Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4265–4269, July 1982
Biochemistry

Activation of human erythrocyte Ca^{2+}-dependent Mg^{2+}-activated ATPase by calmodulin and calcium: Quantitative analysis

(calcium pump/multiple equilibria/linked functions)

JOS A. COX, MICHELLE COMTE, AND ERIC A. STEIN†

Department of Biochemistry, University of Geneva, P.O. Box 78 Jonction, 1211 Geneva 8, Switzerland

Communicated by Edmond H. Fischer, March 29, 1982

ABSTRACT The effect of Ca^{2+} and calmodulin on (CaM) on the activation of Ca^{2+}-dependent Mg^{2+}-activated ATPase (Ca^{2+},Mg^{2+}-ATPase; ATP phosphohydrolase, EC 3.6.1.3) has been carried out because of the finding that the CaM dependence of the activation varies with the concentration of free Ca^{2+}, similarly to brain phosphodiesterase and adenylate cyclase. The study was carried out in the absence of chelating agents because they strongly interfere in the enzyme kinetics. Three main conclusions can be drawn (*i*) CaM·Ca_3 and CaM·Ca_4 together are the biochemically active species *in vitro*. (*ii*) These species bind in a noncooperative way to the CaM-binding site of the enzyme with a dissociation constant of 6×10^{-10} M or 1.1×10^{-8} M, depending on whether Ca^{2+} saturates the substrate binding site of the enzyme or not. (*iii*) The binding of CaM·Ca_3 to the enzyme lowers the dissociation constant of the enzyme for Ca^{2+} at the substrate binding site from 51.5 to 2.8 μM. Contrary to general belief, CaM does not induce pronounced positive cooperativity in the binding of Ca^{2+} to the enzyme. Such a cooperativity is seen only when the enzyme is incompletely saturated with the activator, but it disappears in the presence of saturating concentrations of CaM·Ca_3. The rate equation proposed here accurately predicts the extent of enzyme activation over a wide range of Ca^{2+} and CaM concentrations. In healthy erythrocytes the concentrations of Ca^{2+} and CaM are such that the Ca pump works with a minimal dissipation of energy, but a small increase in the intracellular Ca^{2+} concentration leads to a strong amplification of the pumping activity.

Human erythrocytes keep their intracellular Ca^{2+} concentration below 0.25 μM (1). Homeostasis is maintained by low Ca^{2+} permeability of the membranes (2), chelation of Ca^{2+} by intracellular binding sites (3), and extrusion by an ATP-driven Ca pump (4). Of these three components, only the Ca pump is subject to feed-forward regulation—i.e., the increase in free Ca^{2+} concentration accelerates the Ca pump not only by substrate regulation but also by switching on the activator calmodulin (CaM) (5, 6). Hence, the Ca^{2+}-dependent Mg^{2+}-activated ATPase (Ca^{2+},Mg^{2+}-ATPase; ATP phosphohydrolase, EC 3.6.1.3), which is the enzymatic expression of this pump, is stimulated by Ca^{2+} in two ways: at the CaM-binding site and at the catalytic site, which will subsequently be called "substrate Ca-binding site."

The activation of the enzyme by CaM is characterized by two sequential steps. First, at increasing concentrations of Ca^{2+}, the distribution of the species CaM·Ca_1, CaM·Ca_2, CaM·Ca_3, and CaM·Ca_4 shifts to the more saturated forms. At saturation of a critical number of sites, CaM undergoes a conformational change and becomes a biologically active species, named "CaM*Ca_n." In the case of activation of bovine brain phosphodiesterase (7) and adenylate cyclase (8), CaM·Ca_3 and CaM·Ca_4 are the active species. Second, CaM*Ca_n forms a ternary complex with Ca^{2+},Mg^{2+}-ATPase, which activates the latter by increasing the maximal velocity as well as the affinity of Ca^{2+} for the substrate Ca-binding site of the enzyme (9–15). Not only does CaM decrease the K_m of the enzyme for Ca^{2+} but apparently it also induces a marked positive cooperativity in the binding of Ca^{2+} to the substrate binding site (9, 16). Qualitatively, CaM shifts the enzyme from a slow ATP-hydrolyzing state to a fast hydrolyzing one.

No quantitative picture of the activation of erythrocyte Ca^{2+},Mg^{2+}-ATPase by CaM has been presented so far, for the following reasons. (*i*) Most studies were carried out in the presence of organic Ca chelators such as EGTA and *N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid (HEDTA). At millimolar concentrations, CaEGTA lowers the dissociation constant of the enzyme for Ca^{2+} to submicromolar values in the presence as well as in the absence of CaM (17) and prevents a quantitative analysis. (*ii*) The CaM*Ca_n species that is involved in the equilibrium reaction that activates the enzyme had not been identified unambiguously. (*iii*) The increase in affinity and the apparent induction of positive cooperativity of Ca binding to the substrate site of the enzyme upon addition of CaM had not been studied in the presence of saturating levels of CaM*Ca_n.

