Biochimica et Biophysica Acta, 649 (1981) 367–376
Elsevier/North-Holland Biomedical Press

BBA 79516

DECREASE OF APPARENT CALMODULIN AFFINITY OF ERYTHROCYTE (Ca^{2+} + Mg^{2+})-ATPase AT LOW Ca^{2+} CONCENTRATIONS

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(Received 16th July, 1981)

Key words *(Ca^{2+} + Mg^{2+})-ATPase, Calmodulin affinity, Ca^{2+}; (Human erythrocyte)*

The calmodulin activation of the (Ca^{2+} + Mg^{2+})-ATPase (ATP phosphohydrolase, EC 3.6.1.3) in human erythrocyte membranes was studied in the range of 1 nM to 40 μM of purified calmodulin. The apparent calmodulin-affinity of the ATPase was strongly dependent on Ca^{2+} and decreased approx. 1000-times when the Ca^{2+} concentration was reduced from 112 to 0.5 μM. The data of calmodulin (Z) activation were analyzed by the aid of a kinetic enzyme model which suggests that 1 molecule of calmodulin binds per ATPase unit and that the affinities of the calcium-calmodulin complexes (Ca_iZ) decreases in the order of $Ca_3Z > Ca_4Z > Ca_2Z \geqslant CaZ$. Furthermore, calmodulin dissociates from the calmodulin-saturated Ca^{2+}-ATPase in the range of 10^{-7}–10^{-6} M Ca^{2+}, even at a calmodulin concentration of 5 μM. The apparent concentration of calmodulin in the erythrocyte cytosol was determined to be 3 to 5 μM, corresponding to 50–80-times the cellular concentration of Ca^{2+}-ATPase, estimated to be approx. 10 nmol/g membrane protein. We therefore conclude that most of the calmodulin is dissociated from the Ca^{2+}-transport ATPase in erythrocytes at the prevailing Ca^{2+} concentration (probably 10^{-7}–10^{-8} M) in vivo, and that the calmodulin-binding and subsequent activation of the Ca^{2+}-ATPase requires that the Ca^{2+} concentration rises to 10^{-6}–10^{-5} M.

Introduction

The (Ca^{2+} + Mg^{2+})-ATPase, which is part of the Ca^{2+} pump in erythrocytes [1–6], is stimulated by an endogenous protein activator [7–9]. It has been demonstrated that the activator stimulates the ATPase by Ca^{2+}-dependent binding to the enzyme [10–14], and the activator has been shown to be identical with calmodulin [15–17].

Before this demonstration it was shown that, depending on the absence or presence of Ca^{2+} during membrane preparation, two different types of (Ca^{2+} + Mg^{2+})-ATPase can be obtained in membranes from human erythrocytes [9,18]. The two kinetic types correspond to membrane ATPase deficient in or saturated with calmodulin [11,19], and it has recently been shown that purified erythrocyte (Ca^{2+} + Mg^{2+})-ATPase shows two similar types of kinetics in the absence and presence of calmodulin [6].

The two kinetic types of the (Ca^{2+} + Mg^{2+})-ATPase have been suggested to reveal the existence of a resting and an active state of the Ca^{2+}-pump in vivo [9, 19]. However, in the intact erythrocyte the calmodulin concentration is apparently much higher than needed for saturation of the ATPase and, therefore, the existence of a calmodulin-deficient state of the ATPase in vivo has been questioned [20].

We have now investigated the influence on the membrane-bound (Ca^{2+} + Mg^{2+})-ATPase of calmodulin in the physiological range of concentrations, and we find that calmodulin, even under these conditions, dissociates from the ATPase when the Ca^{2+} concentration is lowered to about 10^{-7} M, because the apparent affinity of calmodulin is drastically reduced at low Ca^{2+} concentrations.

Abbreviations. SDS, sodium dodecyl sulfate; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid.

Methods

Preparation of erythrocyte membranes. Ghosts were prepared from bank blood as described previously [9], centrifuging the membrane suspensions in a Sorvall continuous-flow device which ensures full accessibility of the $(Ca^{2+} + Mg^{2+})$-ATPase [21]. Two types of membrane were prepared.

A-membranes. The erythrocytes were hemolyzed with 9 vol of a solution containing 6.7 mM sodium phosphate and 1.0 mM EGTA, pH 7.4.

B-membranes. The erythrocytes were hemolyzed with a solution containing 6.7 mM sodium phosphate and a calcium-nitrilotriacetic acid buffer, pH 7.4. The concentration of ionized calcium was about 30 μM.

The hemolysis was succeeded by two washings with Tris-HCl buffer and the membranes were stored at −25°C.

The membrane content of 1 L packed erythrocytes was determined by careful preparation of A-membranes, ensuring complete recovery of membranes by recentrifugation of supernatants.

Purification of calmodulin. Calmodulin from bovine brain was purified by the method of Watterson et al. [22], modified as follows (in part according to Gietzen, K., personal communication). Step 1: homogenization in 20 mM Tris-HCl/2 mM EDTA, pH 7.5. Step 2 and 4 precipitation by 60% ammonium sulfate, pH 4.0. Step 3 and 6: DEAE-cellulose column chromatography (Whatman DE-52) with linear salt gradients (50–600 mM and 200–500 mM, respectively) in 10 mM imidazole-HCl, pH 6.5. Step 5: gel filtration performed on an Ultrogel AcA 44 column eluted with 50 mM ammonium bicarbonate, pH 8.5.

