Effects of Ca^{2+} , Mg^{2+} and calmodulin on the formation and decomposition of the phosphorylated intermediate of the erythrocyte Ca^{2+} -stimulated ATPase

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Formation of the phosphorylated intermediate (ECaP) of the human erythrocyte Ca^{2+} -stimulated ATPase (Ca^{2+} -ATPase) was more rapid and reached steady state sooner at $400\ \mu\text{M}\text{-Ca}^{2+}$ than at $1\ \mu\text{M}\text{-Ca}^{2+}$. Calmodulin increased the apparent rate of ECaP formation at $1\ \mu\text{M}\text{-Ca}^{2+}$, whereas at $400\ \mu\text{M}\text{-Ca}^{2+}$, calmodulin decreased the steady-state level of the ECaP without affecting its apparent rate of formation. Removal of endogenous Mg^{2+} with *trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid, which decreased both the velocity and Ca^{2+} -sensitivity of the Ca^{2+} -ATPase, did not alter the Ca^{2+} -sensitivity or the apparent rate of formation of ECaP. ECaP formation at high Ca^{2+} concentrations was not affected by Mg^{2+} concentrations as high as 1 mM, and the ECaP could be dephosphorylated by ADP and ATP along either the forward or reverse pathways. The results suggest that high Ca^{2+} concentrations inhibit Ca^{2+} -ATPase activity by preventing dephosphorylation of the E_2P complex, rather than by inhibition of the transformation from E_1CaP ('high- Ca^{2+} -affinity' ECaP) to E_2CaP ('lower-energy' ECaP).

INTRODUCTION

The reaction mechanism of the erythrocyte-membrane Ca^{2+} -ATPase may be broken down into a series of steps or 'partial reactions'. The high- Ca^{2+} -affinity (E_1) form of the enzyme is considered to bind Ca^{2+} at the cytosolic membrane surface, thereby promoting reaction with ATP and formation of the intermediate complex, E_1CaP . The conformational transition of the 'high-energy' phosphoprotein, E_1CaP , to its 'lower-energy' form, E_2CaP , is believed to result in a decreased affinity of the binding site(s) for Ca^{2+} and reorientation of the site(s) toward the exterior surface of the erythrocyte membrane. This conformational change appears to be stimulated by micromolar concentrations of Mg^{2+} (Rega & Garrahan, 1978). Translocation of Ca^{2+} is thought to result from the displacement of Ca^{2+} from this low-affinity state, producing E_2P . Rega & Garrahan (1975) have suggested that, in the E_2P form, the acyl-phosphate bond is more accessible to water, and is therefore rapidly hydrolysed to E_2 plus P_i . E_2 then undergoes a conformational transition back to E_1 .

At high concentrations [above $(1\text{--}5) \times 10^{-4}\ \text{M}$], Ca^{2+} inhibits Ca^{2+} -ATPase activity (Klinger *et al.*, 1980; Vincenzi *et al.*, 1980; Graf & Penniston, 1981; Schatzmann, 1982; Allen *et al.*, 1986) and produces a large build-up of the phosphorylated intermediate (Lichtner & Wolf, 1980; Allen *et al.*, 1986). Partial digestion of the enzyme with trypsin, which is considered to cleave off a regulatory domain of the enzyme, does not affect Ca^{2+} -dependent inhibition (Stieger & Schatzmann, 1981; Roufogalis *et al.*, 1982), indicating that the site of inhibition likely lies within the catalytic domain. It has

been suggested that, at high concentrations, Ca^{2+} competes with Mg^{2+} at an 'Mg $^{2+}$ -specific' site (see Schatzmann, 1982) to inhibit the conformational change from E_1CaP to E_2CaP .

