bond and Clough the effect of the activator from B-membranes was completely destroyed by treatment with trypsin.

This type of experiment was mainly performed by continuous recording of ADP production. The activities (v), which were time-dependent in the presence of activator, were determined at the end of the incubation when v was approximately constant. Nevertheless, the ATPase activities of the non-preincubated A-membranes were much lower than the activity of the preincubated A-membranes at Ca^{2+} concentrations below 20 μM during assay. This is in accordance with the low reversing effect of low Ca^{2+} concentrations during preincubation (Fig. 2).

When no activator was added during ATPase assay, the time-dependence of v was insignificant.

The activator itself showed no Ca^{2+}-ATPase activity in the absence of membranes.

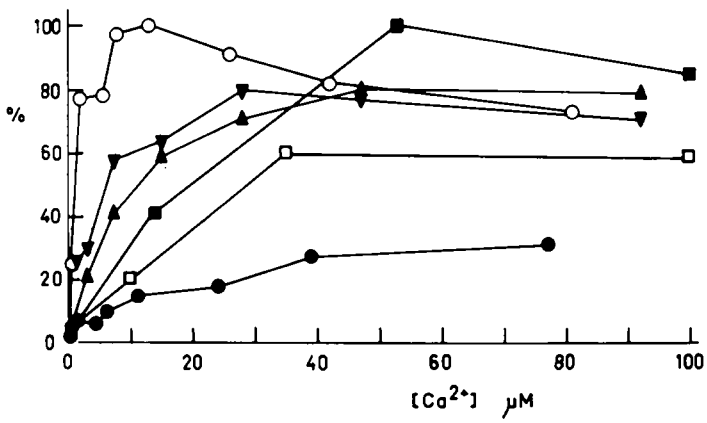

Fig. 4. Relative Ca^{2+}-stimulated ATPase activities of A-membranes vs. Ca^{2+} concentration during ATPase assay, in the presence of various activator preparations. The results are of a single experiment. ATPase assay as in Figs. 1 or 3, including various additions. ●, no addition; □, added activator (cf. Methods, 35 mg dry matter per 1 medium); ■, added activator (350 mg dry matter per l); ▲, medium contained 50% of activator prepared according to Luthra et al. [4]; ▼, medium containing 50% of dialyzed, membrane-free hemolysate prepared according to Bond and Clough [3]; ○, preincubated A-membranes prepared as in Fig. 1, serving as reference, i.e., V is 100%.

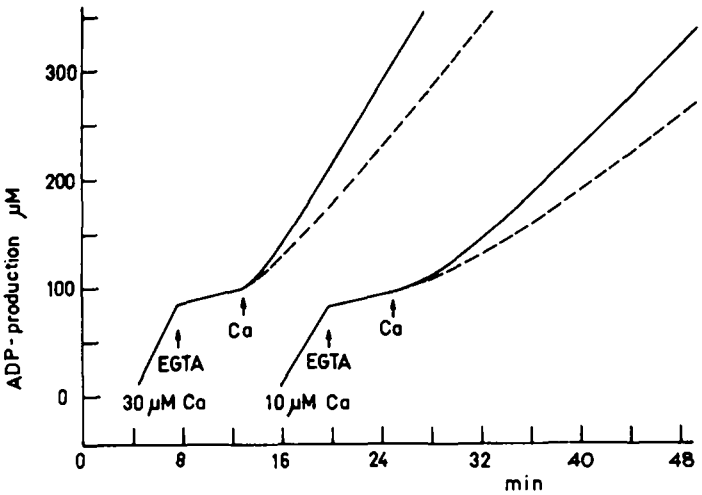

Fig. 5. Time-dependent changes of ATPase activity of B-membranes, produced by changes of Ca^{2+} concentration. The results are of a single experiment. ATPase assay as in Fig. 3, 0.7 g dry membrane per l ATPase medium. Initial Ca^{2+} concentrations were 30 and 10 µM, as indicated. Enzyme reaction was started by addition of membrane suspension. Solid line, activator present during assay (400 mg dry matter per l medium); dotted line, no activator added; EGTA, addition of 24 mM EGTA/0.1 M Tris · HCl, pH 7.3, reducing Ca^{2+} concentration to below 10^{-7} M; Ca, addition of $CaCl_2$ solution, restoring initial Ca^{2+} concentration.