The purpose of this work was to identify, without the use of organic Ca-chelating buffers, the active CaM*Ca_n species and to determine its affinity for the Ca^{2+},Mg^{2+}-ATPase. Our aim also was to determine the affinity of Ca^{2+} for the substrate binding site of the enzyme in the presence and absence of saturating levels of the active CaM*Ca_n species and to provide a quantitative picture for the activation of the enzyme by Ca^{2+} and CaM.

EXPERIMENTAL PROCEDURES

Erythrocyte Membrane Preparation. Fresh human blood was collected in acid/citrate/dextrose solution. The erythrocytes were spun down and washed four times by centrifugation at 400 × *g* for 5 min in 0.15 M NaCl. Hemolysis was induced by diluting the packed cells in 10 vol of 15.3 mM Tris·HCl, pH 7.5/1 mM EGTA with stirring. After 5 min on ice, the membranes were centrifuged at 25,000 × *g* for 40 min and the hemolysate as well as a white firmly attached pellet ["whitish button" (18)] were carefully removed. The membranes were washed twice in the same buffer.

In order to improve the activability by CaM (18)—i.e., to decrease the content of Ca^{2+}-independent Mg^{2+}-activated

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: CaM, calmodulin; Ca^{2+},Mg^{2+}-ATPase, Ca^{2+}-dependent Mg^{2+}-activated ATPase.

† To whom reprint requests should be addressed.

ATPases—the membrane pellet was resuspended in 1 mM Tris·HCl, pH 8.0/0.1 mM K_2EDTA, incubated at 37°C for 20 min, and centrifuged at 40,000 × *g* for 20 min. The pellet was repeatedly washed with sucrose solutions as described by Jarrett and Kyte (19). This ATPase activity was <1% of that of Ca^{2+},Mg^{2+}-ATPase in the absence of CaM. The content of endogenous CaM was assayed by the phosphodiesterase method (7) on a sample of erythrocyte membranes that had been heated previously for 2 min at 100°C and clarified by centrifugation. The membranes contained 43 ng of CaM per mg of protein. This corresponds to 0.1 nM endogenous CaM in the enzyme assay, which is negligible for the type of studies presented under *Results*. After addition of glycerol to a final concentration of 50% (vol/vol), the suspension (2.5 mg of membrane proteins per ml) was stored at −20°C. The Ca^{2+},Mg^{2+}-ATPase activity and activability by CaM were unaffected over a period of 6 months.

Ca^{2+},Mg^{2+}-ATPase Activity. The Ca^{2+},Mg^{2+}-ATPase activity was measured by means of a coupled enzyme assay (20), the sensitivity of which was greatly increased by an adaptation to fluorescence photometry. The medium (final volume, 200 μl) contained 30 mM imidazole·HCl (pH 7.0), 120 mM KCl, 4 mM $MgCl_2$, ouabain at 0.1 mg/ml, 0.94 mM phospho*enol*pyruvate, 0.3 mM NADH, lactate dehydrogenase at 10 μg/ml, pyruvate kinase at 20 μg/ml, 2 μM ionophore A 23187, bovine serum albumin at 0.2 mg/ml, ≈50 μg of erythrocyte membrane proteins per ml, and the indicated concentrations of metal-free bovine brain CaM and of Ca^{2+}. After preincubation for 20 min the reaction was started by addition of ATP to a final concentration of 1 mM. The samples were incubated for 60 min at 37°C with continuous stirring and the reaction was stopped by addition of 50 μl of a "stop" solution containing 1 M HCl and 140 mM $MgCl_2$.

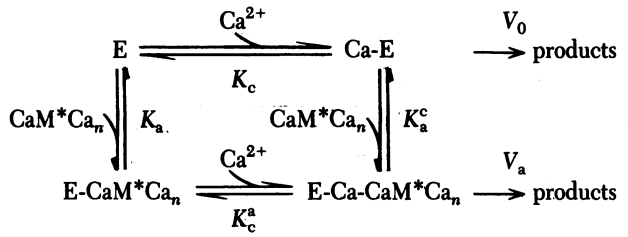
The amount of NAD formed was measured by a fluorescence photometric method modified from that described by Trautschold *et al.* (21). After addition of the stop solution, the reaction mixture was kept for 20 min at room temperature to destroy nonconverted NADH. Then, 250 μl of 12 M NaOH was added and the mixture was incubated for 30 min at 37°C to develop the fluorescent products. The solution was diluted 1:20 with water and cleared by centrifugation. The emission fluorescence was measured at 470 nm (excitation at 365 nm) on a Baird Atomic FC 100 spectrofluorimeter and compared to a standard solution of 50 μM NAD treated with the stop and alkaline solutions as above. Appropriate blank values, obtained in the absence of CaM or Ca^{2+}, were subtracted.