Calmodulin from bovine brain and human erythrocytes, purified by a modification of the method of Jarrett and Penniston [17], was supplied by Dr. Berit Kristensen.

The calmodulin preparations were homogeneous as judged by SDS electrophoresis in 12% acrylamide gels [23].

The amounts of calmodulin used for the experiments were converted to molar concentrations assuming 100% purity and a molecular weight of 16 680.

ATPase assay. The ATPase activity was measured at 37°C as ADP production which was linked to oxidation of NADH and monitored continuously at 366 nm. The assay medium contained 70 mM Tris-HCl, pH 7.2 (determined at 37°C), 1.5 mM ATP, 2.5 mM $MgCl_2$, 60 mM KCl, 1 mM of either EGTA, HEDTA or nitrilotriacetic acid, various concentrations of $CaCl_2$, 0.1 mM G-strophantin, 2.3 mM phospho*enol*-pyruvate, 0.45 mM NADH, 90 μg/ml medium of pyruvate kinase/lactate dehydrogenase (3 1, in 50% glycerol, Boehringer), and unless otherwise stated approximately 0.5 mg membrane protein/ml medium. Mg^{2+}-stimulated ATPase was assayed with 1 mM EGTA and no addition of Ca^{2+}. Ca^{2+}-ATPase activity was determined as the difference between $(Ca^{2+} + Mg^{2+})$-dependent and Mg^{2+}-stimulated activity and expressed as $\mu mol \cdot min^{-1}$ per g protein.

Apparent calmodulin concentration in erythrocytes. Cytosol. Supernatant obtained from the first centrifugation of A-membranes was heated in a boiling water bath for 1 min with stirring, cooled and centrifuged at 100 000 × *g* for 15 min at 0–5°C. The amount of supernatant necessary for half-maximum activation of the Ca^{2+}-ATPase at 112 μM Ca^{2+} was determined and the apparent calmodulin concentration of the cytosol was calculated (see Results and Discussion).

Membranes. The apparent calmodulin concentrations of suspensions of A- and B-membranes containing approximately 10 mg membrane protein/ml were determined after heat treatment and centrifugation as above.

Contrary to the findings of Klinger et al. [24], we detected no reduction of calmodulin activity due to heat treatment when purified bovine calmodulin was added to the cytosol or the membranes.

Preincubation experiments. B-membranes (5.7 mg protein/ml) were preincubated for 1 h at 37°C in a volume of 600 μl with about 5 μM bovine brain calmodulin in the presence of 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.2, 1 mM of either EGTA or HEDTA and various concentrations of $CaCl_2$. The suspensions were cooled and centrifuged at 45 000 × *g* for 10 min at 0–5°C. 400 μl supernatant were removed and the membranes resuspended in the remaining supernatant. The preincubated membranes were diluted 1 : 42 in the ATPase medium containing 0.5 μM Ca^{2+}, and the calcium buffer from the preincubation medium did not change the Ca^{2+} concentration of the assay medium significantly.

Calcium determination. In all media of preincuba-

tions and assays the Ca^{2+} concentrations were buffered by addition of either EGTA, HEDTA or nitrilotriacetic acid, and the concentrations of ionized calcium were measured with a calibrated calcium-selective electrode (Radiometer F2112Ca), as described previously [25]. The total calcium in preparations of membranes and calmodulin (1–2 mol calcium per mol calmodulin), determined by atomic absorption spectrophotometry, was taken into account in the designing of the calcium buffers, and the Ca^{2+} concentrations throughout this investigation refer to free ionized calcium.

Protein determination. Protein was determined according to the method of Lowry et al. [26] with bovine serum albumin (Sigma) as standard of reference.

Results and Discussion

Enzyme model

The Ca^{2+}-dependent activation by calmodulin of erythrocyte Ca^{2+}-ATPase may be described shortly by a two-step model [2,13,19]:

$$i\,Ca^{2+} + j\,Mg^{2+} + Z \overset{K_i}{\rightleftharpoons} Ca_iMg_jZ^* \qquad (1)$$

$$E_A + m\,Ca_iMg_jZ^* \overset{K_{zi}}{\rightleftharpoons} E_B(Ca_iMg_jZ^*)_m \qquad (2)$$

where Z and Z^* refer to calmodulin that is non-activated and activated, respectively, E_A and E_B refer to Ca^{2+}-ATPase in A and B state, the binding constants are defined as $K_i = [Ca_iZ^*]/[Ca]^i \cdot [Z]$ and $K_{zi} = [E_B(Ca_iZ^*)_m]/[E_A] \cdot [Ca_iZ^*]^m$ (since in this investigation [Mg] is constant, Mg can be omitted from the equations), and m is the number of mol calmodulin binding per mol enzyme.