It is now well established that calmodulin stimulates the rate and enhances the Ca^{2+} affinity of Ca^{2+} transport and Ca^{2+} -stimulated ATP hydrolysis (for reviews, see Roufogalis, 1979; Sarkadi, 1980; Schatzmann, 1982, 1985; Penniston, 1983; Al-Jobore *et al.*, 1984). The suggested steps in the transport cycle of the Ca^{2+} pump where calmodulin exerts regulatory effects include both phosphorylation (Enyedi *et al.*, 1980; Muallem & Karlsh, 1980; Rega & Garrahan, 1980) and dephosphorylation (Rega & Garrahan, 1980; Jeffery *et al.*, 1981). However, there is very little known regarding the exact mechanism by which these effects are produced.

In the present study we have examined the interactions between Ca^{2+} and either Mg^{2+} or calmodulin on the partial reactions of the erythrocyte Ca^{2+} -ATPase.

MATERIALS AND METHODS

Sources of materials

Tris-ATP, substantially vanadate-free, was obtained from Sigma Chemical Co. All inorganic chemicals used were of reagent grade. Aquasol and $[2,8\text{-}^3\text{H}]\text{ATP}$ (sp. radioactivity 20–40 Ci/mmol) were obtained from New England Nuclear and ICN respectively. Calmodulin was purchased from Calbiochem, and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 10–40 Ci/mmol) was purchased from Amersham International.

Abbreviations used: Ca^{2+} -ATPase, Ca^{2+} -stimulated ATPase; ECaPs, phosphorylated intermediates believed to occur in the reaction sequence of Ca^{2+} -ATPase (E_1CaP , 'high- Ca^{2+} -affinity' ECaP; E_2CaP , 'lower-energy' ECaP); E_2P , 'lower-energy' phosphoprotein; CDTA, *trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid.

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Membrane preparation

Blood samples were collected into Vacutainers (Becton-Dickinson Canada) containing EDTA, and stored at 4 °C until they were processed the following morning. Fragmented membranes, depleted of calmodulin, were prepared by hypo-osmotic lysis by the method of Carafoli *et al.* (1980), frozen in liquid N₂ and stored at -80 °C for no more than 5 days. Just before an assay, membranes were resuspended and washed three times in a solution of 40 mM-Hepes/KOH, pH 7.4, at 10 °C. The final pellet was resuspended in this solution to a protein concentration of 2–4 mg/ml and used immediately.

Determination of phosphoenzyme and ATPase activity

Phosphoenzyme formation and ATPase activity were estimated as recently described (Allen *et al.*, 1986). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. Free Ca²⁺ concentrations were determined by using the computer program CATIONS (Goldstein, 1979). Equilibrium constants for cations and ligands were obtained from Martell & Smith (1979) and were corrected for ionic strength, pH and temperature. Equilibrium constants for mono-protonated species were calculated by the procedure of Blinks *et al.* (1982). Statistics were done by analysis of variance (Gregg & Bjerring, 1980), covariance being used to correct for inter-assay variability. Newman-Keuls multiple-range tests were used to examine differences at the 0.01 and 0.05 levels of significance. Rate constants for breakdown of the phosphorylated intermediate were calculated by assuming first-order kinetics.