Determination of Free Ca^{2+} Concentrations and Distribution of the CaM·Ca^{2+} Species. After the incubation period, the reaction mixture was centrifuged at 35,000 × *g* for 10 min and total Ca in the supernatant was measured by atomic absorption with Titrisol standards (Merck, Darmstadt, West Germany). The presence of the ionophore A 23187 prevented the sequestration of Ca^{2+} inside the membrane vesicles and kept the free Ca^{2+} concentrations constant, even at levels as low as 4 μM free Ca^{2+}. The latter were calculated iteratively with a computer program (7), taking into account the interactions of H^+, Ca^{2+}, Mg^{2+}, and K^+ with ATP and CaM. For these calculations, the association constants of Donaldson and Kerrick (22) were used for complexes of Ca^{2+},Mg^{2+}, and K^+ with ATP, and those of Cox *et al.* (7) were used for complexes of Ca^{2+} with CaM. A control experiment was carried out in the enzyme incubation medium at various concentrations of total Ca^{2+} by means of a Ca-selective electrode (IS 561, Philips, Eindhoven, Netherlands); the measured values of free Ca^{2+} concentrations agreed within less than 5% with the calculated ones. The distribution of the four complex species CaM·Ca_1, CaM·Ca_2, CaM·Ca_3, and CaM·Ca_4 as a function of free Ca^{2+} was calculated as described (7).

RESULTS

A prerequisite for a quantitative analysis of the activation of Ca^{2+},Mg^{2+}-ATPase by Ca^{2+} and CaM is that CaM does not significantly change the Michaelis constant of the enzyme for ATP. In the range 10–100 μM free Ca^{2+}, the K_m for ATP, determined in triplicate, was 48 μM ± 5 (mean ± SEM) in the presence of CaM and 41 μM ± 8 in its absence, in both cases with no cooperativity. A slight decrease in affinity in the presence of CaM was noticed by Scharff and Foder (9) and by Waisman *et al.* (13). In order to overcome possible problems arising from small variations of K_m, the Ca^{2+},Mg^{2+}-ATPase assay was always carried out in the presence of excess ATP (1 mM).

The quantitative analysis is based on the assumption that the initial velocity, v_i, results from an enzyme that hydrolyzes ATP through two different pathways, depending on whether CaM is present or not (11). The kinetic scheme is as follows:



where E is free Ca^{2+},Mg^{2+}-ATPase (i.e., free of Ca^{2+} and of CaM*Ca_n in its catalytic and regulatory sites, respectively); V_a and V_0 correspond to the maximal velocities of the enzyme in the presence and absence of saturating amounts of CaM*Ca_n, respectively. For this system, Jarrett and Kyte (19) have shown that the difference between the initial velocity in the presence of CaM and that in its absence, Δv_i, at any Ca^{2+} concentration, is given by:

$$\Delta v_i = \Delta V_{max} [CaM^*Ca_n]/(K_{app} + [CaM^*Ca_n]) \quad [1]$$

where

$$\Delta V_{max} = V_a (1 + K_c^a/[Ca^{2+}]) - V_0 (1 + K_c/[Ca^{2+}]) \quad [2]$$

and

$$K_{app} = K_a^c (K_c + [Ca^{2+}])/(K_c^a + [Ca^{2+}]). \quad [3]$$

Eq. 1 is a rectangular hyperbola as a function of [CaM*Ca_n]. Eqs. 2 and 3 show that the maximal velocity and the apparent Michaelis constant still depend on free [Ca^{2+}].

To solve Eq. 3, the values of K_c and K_c^a must be known. The determination of K_c^a was performed in the presence of saturating concentrations of CaM*Ca_n—i.e., the amount of CaM added was such that the concentration of CaM*Ca_3 was at least 10-fold higher than the value of K_a^c given below. Ca binding to the substrate site of Ca^{2+},Mg^{2+}-ATPase occurred without cooperative effects, both in the presence and absence of CaM (Fig. 1). V_a and V_0 correspond to 0.071 and 0.020 mol/min per mg of membrane protein, respectively. The dissociation constants are 51.5 μM in the absence (K_c) and 2.8 μM in the presence (K_c^a) of CaM. These values are of the same order of magnitude as those reported by other investigators (9, 12, 15); however, these reports and others (16) claimed pronounced positive cooperativity in the binding of Ca^{2+} to Ca^{2+},Mg^{2+}-ATPase provided that CaM is present. The positive cooperativity apparently was due to the use of EGTA as Ca^{2+} buffer, which leads to erroneous results (13), or to the fact that saturating amounts of CaM*Ca_n were not present. It should be noted that, at concentrations of Ca^{2+} higher than those used in Fig. 1, Ca^{2+},Mg^{2+}-ATPase is inhibited. In the presence of CaM inhibition starts at 130 μM free