Eqn. 2 describes how the binding of calmodulin converts the enzyme from A state to B state. It has been shown previously [19,27] that the A state is characterized by low maximum activity, high affinity for ATP (at least at low Ca^{2+} concentration), low affinities for calcium and monovalent cations, in contrast with the B state which shows high maximum activity, both high and low affinities for ATP, and high affinities for the cations.

Assuming that the catalytic effect of all B state complexes of enzyme-calmodulin, irrespective of the value of i (cf. Eqn. 2), can be characteristized by a common rate constant, the relative activity $\Delta v/\Delta V$ can be defined as

$$\Delta v/\Delta V = \Big(\sum_{i=0}^{i=4} [E_B(Ca_iZ^*)_m]\Big)/([E_A]) + \sum_{i=0}^{i=4} [E_B(Ca_iZ^*)_m]\Big) \qquad (3)$$

where Δv and ΔV refer to the differences in enzyme activity and maximum activity, respectively, between B and A state. Setting $\varphi_1 = \Sigma_i K_{zi}K_i^m[Ca]^{mi}$, where $K_0 = 1$, and assuming that when calmodulin binds, the m sites on the enzyme are occupied simultaneously (i.e., the binding and activation are strongly cooperative), it follows that $\Sigma_i[E_B(Ca_iZ^*)_m] = \varphi_1[E_A] \cdot [Z]^m$, and therefore

$$\Delta v/\Delta V = \varphi_1/([Z]^{-m} + \varphi_1) \qquad (4)$$

Setting $\varphi_2 = \Sigma_i K_i[Ca]^i$, the total calmodulin concentration (Z_t) is $Z_t = \varphi_2[Z] + m\varphi_1[E_A] \cdot [Z]^m$, and since $[E_A]$ can be replaced by $E_t/(1 + \varphi_1[Z]^m)$, E_t being the total enzyme concentration, [Z] can be obtained from Eqn. 5, for instance by iteration:

$$[Z]^{m+1} + [Z]^m(mE_t - Z_t)/\varphi_2 + [Z]/\varphi_1 - Z_t/\varphi_1\varphi_2 = 0 \qquad (5)$$

In our notation [Z] refers to free calmodulin, not complexed by calcium or enzyme, whereas for instance, Jarrett and Kyte's [28] concentrations of free calmodulin should be compared with our $\varphi_2[Z]$ values, representing all calmodulin species, not complexed by enzyme.

At the calmodulin concentration ($Z_t = K_{0.5}$), corresponding to half-maximum activation of the ATPase ($\Delta v/\Delta V = 0.5$), the expression for Z_t, combined with Eqn. 4, is converted to

$$K_{0.5} = \varphi_2/(\varphi_1)^{1/m} + 0.5\,mE_t \qquad (6)$$

where $\varphi_2/(\varphi_1)^{1/m}$ is a constant at a fixed Ca^{2+} concentration and represents the apparent dissociation constant of the enzyme-calmodulin complex, as defined by Jarrett and Kyte [28].

Enzyme concentration

The activation of the Ca^{2+}-ATPase by calmodulin in the presence of calcium depends on the concentration of erythrocyte membrane during assay, as shown previously [11,28]. Fig. 1A shows that calmodulin concentrations needed for half-maximum activation increased when the concentration of membrane protein was raised from 0.12 to 1.45 g/l. Fig. 1B shows $K_{0.5}$ values determined from curves such as those in Fig. 1A in dependence on the concentration of membrane protein during assay and it appears that, apart from the highest concentration, the points can be fitted by a linear regression, in accordance with Eqn. 6 and previous results [28]. The slope of the line is similar to that found by Jarrett and Kyte [28], whereas our intercept is much lower (2.1 vs. 15.6 nM). The deviation of the highest membrane concentration (Fig. 1B) was not eliminated by prolonged preincubation of the membranes in the presence of calmodulin, and the cause of the deviation is not known.

According to Eqn. 6 an estimate of the number of sites (mE_t) can be calculated from the slope of the linear regression in Fig. 1B. If m is unity, E_t is 10 nmol per g membrane protein. However, it cannot be ruled out that other calmodulin-binding sites compete with those of the Ca^{2+}-ATPase. Therefore, the E_t value obtained from Fig. 1B has to be considered as a maximum value of the Ca^{2+}-ATPase concentration in the membrane. Previously, the Ca^{2+}-ATPase concentration was estimated to be 10–20 nmol/g membrane protein [28–30].

The catalytic unit of erythrocyte Ca^{2+}-ATPase seems to be a $(1.4–1.5) \cdot 10^5$ dalton peptide [6,31–34]. If human erythrocytes contain approx. $6.5 \cdot 10^{-13}$ g membrane protein per cell [35,36], E_t = 10 nmol/g corresponds to approx. 4000 ATPase units per red cell. Possibly, a number (n) of ATPase units