RESULTS

Effect of Ca²⁺ and calmodulin on Ca²⁺-ATPase and phosphoprotein formation

As shown in our previous studies (Allen *et al.*, 1986) and in Fig. 1, the effect of Ca²⁺ concentration on ATPase activity was 2-fold: there was stimulation of hydrolysis at low concentrations ($K_1 = 4 \times 10^{-7}$ M), but inhibition of activity at concentrations greater than $(4-6) \times 10^{-5}$ M ($K_2 = 2 \times 10^{-3}$ M). The Ca²⁺-dependent formation of ECaP was biphasic; a high-affinity component was associated with low levels of ECaP, whereas in the region of the low-affinity component, ECaP rose sharply to reach plateau levels which were 10 times higher than observed in the high-affinity region of the Ca²⁺ curve. Autoradiograms of phosphorylated erythrocyte membranes (not shown) revealed that, at all Ca²⁺ concentrations tested, most of the ³²P label was incorporated into a protein with an apparent M_r of 135000–145000; phosphorylation of this protein was calcium-dependent and was decreased by treatment with hydroxylamine. Calmodulin (10 µg/ml) increased the apparent maximum velocity nearly 2-fold, and the apparent affinity of the (Ca²⁺ + Mg²⁺)-ATPase for Ca²⁺, but it did not affect Ca²⁺-dependence of inhibition of ATPase activity (Fig. 1a). Calmodulin had no significant effect on the Ca²⁺-dependence of phosphoprotein formation (measured at 15 s) at Ca²⁺ concentrations ranging from 10⁻⁸ to 10⁻² M (Fig. 1b). Further analysis of phosphoprotein formation by both non-linear regression and by an Eadie-Hofstee plot (not shown) showed that calmodulin did not change either the high- or low-affinity Ca²⁺

Fig. 1. Effect of calmodulin on Ca²⁺-ATPase activity (a), calcium-dependent phosphoprotein formation (b) and turnover (b, inset) in calmodulin-depleted erythrocyte membrane fragments at various Ca²⁺ concentrations

Erythrocyte membranes (2–4 mg/ml) were incubated for 15 s (phosphorylation) or 10 min (ATP hydrolysis) at 10 °C in the presence of (final vol. 0.2 ml): 40 mM-Hepes/KOH, pH 7.4, at 10 °C, 0.025 M-MgCl₂, 0.05 mM-ouabain, 0.1 mM-EGTA/Tris, and 0.002 mM-ATP containing [³²P]ATP (final radioactivity 1 Ci/mmol) and [2,8-³H]ATP (final radioactivity 1 Ci/mmol). Calmodulin-stimulated (10 µg/ml) (■) and basal (●) activities refer to the Ca²⁺ component after subtraction of the values measured in the presence of EGTA (0.1 mM) alone. 'Turnover' is the ratio of ATPase activity to phosphoprotein formation. The results shown are means ± S.E.M. for at least five membrane preparations; each was assayed in triplicate. The asterisk indicates significantly different from basal activity ($P < 0.01$).

Fig. 2. Effect of Ca^{2+} concentration on Ca^{2+} -ATPase activity and the time course of Ca^{2+} -dependent phosphoprotein formation in erythrocyte membrane fragments

ATPase activity and phosphoprotein formation were measured in the presence of either $1 \mu\text{M}$ (●) or $400 \mu\text{M}$ (■) free Ca^{2+} . The result shown is the mean $\pm$ S.E.M. of observations from five membrane preparations; each was assayed in triplicate. ATPase activity and phosphoprotein formation were determined in parallel. A single asterisk indicates significantly different from control at $P < 0.05$. Double asterisks indicate significantly different from control at $P < 0.01$.

dissociation constants. Hence the 'turnover' of the enzyme (velocity/E CaP) was markedly increased by calmodulin only at the very low Ca^{2+} concentrations (Fig. 1*b*, inset), this difference being significant ($P < 0.01$) only at the lowest free Ca^{2+} concentration examined.

Effect of Ca^{2+} and calmodulin on the rate of phosphoprotein formation

The effect of Ca^{2+} concentration on the rate of formation and steady-state level of E CaP was studied at two Ca^{2+} concentrations. In the presence of $4.0 \times 10^{-4} \text{ M}$ - Ca^{2+} , E CaP reached steady-state levels within 15–20 s, whereas in the presence of $1.0 \times 10^{-6} \text{ M}$ - Ca^{2+} , steady state was not reached even after 30 s (Fig. 2). The effect of calmodulin on the time course of formation of the E CaP complex was studied at these two Ca^{2+} concentrations. In the presence of $1.0 \times 10^{-6} \text{ M}$ - Ca^{2+} , calmodulin stimulated Ca^{2+} -ATPase activity by 64% and increased the apparent rate of formation of E CaP (Fig. 3*a*). However, a steady state was not established within the reaction times examined. At the higher Ca^{2+} concentration ($4.0 \times 10^{-4} \text{ M}$), calmodulin enhanced ATPase activity by 48%, but had no effect on the apparent rate of formation of E CaP , and it reduced the steady-state level of E CaP by 25% (Fig. 3*b*).