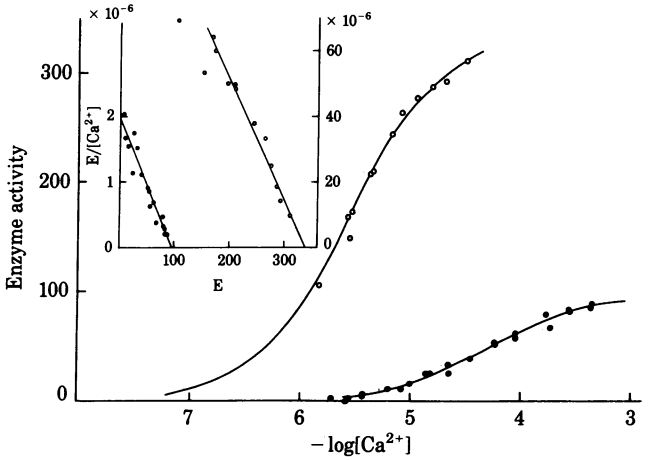


FIG. 1. Activity of erythrocyte Ca^{2+},Mg^{2+}-ATPase as a function of the free Ca^{2+} concentration in the presence (○) or absence (●) of 0.94 μM CaM and the corresponding Scatchard plots (*Inset*). Enzyme activity (E) is in arbitrary fluorescence units. The amount of added metal-free CaM was chosen so that at the lowest free Ca^{2+} concentration, the CaM·Ca_3 concentration was 10-fold higher than the dissociation constant of the enzyme for CaM·Ca_3 (see text). The chelating effect of added CaM was taken into account for the determinations of free Ca^{2+}. The lower part of the activation curve in the presence of CaM could not be determined because no chelators such as EGTA were used to lower the free Ca^{2+} concentration. The lines represent the theoretical activation curves as calculated from the Scatchard plots.

Ca^{2+}; it starts at 630 μM in its absence (results not shown). Because at these concentrations the activation by Ca^{2+} is essentially completed, we consider that this inhibitory effect does not significantly influence the activation pattern.

Fig. 2 shows the activation of Ca^{2+},Mg^{2+}-ATPase as a function of total CaM concentration at four selected concentrations of free Ca^{2+}. As expected (7), less CaM was needed to induce half-maximal activation of the enzyme at higher concentrations of free Ca^{2+}. It should be noted that, in the range of Ca^{2+} concentrations studied, the inhibitory effect of CaATP can be neglected because its concentration was never higher than 1/10th the inhibition constant (11). For 11 experiments at different concentrations of free Ca^{2+}, the concentrations of the different free CaM-Ca species at half maximal activation of Ca^{2+},Mg^{2+}-ATPase were calculated as described (7) and are

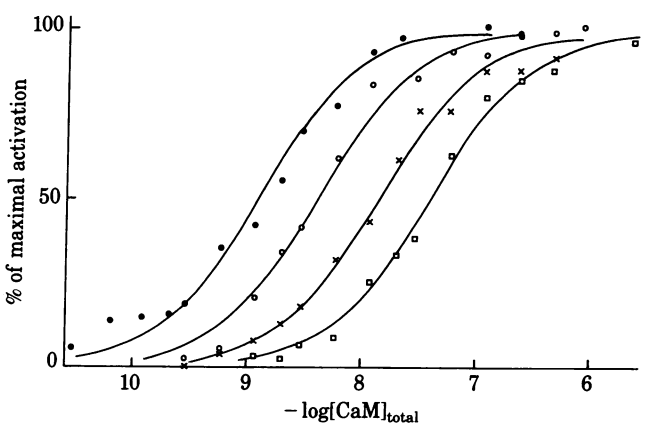


FIG. 2. Activation of erythrocyte Ca^{2+},Mg^{2+}-ATPase as a function of the total concentration of CaM at four selected concentrations of free Ca^{2+}. The curves were normalized to percentage of maximal activability. Free Ca^{2+} concentration: ●, 50.5 μM; ○, 20.6 μM; ×, 9.2 μM; □, 5.1 μM. The lines represent the theoretical activation curves, assuming classical Michaelis–Menten kinetics.