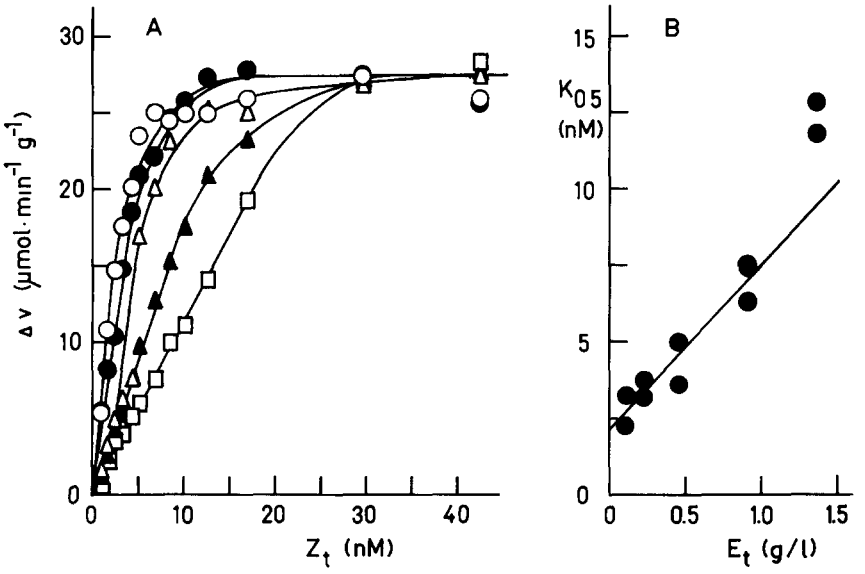

Fig. 1A. Ca^{2+}-stimulated ATPase activity (Δv) vs. total calmodulin concentration (Z_t) at various enzyme concentrations, expressed as g membrane protein/l: 0.12 (○), 0.24 (●), 0 48 (△), 0.96 (▲) and 1.45 (□). ATPase activity was assayed at 112 μM free Ca^{2+} as described in Methods and expressed as μmol min^{-1} per g protein. Activity of A-membranes without the addition of calmodulin (mean ± S.E. of the five determinations: 20.8 ± 0.4 μmol · min^{-1} · g^{-1}) is subtracted. Single experiment. Fig. 1B. The calmodulin concentration required for half-maximum activation ($K_{0.5}$) vs. total enzyme concentration (E_t), here expressed as g membrane protein/l Two or three experiments as in Fig. 1A. $K_{0.5}$ values were determined from Hill plots, i.e., $\log(\Delta v/(\Delta V - \Delta v))$ vs. $\log Z_t$ and the maximum activity (ΔV) was determined from Lineweaver-Burk plots. The line was obtained by linear regression analysis using membrane concentrations between 0.12 and 0 96 g protein/l ($y = 5.1 \cdot x + 2.1$, $r = 0.956$). E_t was determined from the slope to 10.2 nmol Ca^{2+}-ATPase/g membrane protein (see Results and Discussion).

are assembled in an oligomeric enzyme molecule, whereby the number of pump copies are reduced by a factor of n. The maximum ATPase activity of 60 μmol · min^{-1} per g protein (see Table II) corresponds to a turnover number of approx. 6000/min per ATPase unit.

Calmodulin activation dependent on Ca^{2+} concentration

Fig. 2A shows the Ca^{2+}-ATPase activity in dependence on the concentration of calmodulin during assay at various Ca^{2+} concentrations (0.5–17 μM). It is noticed that the ΔV values at saturating calmodulin concentration (Z_t) depend on the Ca^{2+} concentration. Therefore, it has to be assumed that Ca^{2+}, besides its effect via calmodulin, activates the ATPase via Ca^{2+} sites located on the enzyme molecule itself, as suggested previously [19].

Fig. 2B shows that the concentration of calmodulin required for half-maximum activation ($K_{0.5}$) increases from about 5 nM at high Ca^{2+} concentration (10^{-4} M, cf. Fig. 1A) to approx. 2000 nM at low Ca^{2+} ($5 \cdot 10^{-7}$ M). The last $K_{0.5}$ value is uncertain because in the presence of $5 \cdot 10^{-7}$ M Ca^{2+} the enzyme was not saturated even in the presence of 42.5 μM calmodulin, and therefore the determination of the V value was uncertain.

Similar changes of $K_{0.5}$ values with decreasing Ca^{2+} concentration were found with membranes from three different blood specimens, with bovine brain calmodulin purified by a different method (see Methods), and with purified erythrocyte calmodulin. The $K_{0.5}$ value of erythrocyte calmodulin was 5.9 nM at 112 μM Ca^{2+}, compared to 5.3 ± 0.5 nM (S.E., $n = 7$) for bovine brain calmodulin.

Roufogalis and Mauldin [37] also found that more bovine brain calmodulin was needed to saturate the (Ca^{2+} + Mg^{2+})-ATPase activity at low Ca^{2+} concentrations than at high Ca^{2+} concentrations, but $K_{0.5}$ values were not calculated.