Effect of Mg^{2+} on Ca^{2+} -ATPase activity and phosphoprotein formation

Although several authors (Rega & Garrahan, 1975; Schatzmann & Bürgin, 1978; Szasz *et al.*, 1978) have

Fig. 3. Effect of calmodulin on Ca^{2+} -ATPase activity and the time course of Ca^{2+} -dependent phosphoprotein formation in erythrocyte-membrane fragments in the presence of either $1 \mu\text{M}$ (a) or $400 \mu\text{M}$ (b) free Ca^{2+}

Calmodulin-stimulated ($10 \mu\text{g/ml}$) (■) and basal (●) activities were determined as described in the text. The results shown here are means $\pm$ S.E.M. for five membrane preparations; each was assayed in triplicate. ATPase activity and phosphoprotein formation were determined simultaneously. An asterisk indicates significantly different from the basal activity ($P < 0.01$).

shown that formation of the E CaP complex does not require the presence of Mg^{2+} , it was suggested (see Schatzmann, 1982) that Mg^{2+} may be required at the phosphorylation step. In the present study, membranes were washed three times in a Mg^{2+} -free buffer, then assayed in the presence of 0.1 mM -CDTA to chelate residual Mg^{2+} . The maximum velocity of ATP hydrolysis was reduced from $25.0 \text{ pmol/min per mg}$ in the presence of $25 \mu\text{M}$ - Mg^{2+} to $17.4 \text{ pmol/min per mg}$ in the presence

Fig. 4. Effects of Mg^{2+} on Ca^{2+} -ATPase activity (a), Ca^{2+} -dependent phosphoprotein formation (b) and turnover (b, inset) in erythrocyte membrane fragments at various free Ca^{2+} concentrations

Activities were assayed in the presence of either 0.025 mM-magnesium (■) or 0.1 mM-CDTA (●). ATPase activity and phosphoprotein formation refer to the Ca^{2+} component after subtraction of the values measured in the presence of EGTA (0.1 mM) alone. The 'turnover' is the ratio of ATPase activity to phosphoprotein formation. The results shown are means $\pm$ S.E.M. for observations on at least four membrane preparations; each was assayed in triplicate.

of CDTA and a lower- Ca^{2+} -affinity activation component was observed under these conditions (Fig. 4a). By contrast, the calcium-dependence of ECaP formation was unaltered by the absence of Mg^{2+} (Fig. 4b). Hence, the turnover of the ATPase per unit of protein was considerably decreased in the absence of Mg^{2+} at all Ca^{2+} concentrations examined (Fig. 4b, inset). In time-course studies of enzyme phosphorylation, where the free Ca^{2+} concentration was either 1.0×10^{-6} M (Fig. 5a) or 4.0×10^{-4} M, the absence of Mg^{2+} had no statistically significant effect on either the apparent rate of formation or the steady-state level of ECaP (Fig. 5b).