Table 1. Concentrations of different CaM·Ca_n species at half-maximal activation of erythrocyte Ca^{2+},Mg^{2+}-ATPase at different free Ca^{2+} concentrations

Ca^{2+}, μM	Total CaM, nM	CaM·Ca_n, nM $\sum_{n=1}^{n=4}$	$\sum_{n=2}^{n=4}$	$\sum_{n=3}^{n=4}$	$n = 4$
3.87	74	57.7	25.8	4.85	0.084
5.06	38	32.1	17.0	3.97	0.090
5.41	40.9	25.1	19.3	4.74	0.115
9.2	13.5	12.7	9.02	3.25	0.132
14.0	7.35	7.16	5.84	2.73	0.165
18.7	4.12	4.06	3.55	1.93	0.153
20.6	4.06	4.01	3.58	2.04	0.176
39.8	1.76	1.76	1.69	1.24	0.192
50.5	1.21	1.21	1.18	0.926	0.174
84.9	0.94	0.94	0.93	0.814	0.228
153.0	1.29	1.29	1.29	1.21	0.498

shown in Table 1 (it was assumed that, if CaM*Ca_n is the biologically active species, those species with more than n Ca^{2+} are also active). These concentration values are equal to K_{app} (see Eq. 1) and should obey Eq. 3 only for the biologically active CaM*Ca_n species. The values of K_c and K_c^a are known from independent experiments, so the K_{app} values can be plotted against $(K_c + [Ca^{2+}])/(K_c^a + [Ca^{2+}])$ and should yield a straight

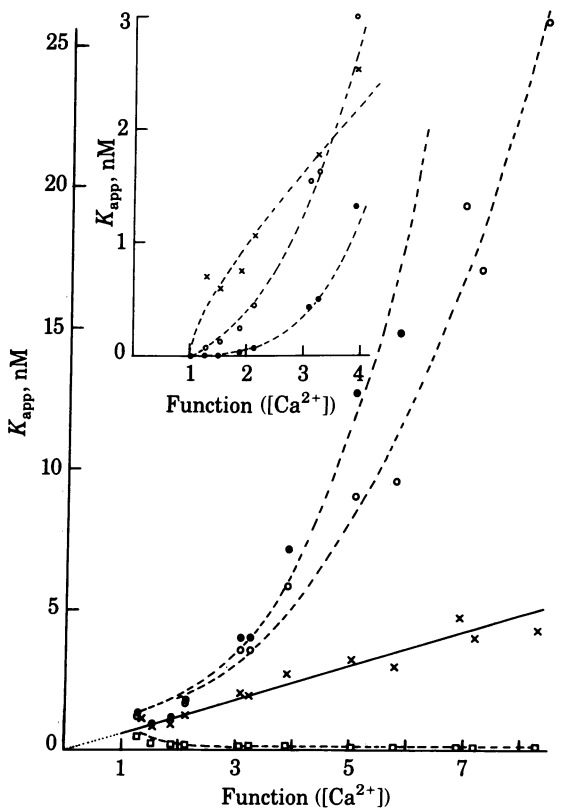


FIG. 3. Determination of the stoichiometry of the biologically active CaM*Ca_n species. Data from Table 1 are plotted according to Eq. 3. Function $([Ca^{2+}]) = (K_c + [Ca^{2+}])/(K_c^a + [Ca^{2+}])$. Values were calculated with $\Sigma_{n=1}^{n=4}$ CaM·Ca_n (●), $\Sigma_{n=2}^{n=4}$ CaM·Ca_n (○), $\Sigma_{n=3}^{n=4}$ CaM·Ca_n (×), CaM·Ca_4 concentration (□). The solid line represents the theoretical curve calculated from Fig. 3 with the experimental values of K_c and K_c^a for the case of $\Sigma_{n=3}^{n=4}$ CaM·Ca_n; the dashed lines are calculated for other combinations. The abscissa term cannot be <1; the extrapolation toward zero is represented as a dotted line. (*Inset*) Values calculated for a single active CaM*Ca_n species: ●, $n = 1$; ○, $n = 2$; ×, $n = 3$. Only the measurements at high Ca^{2+} concentration are presented.

line, passing through the origin, provided a correct value for n in CaM*Ca_n is used.

Fig. 3 shows this relationship for the different species. Only when calculated on the basis of CaM*Ca_3 + CaM*Ca_4 do all experimental points follow a linear relationship with a correlation coefficient of 0.98; moreover, the corresponding straight line passes through the origin. Neither of these two conditions is fulfilled in the case of other combinations. As illustrated in Fig. 3 *Inset*, the plot of the data with CaM·Ca_3 alone as active species does not yield a line passing through the origin for free Ca^{2+} concentrations >40 μM. This demonstrates that only the sum of CaM*Ca_3 and CaM*Ca_4 concentrations obeys the general equation and that CaM*Ca_4 is as active as CaM*Ca_3. *In vivo*, however, the concentration of CaM*Ca_4 is negligible and activation is brought about solely by CaM*Ca_3.