The low apparent affinity for calmodulin was not due to reduced accessibility of the membrane-bound enzyme, since Ca^{2+}-ATPase solubilized by Triton X-100 (see Ref. 27) exhibited the same low affinity

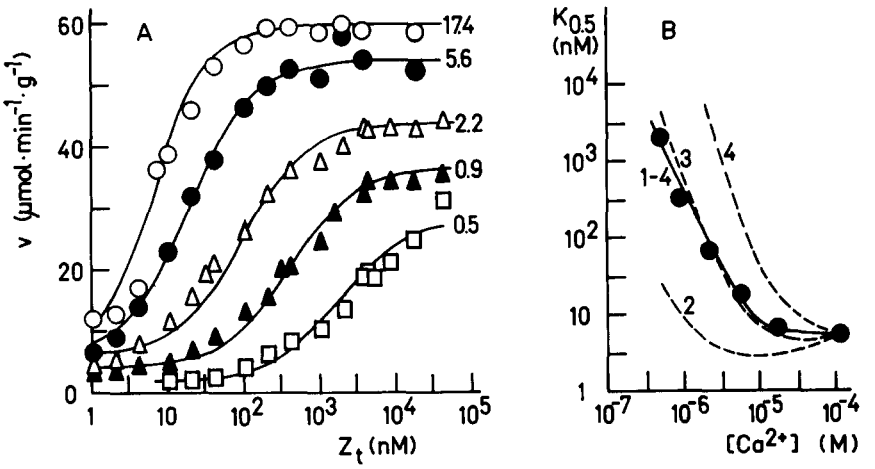

Fig. 2A. Ca^{2+}-stimulated ATPase activity (v) vs. total calmodulin concentration (Z_t) at various free Ca^{2+} concentrations during assay. Single experiment. $K_{0.5}$ values were determined as in Fig. 1B and the following values were obtained: 6.6, 18.2, 68, 332 and 2000 nM calmodulin at 17.4, 5.6, 2.2, 0.9 and 0.5 μM Ca^{2+}, respectively. The curves are calculated by using the enzyme model described in Results and Discussion. Fig. 2B. The calmodulin concentration required for half-maximum activation ($K_{0.5}$) vs. free Ca^{2+} concentration during assay. Same experiment as in Fig. 2A. Dashed curves (2, 3 and 4) are calculated from the enzyme model (see Results and Discussion), assuming that the only activating calcium-calmodulin complex is. Ca_2Z (2), Ca_3Z (3) and Ca_4Z (4). The binding constants (nM^{-1}) were $K_{Z2} = 5.2$, $K_{Z3} = 1.3$ and $K_{Z4} = 0.5$, respectively. The solid curve (1–4) is calculated, using the binding constants (nM^{-1}) $K_{Z0} = 0$, $K_{Z1} = 0.0007$, $K_{Z2} = 0.042$, $K_{Z3} = 0.72$ and $K_{Z4} = 0.22$ Further, in all curves the following parameters (see Results and Discussion) were used: $K_0 = 1$, $K_1 = 1.9\ 10^5$ M^{-1}, $K_2 = 4\ 0\ 10^{10}$ M^{-2}, $K_3 = 1.6 \cdot 10^{15}$ M^{-3}, $K_4 = 4.2 \cdot 10^{19}$ M^{-4}, $E_t = 5$ nmol/l medium and $m = 1$.

for calmodulin (not shown) at this Ca^{2+} concentration (5 10^{-7} M)

The curves shown in Fig. 2B are calculated by the use of Eqn 6. Values of φ_2, corresponding to the different Ca^{2+} concentrations, have been calculated from $K_t = a_1 \cdot a_2 \cdots a_t$, where the a_t values are the macroscopic binding constants for calcium-calmodulin complexes at 3 mM Mg^{2+} found by Crouch and Klee [38]. Similar a_t values have been reported recently by Huang et al. [39]. Different values of φ_1 have been calculated, assuming that either one or several of the possible calcium-calmodulin complexes bind to and activate the Ca^{2+}-ATPase (cf. Eqn. 2). By using the E_t value obtained from Figs. 1A and B, and by setting $m = 1$, $K_{0.5}$ curves corresponding to activation by either Ca_2Z, Ca_3Z or Ca_4Z were calculated (Fig. 2B, curves 2, 3 and 4) The best fit (curve 1–4), however, was obtained by assuming that all of the calcium-binding calmodulin complexes activate, and that the binding constants K_{Z4}, K_{Z3}, K_{Z2}, K_{Z1} and K_{Z0} (cf. Eqn. 2) are related to each other as 0.3, 1.0, 0.06, 0.001 and zero, respectively. The value of unity corresponds to a dissociation constant ($1/K_{Z3}$) of 1.4 nM.

The parameters used for calculation of curve 1–4 (Fig. 2B) were also inserted in Eqns. 4 and 5 and used for calculation of the curves in Fig. 2A The calculated values of $\Delta v/\Delta V$ were combined with values of ΔV and v_A (activity of A-state ATPase without addition of calmodulin) obtained from the experiments in Fig 2A ($v = v_A + \Delta v$). It appears from Fig. 2A that the enzyme model fits the results reasonably well Corresponding curves were calculated, setting $m = 2$. These curves, however, were much steeper (not shown) and did not fit the results.

Therefore, it is concluded from our results in combination with the used enzyme model that m is unity, i.e , that only one molecule of calmodulin binds per ATPase unit. Furthermore, it is concluded that the affinities of the calcium-calmodulin complexes decrease in the order of $Ca_3Z > Ca_4Z > Ca_2Z \gg CaZ$, and the model implies that the activating effect of all complexes is the same because of the common rate constant (cf. enzyme model). Obviously, these conclusions have to be modified if it turns out that the enzyme model presented is too simplified, or that the membrane preparations contain a considerable amount of other calmodulin-binding sites, competing with those of the Ca^{2+}-ATPase.