Time-dependent shift between two states of Ca^{2+}-ATPase

Fig. 5 shows that the ATPase activity of B-membranes was reduced rapidly as a result of the addition of EGTA which reduces the Ca^{2+} concentration to below 10^{-7} M. In re-establishing the Ca^{2+} concentration by addition of Ca^{2+}, both the recovery of the ATPase activity and the time elapsing until this recovery took place depended on the concentrations of Ca^{2+} and activator (Fig. 5 and Table III). With addition of activator, the time increased with decreasing Ca^{2+} concentrations. With no activator added the time increased to

TABLE III

TIME-DEPENDENT RECOVERY OF Ca^{2+}-STIMULATED ATPase ACTIVITY IN B-MEMBRANES AFTER EGTA EXPOSURE

Experimental procedures as in Fig. 5. Recovery was the activity at the shown Ca^{2+} concentrations obtained after the indicated time following exposure to excess of EGTA in per cent of activity at the same Ca^{2+} concentration before the increase of EGTA concentration. Ca^{2+} concentrations of the used Ca^{2+}-EGTA buffers were measured as described previously [1]. Mean of three experiments ± S.E.

Ca^{2+} concentration (µM)	No activator added		Activator added	
	Recovery (%)	Time to recovery (min)	Recovery (%)	Time to recovery (min)
1.4 ± 0.1	8 ± 2	15.1 ± 1.0	28 ± 6	13.5 ± 0.8
10.9 ± 0.4	42 ± 6	16.6 ± 2.3	73 ± 5	10.2 ± 4.5
35.3 ± 2.7	67 ± 3	12.2 ± 1.4	80 ± 3	4.3 ± 0.7
106.3 ± 4.3	74 ± 3	14.1 ± 6.8	89 ± 6	1.0 ± 1.7

about 15 min and the activity reached an approximately constant value. Below 10 μM Ca^{2+} the activity did not become constant within the investigated period whether the activator was added or not. The recovery in the presence of Ca^{2+} but with no activator added may be explained by the presence of activating factor arising from the B-membranes. The initial activities were not influenced by the addition of activator. Comparing Fig. 4 and Table III with Fig. 2, it would appear that lower Ca^{2+} concentrations were needed to reverse the A-state to B-state during the ATPase reaction than in the presence of only activator and Ca^{2+}. However, more experiments are needed for elucidation.

These experiments indicate that the recovery of the Ca^{2+}-stimulated ATPase activity of B-membranes which have been exposed to EGTA for a few minutes proceeds extremely slowly under the investigated conditions. Preliminary experiments showed that the recovery was slow even if the EGTA exposure lasted only a few seconds.

Discussion

Reversible shift between two states

The dependence on Ca^{2+} of the kinetic state of Ca^{2+}-ATPase was demonstrated previously [1,2,5]. The present results show that another factor, besides Ca^{2+}, is involved in a reversible shift between the two states of the ATPase. The activating factor is probably a protein (Ap) which may bind to the ATPase (E) together with Ca^{2+}, changing the conformational state of the enzyme. Eqn. 1 represents a possible overall reaction for the reversible shift. The experiments allow no conclusions concerning the sequence of binding of E, Ap or Ca^{2+}.

$$E_A + m\mathrm{Ap} + n\mathrm{Ca} \rightleftharpoons E_B \cdot \mathrm{Ap}_m \cdot \mathrm{Ca}_n \qquad (1)$$

m and n are the number of sites. The value of n was shown to be at least two [1], whereas m may be one or more (cf. Fig. 3). The B-state of the Ca^{2+}-ATPase with K_{Ca} about 1 μM and high V may be represented by $E_B \cdot \mathrm{Ap}_m \cdot \mathrm{Ca}_n$. The A-state showing high K_{Ca} and low V may correspond to E_A. However, it cannot be excluded that the A-membranes include som residual activator, i.e. the A-state may be a mixture of E-species.