The slope of the line yields a dissociation constant for the Ca-saturated enzyme for CaM·Ca_3 + CaM·Ca_4 of 0.6×10^{-9} M. The interaction of the enzyme with CaM·Ca_3 + CaM·Ca_4 is noncooperative as can be inferred from the behavior of the curves of Figs. 1 and 2.

DISCUSSION

The influence of CaM on the affinity of the enzyme for Ca^{2+} and vice versa has been quantitatively analyzed in terms of the theory of linked functions (23). It should be pointed out that our analysis (7, 8) implies that in the equilibrium reaction

$$\text{enzyme}_{\text{basal}} + \text{CaM*Ca}_n \rightleftharpoons \text{complex}_{\text{activated}}$$

the concentration of free CaM*Ca_n regulates the proportions of the enzyme found in the basal and in the activated state. Even if the affinity of CaM for Ca^{2+} changes when CaM forms a complex with the enzyme, the Ca^{2+} content and the affinity of the latter complex do not need to be considered in our analysis.

The analysis implies that the concentration of the enzyme or other CaM-binding proteins should be small compared to that of CaM*Ca_n at half-maximal activation. According to calculations based on the data of Jarrett and Kyte (19), the concentration of Ca^{2+},Mg^{2+}-ATPase in our enzyme assays amounts to no more than 10^{-12} M—i.e., 1/600th of the dissociation constant for CaM·Ca_3. Spectrin has been reported to be an abundant CaM-binding protein (24) but does not interfere in the assays because our membrane preparation was stripped of spectrin, and the affinity of spectrin for calmodulin is no more than 1/1,000th that for the Ca,Mg^{2+}-ATPase (24).

The CaM*Ca_n species in equilibrium with the activated enzyme proved to be the one that binds at least three Ca^{2+}. Because a similar stoichiometry was obtained with phosphodiesterase (7), adenylate cyclase (8), and CaM-binding to synaptosomal membranes (8), it seems that the Ca-dependent exposure of the hydrophobic region on CaM, which is thought to be the key event in the activation of CaM by Ca^{2+} (25, 26), is concomitant with the appearance of the species CaM·Ca_3. Contrary to our results, Huang *et al.* (27) and Blumenthal and Stull (28) proposed that the activation of CaM requires the binding of four Ca^{2+}. However, when ionic strengths as high as those found *in vivo* were used for enzyme assays, we (7) and Haiech *et al.* (29) found that CaM displays only three high-affinity binding constants for Ca^{2+}, although full enzyme activation is reached. Recently, Foder and Scharff (30) claimed that all CaM·Ca_n complexes activate erythrocyte Ca^{2+},Mg^{2+}-ATPase but have varying affinities for the enzyme. Unfortunately these authors disregarded the fact that K_{app} depends not only on Ca^{2+} concentration but also on K_c and K_a^c. Furthermore, their kinetic analysis is blurred because 1 mM EGTA or another chelator was present throughout.

The dissociation constant of the enzyme for CaM*Ca_3 in the presence of saturating amounts of Ca^{2+} in the substrate binding site amounts to 0.6 nM. On the assumption that any added CaM is active irrespective of the degree of saturating with Ca^{2+}, Raess and Vincenzi (31) reported a K_{app} of 3 nM at 10 μM free Ca^{2+}. Recalculation of their data in terms of CaM·Ca_3 alone yields a K_a^c of 0.75 nM, which is in good agreement with our value. Similar calculations with the data of Jarrett and Kyte (19) and of Graf and Penniston (32) yield K_c^a values of 3.5 nM and 0.1 nM, respectively. Given the experimental errors and discrepancies in the methods, it can be assumed that the dissociation constant is in the nanomolar range and close to those pertaining to brain phosphodiesterase and adenylate cyclase. It should be noted that the affinity of the enzyme for CaM*Ca_3 is not the same when the substrate binding site is saturated with Ca^{2+} as when the site is not saturated. Because $K_c \times K_a^c = K_a \times K_c^a$, the value of K_a—i.e., the dissociation constant of the enzyme for CaM·Ca_3 in the absence of Ca^{2+} at the substrate binding site—can be calculated; it equals 11 nM, about 1/20th that in the presence of substrate Ca^{2+}. This is a quantitative demonstration of the modulation of the affinity of a CaM-dependent enzyme for its activator by the substrate.