Previously it was suggested [19] that the Ca_4Z complex binds to the ATPase without activating the enzyme because calmodulin does not activate at high Ca^{2+} concentrations at the usual assay conditions (cf. Fig. 3). However, recent experiments have shown [27] that higher ATP concentrations (10–15 mM) counteract the inhibitory effect of high concentrations of free ionized calcium (measured in the presence of ATP) on the calmodulin-saturated B-state ATPase. This suggests that at lower ATP concentrations the high Ca^{2+} concentrations inhibit via sites located on the enzyme molecule. The effect of the somewhat lower affinity of Ca_4Z compared to Ca_3Z, predicted by the enzyme model, is counteracted by the higher Ca_4Z fraction of total calmodulin. For instance, the binding constants for the calcium-calmodulin complexes [38,39] imply that with increasing Ca^{2+} concentration the Ca_3Z fraction maximally attains one third of total calmodulin (at 50 μM Ca^{2+}), whereas the Ca_4Z-fraction increases from 50% at 50 μM to 95% at 0.5–1.0 mM Ca^{2+}.

In contrast to the erythrocyte Ca^{2+}-ATPase, both cyclic AMP phosphodiesterase from brain [39] and myosin light chain kinase [40] seem to be activated only by the Ca_4^{2+}-calmodulin complex (Ca_4Z). Kinetic analyses of our results from Fig. 2A according to the methods used by Huang et al. [39] and Blumenthal and Stull [40] suggest that, besides Ca_4Z, at least Ca_3Z and Ca_2Z are involved in the activation of Ca^{2+}-ATPase, in agreement with the kinetic analysis above and with the suggestions made by Vincenzi et al. [20].

Apparent calmodulin concentration in erythrocytes

In order to compare the results obtained in vitro with the conditions in the intact cell we have measured the content of calmodulin in erythrocytes.

Heated cytosol preparations from washed and packed erythrocytes (see Methods), obtained from three donors or from four different bank blood specimens, were able to activate membrane-bound Ca^{2+}-ATPase, and the amount of cytosol preparation needed for half-maximum activation of the Ca^{2+}-ATPase was related to $K_{0.5}$ values of calmodulin in experiments such as those of Fig. 1A. Assuming that only the calmodulin in the cytosol preparations was responsible for the activation of the Ca^{2+}-ATPase, the apparent concentrations of calmodulin in the erythro-

cyte cytosol could now be calculated (Table I). The apparent concentrations of calmodulin were found to be 3 to 5 μmol/l packed cells. The differences between fresh blood and bank blood were not significant. The concentrations of calmodulin have earlier been reported to be 2 to 7 μmol/l cells [17,20].

These results suggest that the Ca^{2+}-transport ATPase in erythrocytes is not saturated with calmodulin when the concentration of ionized calcium is below 10^{-6} M (see Fig. 2A).

Comparison of A- and B-membranes

The results above were obtained with purified calmodulin and erythrocyte membranes from which calmodulin was partly removed (A-membranes). In order to test whether the properties of the brain calmodulin and the A-membranes were changed due to the preparation procedures, their properties were compared with those of erythrocyte membranes which have kept the native erythrocyte calmodulin bound throughout the whole preparation procedure (B-membranes).

It has been shown that heat-treatment of erythrocyte membranes releases bound calmodulin [13]. By comparing the activating effect on the Ca^{2+}-ATPase of the supernatant from membrane suspensions which were heated and then centrifuged (cf. Methods) with the effect of calmodulin (see above) the apparent calmodulin concentration of the two different membrane preparations could be determined (Table I).

It appears that the difference of calmodulin content between B- and A-membranes is 20 nmol/g, which exceeds the apparent enzyme concentration (E_t) found above. Further experiments must show whether the apparent excess of calmodulin-binding sites is real. Extra sites do not necessarily compete with the Ca^{2+}-ATPase for calmodulin at our experimental conditions.

The value of residual calmodulin in A-membranes (5 nmol/g) is lower than those reported by other authors who found 9 to 24 nmol/g protein [13,24, 41], whereas the value of B-membranes (25 nmol/g) agrees with the value of 28 nmol/g found by Niggli et al. [14]. Klinger et al. [24] also found that the calmodulin-content of B-membranes was 5-times that of A-membranes, but their values were 2.5-times as high as our values.

In accordance with earlier results [9,18] the two membrane preparations which differ with respect to calmodulin content (Table I) showed different Ca^{2+}-sensitivities and maximum activities (Fig. 3 and Table II). Furthermore, it appears from Fig. 3 that the calmodulin-deficient A-membranes in the presence of

TABLE I

APPARENT CALMODULIN CONCENTRATION IN ERYTHROCYTES

The apparent concentrations are shown as mean ± S.E., tested by Student's *t*-test, and they refer to the activating effect of heat-treated cytosol and membrane preparations on the Ca^{2+}-ATPase, compared with the $K_{0.5}$ value of purified calmodulin assayed at 112 μM free Ca^{2+} and 0.5 g/l membrane protein (see text).