A similar co-action of a protein activator and Ca^{2+} is known from the activation of 3′,5′-cyclic-AMP phosphodiesterase [10,11] and adenylate cyclase [12].

Hysteretic behaviour of Ca^{2+}-ATPase

Lowering the Ca^{2+} concentration to below 10^{-7} M by addition of EGTA, the ATPase activity decreased rapidly (Fig. 5). Besides interrupting the Ca^{2+} activation, the EGTA addition may release some activating factor from the B-membranes, i.e., the reaction in Eqn. 1 is shifted to the left.

However, increasing the Ca^{2+} concentration after EGTA addition, the activity recovered slowly (Fig. 5 and Table III). According to Frieden [9] who defined hysteretic enzymes as "enzymes which respond slowly to rapid changes in ligand concentration", the slow recovery of the ATPase activity can be characterized as a hysteretic response. This phenomenon may be due to a slow association of enzyme and activating factor together with Ca^{2+}, i.e., the reaction in Eqn. 1 is shifted slowly to the right. This view is supported by the

accelerating effect on the reaction of increasing concentrations of Ca^{2+} and activator (Table III). Furthermore, analysis according to Frieden [9] of the part of the time curves which succeeds the last Ca^{2+} addition in Fig. 5 excluded the possibility of a first order reaction. The kinetics of the shift from A-state to B-state may be rather complicated because the crude activator preparation used in these experiments may contain other cellular components which may interfere with the proposed association of enzyme and activating factor.

If it is assumed that the intracellular Ca^{2+} concentration may fluctuate (cf. ref. 2), at least in part of the life of the erythrocyte, the slow recovery of the ATPase activity, i.e., the hysteretic response, may reflect an important mechanism. Provided that the Ca^{2+}-ATPase is involved in the outward pumping of Ca^{2+} (cf. Schatzmann [13] and Ferreira and Lew [14]), the dissociated and associated states of the ATPase may correspond to a resting and an active state, respectively, of the Ca^{2+} pump, as suggested previously [1]. This theory is supported by Macintyre and Green [15], showing a stimulating effect of membrane-free hemolysate on the Ca^{2+} pump in inside-out vesicles from human erythrocytes. If the intracellular Ca^{2+} concentration is increased by some stimulus, the association of enzyme and activator and the resulting increase of pumping rate may require some time, permitting the development of a Ca^{2+} signal of an appropriate duration.

The question is if the hysteretic response occurs in the intact cell. It has been emphasized by Luthra et al. [4] that the concentration of activator in the intact erythrocyte is about 8 times the concentration which is necessary to saturate the ATPase. This may imply that the association in the cell proceeds faster than in the present experiments. However, this effect is counteracted by the intracellular Ca^{2+} concentration which is probably lower than the Ca^{2+} concentration used in vitro to ensure the association of activator and enzyme. The observation by Lassen et al. [16] that the recovery of the resting potential of *Amphiuma* red cell membranes, following a Ca^{2+}-induced hyperpolarization, lasted several minutes may be interpreted as a result of time-dependent changes of Ca^{2+} pump activity. However, other interpretations are also possible.

Experiments with more concentrated activator preparations combined with physiological concentrations of Ca^{2+} are in progress to study the hysteretic behaviour of the reversible shift which may be essential to the regulation of the activity of Ca^{2+}-ATPase in the intact cell.

Addendum

After completion of this paper, three investigations have appeared concerning an activator of the Ca^{2+}-ATPase from human erythrocytes [17—19]. The activator, which is most probably identical with the activating factor described above, resembles or is identical to [18,19] the activator of cyclic-AMP phosphodiesterase [10,11] and adenylate cyclase [12].

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