Contrary to some reports (9, 16), excess CaM does not induce pronounced positive cooperativity in Ca binding to the substrate site. However, the appearance of apparent positive cooperativity can be predicted from the kinetic mechanism depicted above. Indeed, with all the parameters known, the total initial velocity, v_i, can be expressed as a function of the free Ca^{2+} concentration at different concentrations of total CaM. If at the extreme values of total CaM (10^{-11} and 10^{-3} M) the activation curves are noncooperative, at intermediate concentrations of total CaM, the activation curves do display pronounced positive cooperativity (Fig. 4). Fig. 5 shows the three-dimensional picture of the activation of Ca^{2+},Mg^{2+}-ATPase by Ca^{2+} and CaM. When the free Ca^{2+} concentration is increased at low total CaM, the activity reaches a plateau value which is one-third of that in the presence of high CaM; moreover, the transition from the lower to the higher plateau, induced by the increase in total CaM, tends to be cooperative.

In normal erythrocytes the total concentration of CaM is thought to be 2.5–7 μM (30, 33, 34) and the concentration of free Ca^{2+} is 0.25 μM (1). Hence, the steady-state activity of the Ca pump amounts to 0.2–0.5% of its maximal activation (Fig.

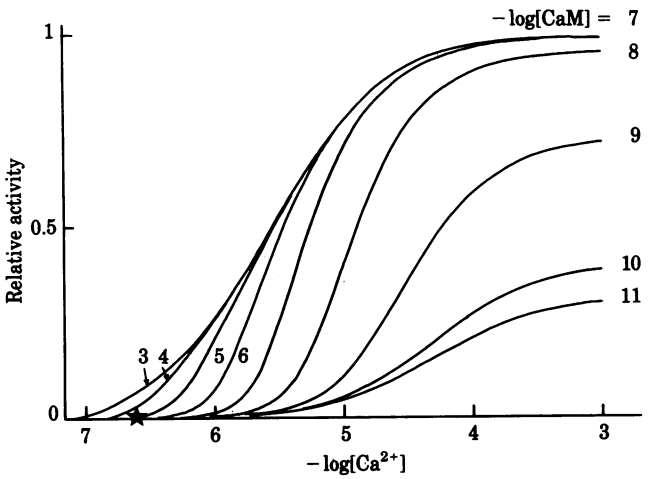


FIG. 4. Activity of erythrocyte Ca^{2+},Mg^{2+}-ATPase as a function of concentrations of free Ca^{2+} and CaM calculated with the experimentally determined parameters. The inhibitory effect of Ca^{2+} at >130 μM in the presence of CaM was not considered here. ★, Ca^{2+},Mg^{2+}-ATPase activity at the free Ca^{2+} and total CaM concentrations presumed to exist in the intact erythrocyte.

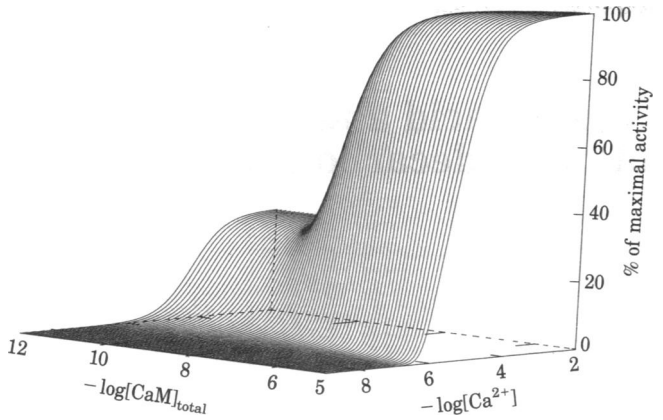


FIG. 5. Three-dimensional picture of the activity of Ca^{2+},Mg^{2+}-ATPase as a function of Ca^{2+} and CaM concentrations. The inhibitory effect of Ca^{2+} at >130 μM in the presence of CaM, and at >630 μM in its absence, was not considered here. The Ca-independent hydrolysis of ATP has been subtracted. The lower plateau represents the maximal Ca-dependent basal activity (V_0 = 0.020 mol/min per mg of protein); the upper plateau represents the maximal Ca- and CaM-dependent activity (V_a = 0.071 μmol/min per mg of protein). The curves at the far left and far right represent the basal and CaM-dependent activities, respectively, as a function of the free Ca^{2+} concentration. Ten lines correspond to 1 order of magnitude of CaM concentration.

4) and therefore represents an economical way of functioning. When the Ca^{2+} permeability increases and the intracellular free Ca^{2+} concentration increases 2-fold, the pumping activity increases more than 6-fold, whereas a 10-fold increase of intracellular Ca^{2+} leads to a 70-fold increase in pumping. If the efficiency of this mechanism decreases, as is apparently the case in sickle cell anemia (35), the rate of Ca pumping slows down rapidly and the intracellular Ca^{2+} concentration increases. This explains why Eaton *et al.* (36) found approximately 8 times as much calcium in erythrocytes of sickle cell patients as in normal cells.