Material	Apparent concentration	Significance
Cytosol (μmol/l cells)		
Fresh blood	4.5 ± 0 2 (3)	–
Bank blood	3.9 ± 0 4 (4)	$P > 0.2$
Membranes (nmol/g protein)		
EGTA-hemolyzed (A)	4 6 ± 0.2 (4)	–
Ca^{2+}-hemolyzed (B)	25 ± 3 (4)	$P < 0.001$

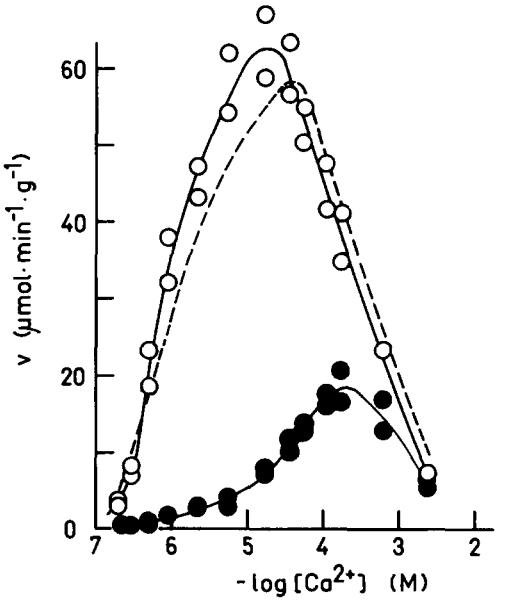

Fig. 3. Ca^{2+}-stimulated ATPase activity (v) vs. free Ca^{2+} concentration during assay. The symbols refer to A-membranes (•, 2 experiments with different blood specimens), A-membranes and 4 μM bovine brain calmodulin (○, 2 experiments with different blood specimens) and B-membranes (– – –, curve based on 8 different blood specimens).

TABLE II

Ca^{2+}-ATPase ACTIVITY OF A- AND B-MEMBRANES

Experiments from Fig. 3 The values are shown as mean ± S.E. The maximum activity (V) was determined from Lineweaver-Burk plots The Ca^{2+} concentrations needed for half-maximum stimulation (K_{Ca}) was determined from Hill plots; i.e., $\log(v/(V-v))$ vs. $\log[Ca^{2+}]$, and n_H refers to the slope of the Hill plot.

Membranes	Number of experiments	V (μmol · min^{-1} per g)	K_{Ca} (μM)	n_H
A	2	21 ± 2	32 ± 1	0 7 ± 0.0
B	8	59 ± 2	1.1 ± 0.1	1 6 ± 0.1
A + 4 μM calmodulin	2	68 ± 5	0.9 ± 0 0	2 7 ± 0.1

4 μM brain calmodulin during assay showed a Ca^{2+}-activation curve that is nearly identical with that of B-membranes. This result indicates that the different procedures did not change the membrane Ca^{2+}-ATPase or the calmodulins. Moreover, the results suggest that the B-membranes are well suited for an examination of the dissociation of calmodulin from the Ca^{2+}-ATPase.

It has been suggested that cellular peptides other than calmodulin participate in the Ca^{2+}-dependent activation of the erythrocyte Ca^{2+}-ATPase [42] However, it appears from Fig. 3 and Table II that the purified brain calmodulin activates the Ca^{2+}-ATPase in the calmodulin-deficient A-membranes to a level that is similar to the Ca^{2+}-dependent activity of B-membranes which have preserved much more of the original erythrocyte protein activator in the bound state. This suggests that calmodulin is the principal protein activator of the Ca^{2+}-ATPase.

Dissociation of calmodulin dependent on Ca^{2+} concentration

Preincubation of B-membranes, i.e., membranes virtually saturated with calmodulin (cf. Fig. 3), in the presence of about 5 μM calmodulin and at Ca^{2+} concentrations ranging from 10^{-8}–10^{-3} M resulted in membranes which showed different Ca^{2+}-ATPase activities when assayed (Fig. 4). The concentration of B-membranes during preincubation was approx 6 g protein per L medium which is equal to the amount of membrane protein in 1 L packed erythrocytes, determined to be 6 g/l (cf Methods). By transfer from preincubation to ATPase assay the membranes and the calmodulin was diluted 42-times. At the resulting concentration (about 100 nM) calmodulin binds rapidly to the ATPase at high Ca^{2+} concentration. It has previously been shown [4,11, 19,20] that the binding of the calmodulin to the Ca^{2+}-ATPase occurs much more slowly at low Ca^{2+} concentrations. Therefore, the initial ATPase activity was assayed at low Ca^{2+} concentration ($5 \cdot 10^{-7}$ M), and the re-binding, if any, of calmodulin during assay was considered by subtracting the initial ATPase activity of calmodulin-deficient membranes (A-membranes), assayed in the presence of 114 nM calmodulin and $5 \cdot 10^{-7}$ M Ca^{2+}.