Mechanism of Ca^{2+} inhibition

In response to suggestions that high concentrations of Ca^{2+} compete at an Mg^{2+} -specific site (see Schatzmann, 1982), thereby inhibiting the conversion of E_1CaP into E_2CaP , the effect of Mg^{2+} on the low- Ca^{2+} -affinity component of ECaP formation was examined. In the absence of Mg^{2+} (0.1 mM-CDTA), the Ca^{2+} -dependence of phosphoprotein formation did not differ from that in the presence of 0.5 mM- Mg^{2+} over the range of high Ca^{2+} concentrations examined (Fig. 6a). In addition, Mg^{2+} concentrations as high as 1 mM had no effect on the level of ECaP formed in the presence of 10 mM- Ca^{2+} (Fig. 6b). To examine the possibility of an effect of high Ca^{2+} concentrations on the Mg^{2+} -stimulated transition from E_1CaP to E_2CaP , membranes were phosphorylated for 15 s in the presence of 10 mM- Ca^{2+} and various Mg^{2+} concentrations up to 1 mM. Dephosphorylation was subsequently initiated by the addition of either ADP or ATP to a final concentration of 1 mM. In the presence of Mg^{2+} , ATP is known to enhance the rate of dephosphorylation along the forward pathway, whereas ADP is considered to stimulate dephosphorylation in both the forward and the reverse directions (Rega & Garrahan, 1978; Schatzmann, 1982). The rate of ADP-stimulated dephosphorylation of the intermediate has been shown to be enhanced by Ca^{2+} , but unaffected by Mg^{2+} (Rega & Garrahan, 1978). In the presence of 10 mM- Ca^{2+} , Mg^{2+} concentrations up to 1 mM had no effect on the rate of phosphoprotein decomposition following a 'chase' with either ATP ($k = 0.19 \text{ s}^{-1}$) or ADP ($k = 0.31 \text{ s}^{-1}$) (Table 1). Although the rate of dephosphorylation in the first 5 s was greater in the presence of ADP, there was no difference in the amount of phosphoprotein remaining after a 15 s exposure to either ADP or ATP (Table 1). These observations suggest that both E_1CaP and E_2CaP forms of the enzyme are present at high concentrations of Ca^{2+} .

DISCUSSION

The activity of the Ca^{2+} -ATPase in human erythrocyte plasma membranes is inhibited by high Ca^{2+} concentrations, but the mechanism of this inhibition has not been clearly established (Klinger *et al.*, 1980; Vincenzi *et al.*, 1980; Al-Jobore & Roufogalis, 1981; Graf & Penniston, 1981). It has been suggested that this inhibition occurs at an ' Mg^{2+} -specific' site, thereby inhibiting dephosphorylation by blocking the conformational change from E_1CaP to E_2CaP (Lichtner & Wolf, 1980; Schatzmann, 1982). However, the present study shows that Mg^{2+} concentrations up to 1 mM had no antagonistic effect on the level of phosphoprotein

Fig. 5. Effect of Mg^{2+} on Ca^{2+} -ATPase activity and the time course of Ca^{2+} -dependent phosphoprotein formation in erythrocyte membrane fragments in the presence of either 1 μM (a) or 400 μM (b) free Ca^{2+}

The Figure shows ATPase activity and phosphoprotein formation in the presence of either 0.025 mM- Mg^{2+} (●) or 0.1 mM-CDTA (■). Other conditions were as described in the legend to Fig. 1. The results shown here are means $\pm$ S.E.M. for four membrane preparations; each was assayed in triplicate. ATPase activity and phosphoprotein formation were determined simultaneously. An asterisk indicates significantly different from the 0.025 mM- Mg^{2+} condition ($P < 0.01$).

formed at 10 mM- Ca^{2+} (Fig. 6). To examine further whether the equilibrium between E_1CaP and E_2CaP was shifted towards E_1CaP by millimolar Ca^{2+} , as would be predicted if Ca^{2+} blocked this transformation, the nucleotide-sensitivity of the phosphoprotein formed at 10 mM- Ca^{2+} was examined. If E_1CaP predominated, only ADP would produce dephosphorylation (in the back-

Fig. 6. Effect of magnesium on (a) the low- Ca^{2+} -affinity component of Ca^{2+} -dependent phosphoprotein formation, and (b) phosphoprotein formation in the presence of 10 mM- Ca^{2+}

(a) Phosphoprotein formation was determined in the presence of either 0.5 mM- Mg^{2+} (■) or 0.1 mM-CDTA (●). Phosphoprotein refers to the Ca^{2+} component after subtraction of the values measured in the presence of EGTA (0.1 mM) alone. The results shown are means $\pm$ S.E.M. for observations on at least three membrane preparations; each was assayed in triplicate. (b) Phosphoprotein formation refers to total phosphorylation. Other assay conditions were as described in the legend to Fig. 1. The result shown is the mean $\pm$ S.E.M. for observations on at least three membrane preparations; each was assayed in triplicate.