The authors are grateful to Dr. J.-J. Combremont (Laboratoire de Chimie Théorique Appliquée, University of Geneva) for computer graphics. This work was supported by Swiss National Science Foundation Grant 3.237.77.

1. Simons, T. J. B. (1976) *J. Physiol.* **256**, 227–244.
2. Ferreira, H. G. & Lew, V. L. (1976) *Nature (London)* **259**, 47–49.
3. Carafoli, E. (1981) *Cell Calcium* **2**, 353–363.
4. Schatzmann, H. J. & Vincenzi, F. F. (1969) *J. Physiol.* **214**, 481–507.
5. Gopinath, R. M. & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1203–1209.
6. Jarrett, H. W. & Penniston, J. T. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1210–1216.
7. Cox, J. A., Malnoë, A. & Stein, E. A. (1981) *J. Biol. Chem.* **256**, 3218–3222.
8. Malnoë, A., Cox, J. A. & Stein, E. A. (1982) *Biochim. Biophys. Acta* **714**, 84–92.
9. Scharff, O. & Foder, B. (1978) *Biochim. Biophys. Acta* **471**, 59–66.
10. Muallem, S. & Karlish, S. J. D. (1980) *Biochim. Biophys. Acta* **597**, 631–636.
11. Muallem, S. & Karlish, S. J. D. (1981) *Biochim. Biophys. Acta* **647**, 73–86.
12. Niggli, V., Adnuyah, E. S., Penniston, J. T. & Carafoli, E. (1981) *J. Biol. Chem.* **256**, 395–401.
13. Waisman, D. M., Gimble, J. M., Goodman, D. B. P. & Rasmussen, M. (1981) *J. Biol. Chem.* **256**, 409–414.
14. Macintyre, J. D. & Gunn, R. B. (1981) *Biochim. Biophys. Acta* **644**, 351–362.
15. Roufogalis, B. D. & Mauldin, D. (1980) *Can. J. Biochem.* **58**, 922–927.
16. Downes, P. & Michell, R. H. (1981) *Nature (London)* **290**, 270–271.
17. Al-Jobore, A. & Roufogalis, B. D. (1981) *Biochim. Biophys. Acta* **645**, 1–9.
18. Mauldin, D. & Roufogalis, B. D. (1980) *Biochem. J.* **187**, 507–513.
19. Jarrett, J. W. & Kyte, J. (1979) *J. Biol. Chem.* **254**, 8237–8244.
20. Barnett, R. E. (1970) *Biochemistry* **9**, 4644–4648.
21. Trautschold, I. & Löffler, G. (1974) in *Methods of Enzymatic Analysis*, ed. Bergmeyer, H. U. (Academic, New York), pp. 229–247.
22. Donaldson, S. K. B. & Kerrick, W. G. L. (1975) *J. Gen. Physiol.* **66**, 427–444.
23. Weber, G. (1975) *Adv. Protein Chem.* **29**, 1–82.
24. Sobue, K., Muramoto, Y., Fujita, M. & Kakiuchi, S. (1981) *Biochem. Biophys. Res. Commun.* **100**, 1062–1070.
25. La Porte, D. C., Wierman, B. M. & Storm, D. R. (1980) *Biochemistry* **19**, 3814–3819.
26. Tanaka, R. & Hidaka, H. (1980) *J. Biol. Chem.* **255**, 11078–11080.
27. Huang, C. Y., Chaun, V., Chock, P. B., Wang, J. H. & Sharma, R. K. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 871–874.
28. Blumenthal, D. K. & Stull, J. T. (1980) *Biochemistry* **19**, 5608–5614.
29. Haiech, J., Klee, C. B. & Demaille, J. G. (1981) *Biochemistry* **20**, 3890–3897.
30. Foder, B. & Scharff, O. (1981) *Biochim. Biophys. Acta* **649**, 367–376.
31. Raess, B. U. & Vincenzi, F. F. (1980) *Mol. Pharmacol.* **18**, 253–258.
32. Graf, E. & Penniston, J. T. (1981) *Arch. Biochem. Biophys.* **210**, 257–262.
33. Hinds, T. R. & Andreasen, T. J. (1981) *J. Biol. Chem.* **256**, 7877–7882.
34. Vincenzi, F. F. & Gopinath, R. M. (1977) in *Calcium and Cell Function*, ed. Cheung, W. Y. (Academic, New York), Vol. 1, pp. 128–165.
35. Vincenzi, F. F. & Gopinath, R. M. (1977) in *Calcium-binding Proteins and Calcium Function*, eds. Wasserman, R. H. *et al.* (Elsevier/North-Holland, New York), pp. 507–509.
36. Eaton, J. W., Skelton, T. D., Swofford, H. S., Kolpin, C. E. & Jacob, H. S. (1973) *Nature (London)* **246**, 105–106.