It appears from Fig. 4 that preincubation of the

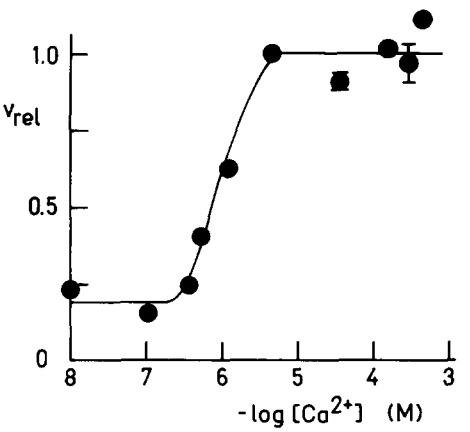

Fig 4. Effect of preincubation at physiological calmodulin concentration and different free Ca^{2+} concentrations on relative Ca^{2+}-stimulated ATPase activity (v_{rel}) of B-membranes B-membranes, preincubated at various Ca^{2+} concentrations and 5 μM calmodulin as described in Methods, were assayed at 114 nM calmodulin arising from the pretreated B-membranes, and 0.5 μM Ca^{2+}. $v_{rel} = 0$ is the activity of A-membranes at 0.5 μM Ca^{2+} and 114 nM calmodulin (5.1 μmol min^{-1} g^{-1}) and $v_{rel} = 1.0$ is 16.4 μmol · min^{-1} g^{-1} Data points are the mean ± S E of three determinations

calmodulin-saturated B-membranes at Ca^{2+}-concentrations below $4 \cdot 10^{-7}$ M ($-\log[Ca^{2+}] = 6.4$) reduces their Ca^{2+}-ATPase activities to a level slightly above the level of the calmodulin-deficient A-membranes, i.e., the calmodulin dissociates from the Ca^{2+}-ATPase at low Ca^{2+} concentrations even when the concentration of calmodulin (5 μmol/l medium) is 80-times that of Ca^{2+}-ATPase (approx. 60 nmol/l medium).

The Ca^{2+}-ATPase was not denatured during the preincubation, since maximum activity could be recovered by raising the Ca^{2+} concentration during assay due to the re-binding of calmodulin.

The enzyme model, together with the binding constants used in curve 1–4 from Fig. 2B, predicted that the v_{rel} curve in Fig. 4 should be displaced approx. 0.4 abscissa units to the left of the curve shown. Possibly the discrepancy may be explained by deviating binding kinetics at high membrane concentrations in vitro (cf. Fig. 1B)

Binding of calmodulin to Ca^{2+}-ATPase in the intact cell

A cytosolic calmodulin concentration of 3–5 μM (cf. Table I) implies that the mol ratio of calmodulin/Ca^{2+}-ATPase is 50–80 in erythrocytes. Nevertheless, at the prevailing Ca^{2+} concentration in erythrocytes, which is probably about 10^{-7} M or lower [43,44], the results above suggest that most of the calmodulin is dissociated from the Ca^{2+}-ATPase. This suggests further that the Ca^{2+}-transport ATPase in vivo is mainly in the calmodulin-dissociated A-state which may represent a resting state of the Ca^{2+}-pump, as suggested previously [9,11].

Apparently, the binding of calmodulin to the ATPase, resulting in the shift from A to B state (cf. Fig. 3), requires that the intracellular Ca^{2+} concentration raises to 10^{-6}–10^{-5} M (cf. Fig. 4).

The possible existence of a resting state of the Ca^{2+} pump in plasma membranes is interesting because the reactions between calmodulin and enzyme (Eqn. 2) seem to occur slowly, as demonstrated in vitro for erythrocyte Ca^{2+}-ATPase [4,11, 19,20] and also for the reaction with purified cyclic nucleotide phosphodiesterase from brain (Wang and coworkers, see Ref. 46). A slow recombination of calmodulin and enzyme implies that in the case of an initiated Ca^{2+} influx into the red cell some time elapses (several seconds or even minutes) before the Ca^{2+} pump is activated by the Ca^{2+}-dependent binding of calmodulin. The activated Ca^{2+}-ATPase pumps out Ca^{2+} and re-establishes the low cytosolic Ca^{2+} concentration. The subsequent dissociation of calmodulin seems also to be slow, but the rate of dissociation in vitro has been shown to increase concurrently with decreasing Ca^{2+} concentrations [19]. The whole reaction constitutes a Ca^{2+} signal of a certain duration (cf. Ref. 11), allowing the activation of other Ca^{2+}-dependent cellular reactions.

Furthermore, the existence of a resting or ground state of the Ca^{2+} pump with strongly reduced pumping activity and low Ca^{2+} sensitivity, combined with a low rate of passive Ca^{2+} influx in the resting cell, may imply a higher intracellular concentration of ionized calcium than expected in the case of a Ca^{2+} pump that, as suggested by Lew and Ferreira [45], permanently exhibits high Ca^{2+} sensitivity.

Finally, the existence of a calmodulin-deficient state in vivo may increase the positive cooperativity of the Ca^{2+} activation of the Ca^{2+} pump, i.e., the activation occurs within a narrow range of Ca^{2+} concentrations.

Acknowledgements

We wish to thank Dr. Berit Kristensen (University of Copenhagen) for supplying two of the calmodulin preparations and for performing the acrylamide gel electrophoresis. We also thank Anette Svanberg for excellent technical assistance.

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