Table 1. Effect of ADP and ATP on the phosphorylated intermediate

Membrane fragments were phosphorylated for 15 s in the presence of 10 mM-Ca²⁺ and various Mg²⁺ concentrations, then dephosphorylation was initiated by addition of either ADP or cold ATP (1 mM final concn.) and the reaction terminated after either 5 or 15 s by the rapid addition of ice-cold 5% (w/v) trichloroacetic acid. The results shown are the means $\pm$ S.E.M. for the number of membrane preparations shown in parentheses. Total phosphoprotein refers to the amount formed in 15 s.

[Mg ²⁺]	Fraction of total phosphoprotein remaining (%)			
	ADP		ATP	
	5 s (3)	15 s (4)	5 s (3)	15 s (4)
None (0.1 mM-CDTA)	25 $\pm$ 3	9 $\pm$ 3	38 $\pm$ 3*	11 $\pm$ 5†
25 μ M	21 $\pm$ 2	12 $\pm$ 2	36 $\pm$ 3*	16 $\pm$ 14†
0.5 mM	18 $\pm$ 2	9 $\pm$ 2	39 $\pm$ 4*	9 $\pm$ 1†
1.0 mM	24 $\pm$ 2	18 $\pm$ 4	45 $\pm$ 7*	11 $\pm$ 4†

* Signifies difference from 5 s treatment with ADP ($P \leq 0.01$).
† Signifies difference from 5 s treatment with ATP ($P < 0.01$).

ward direction; Rega & Garrahan, 1978), whereas dephosphorylation was enhanced by both ATP and ADP chases (Table 1), both of which stimulate dephosphorylation in the forward direction (see Schatzmann, 1982). This result indicates that the E₁CaP-to-E₂CaP step is not sufficiently inhibited by 10 mM-Ca²⁺ to account for the low turnover (1–5 min⁻¹) observed under these conditions. Furthermore, the lack of effect of increasing Mg²⁺ concentration in dephosphorylation by either ATP or ADP (Table 1), as well as the lack of antagonism of the level of phosphoenzyme formed (Fig. 6b), are not consistent with inhibition by Ca²⁺ at an Mg²⁺-specific site. These results, taken together with studies demonstrating reversal of the Ca²⁺ pump by millimolar Ca²⁺ (Rossi *et al.*, 1978; Wüthrich *et al.*, 1979), and the recent observation that Ca²⁺ inhibits phosphoenzyme cleavage (Kosk-Kosicka *et al.*, 1986), show that the inhibition by high Ca²⁺ is due to accumulation of the low-Ca²⁺-affinity state, E₂P, and blockade of subsequent dephosphorylation while Ca²⁺ remains bound to this site.

The failure of ECaP formation to reach steady state in the presence of 10⁻⁶ M-Ca²⁺ indicates that the true rate of formation of the phosphorylated intermediate complex was slow at this low Ca²⁺ concentration. However, the high Ca²⁺-ATPase activity observed under the same conditions suggests the following. (1) In the presence of 10⁻⁶ M-Ca²⁺, formation of the E₁CaP complex was a rate-limiting step either directly or by virtue of a rate-limiting E₂ $\rightarrow$ E₁ transition. (2) Though turnover of the phosphorylated intermediate was high, the failure of phosphorylation to reach a steady state suggests that the rate of formation of the ECaP complex must have been similar to the rate of dephosphorylation. (3) All partial reactions which occur between phosphorylation and dephosphorylation of the enzyme were at least as rapid as the formation and breakdown of the intermediate. At

a higher Ca²⁺ concentration (4.0 $\times$ 10⁻⁴ M), both the apparent rate of formation and the steady-state levels of ECaP were increased relative to those at 10⁻⁶ M-Ca²⁺, whereas Ca²⁺-ATPase activity was slightly inhibited. The greater apparent rate of phosphoprotein formation may be attributed to greater saturation of the high-affinity Ca²⁺-binding sites on the enzyme, which have a dissociation constant of approx. 10⁻⁶–10⁻⁷ M (Schatzmann, 1973; Ferreira & Lew, 1976; Larsen *et al.*, 1978; Kosk-Kosicka & Inesi, 1985). The increase in the steady-state level of phosphoprotein may have resulted from the inhibition of dephosphorylation by Ca²⁺, forcing the system into a new equilibrium, where the rates of formation and breakdown of the phosphorylated intermediate were similar once again.

Calmodulin increases both the turnover and the Ca²⁺-sensitivity of the Ca²⁺-ATPase, but it is not clear if both these actions are due to its kinetic effect on the phosphorylation and dephosphorylation steps in the reaction sequence or a direct effect of calmodulin on the Ca²⁺ affinity of the binding site (Muallem & Karlsh, 1980, 1981). In the present study the effect of calmodulin on the kinetics of ECaP formation was shown to depend on the Ca²⁺ concentration. Calmodulin enhanced the rate of phosphorylation of the Ca²⁺-ATPase only when this was rate-limiting, i.e., at low Ca²⁺ concentrations. Since ECaP levels at 15 s were largely unaltered, calmodulin greatly enhanced the turnover of the Ca²⁺-ATPase only at low Ca²⁺ levels (Fig. 1). As calmodulin also enhances the rate of dephosphorylation (Jeffery *et al.*, 1981; Rega & Garrahan, 1980), it is likely that calmodulin increases the Ca²⁺ affinity of the Ca²⁺-binding site on the Ca²⁺-ATPase, leading to its greater saturation at low Ca²⁺ levels. Direct evidence that calmodulin lowers the Ca²⁺ binding constant was recently obtained on the purified Ca²⁺-ATPase (Kosk-Kosicka & Inesi, 1985), and it has been found that polyanions increase Ca²⁺-sensitivity of the ATPase without affecting its maximum velocity (Minocherhomjee *et al.*, 1982). On the other hand, calmodulin did not alter the Ca²⁺-sensitivity of inhibition of the Ca²⁺-ATPase, as also found previously (Klinger *et al.*, 1980; Schatzmann, 1982), or of ECaP formation at high Ca²⁺ concentrations, suggesting that it does not affect Ca²⁺ binding to the E₂ state of the enzyme.

When the formation of the ECaP complex was examined in the absence of Mg²⁺, there was no apparent change in either its Ca²⁺-dependency or the apparent rate of its formation, although the overall Ca²⁺ affinity and maximum velocity of the Ca²⁺-ATPase activity were reduced. These results may be explained if Mg²⁺ simultaneously stimulates both formation (Rega & Garrahan, 1975; Garrahan & Rega, 1978; Schatzmann & Bürgin, 1978; Larocca *et al.*, 1981) and breakdown (Rega & Garrahan, 1975; Carafoli *et al.*, 1980; Jeffery *et al.*, 1981) of the intermediate.

In summary, these current studies indicate that the inhibitory action of high Ca²⁺ on erythrocyte Ca²⁺-ATPase activity may be due to prevention of the Ca²⁺-unloading step of the reaction sequence. Calmodulin, which stimulates both the velocity and the Ca²⁺ affinity of the enzyme system, may do so by stimulating the turnover of the reaction sequence and by a direct effect on the high-affinity calcium binding site(s). Finally, along with other recent reports, our data indicate that Mg²⁺ is not an absolute requirement for Ca²⁺-ATPase

activity, but may facilitate the rapid turnover of the reaction sequence.

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