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STUDIES ON THE MECHANISM OF REGULATION OF THE RED-CELL Ca^{2+} PUMP BY CALMODULIN AND ATP

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(1) The effects of calmodulin binding on the rates of Ca^{2+}-dependent phosphorylation and dephosphorylation of the red-cell Ca^{2+} pump, have been tested in membranes stripped of endogenous calmodulin or recombined with purified calmodulin. (2) In Mg^{2+}-containing media, phosphorylation and dephosphorylation rates are accelerated by a large factor (at 0°C), but the steady-state level of phosphoenzyme is unaffected by calmodulin binding (at 0°C and 37°C). In Mg^{2+}-free media, slower rates of phosphoenzyme formation and hydrolysis are observed, but both rates and the steady-state phosphoenzyme level are raised following calmodulin binding. (3) At 37°C and 0°C, the rate of (Ca^{2+} + Mg^{2+})-ATPase activity is stimulated maximally by 6–7-fold, following calmodulin binding. At 37°C the apparent Ca^{2+} affinity for sustaining ATP hydrolysis is raised at least 20-fold, $K_{m_{(Ca)}} \simeq 10$ μM (– calmodulin) and $K_{m_{(Ca)}} < 0.5$ μM (+ calmodulin), but at 0°C the apparent Ca^{2+} affinity is very high in calmodulin-stripped membranes and little or no effect of calmodulin is observed ($K_{m_{(Ca)}} \simeq 3–4 \cdot 10^{-8}$ M). (Ca^{2+} + Mg^{2+})-ATPase activity in calmodulin activated membranes and at saturating ATP levels, is sharply inhibited by addition of calcium in the range 50–2 000 μM. (4) A systematic study of the effects of the nucleotide species MgATP, CaATP and free ATP on (Ca^{2+} + Mg^{2+})-ATPase activity in calmodulin-activated membranes reveals: (a) In the 1–10 μmolar concentration range MgATP, CaATP and free ATP appear to sustain (Ca^{2+} + Mg^{2+})-ATPase activity equally effectively. (b) In the range 100–2 000 μM, MgATP accelerates ATP hydrolysis ($K_{m_{(MgATP)}} \simeq 360$ μM), and CaATP is an inhibitor ($K_{i_{(CaATP)}} \simeq 165$ μM), probably competing with MgATP for the regulatory site. (5) The results suggest that calmodulin binding alters the conformational state of the Ca^{2+}-pump active site, producing a high (Ca^{2+} + Mg^{2+})-ATPase activity, high Ca^{2+} affinity and regulation of activity by MgATP.

Introduction

The ATP-fuelled Ca^{2+} pump of human erythrocyte membranes maintains low cytoplasmic Ca^{2+} concentrations. It is now clear that the red cell Ca^{2+} pump activity, like that of many Ca^{2+}-dependent processes in many cells is greatly influenced by interaction with the Ca^{2+}-binding protein calmodulin [1,2]. Binding of calmodulin to red cell membranes increases the rate of (Ca^{2+} + Mg^{2+})-ATPase activity and in general reduces the concentration of Ca^{2+} required for activating the enzyme [3–7]. However, the detailed mechanism of action of calmodulin remains largely unknown. Investigating this problem constitutes the major aim of the present paper.

In a preliminary communication [8] we reported that binding of pure calmodulin to red-cell membranes, thoroughly stripped of their endogenous calmodulin produced a large acceleration in the rate

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

of Ca^{2+}-dependent phosphorylation of the (Ca^{2+} + Mg^{2+})-ATPase. In media containing Mg^{2+}, the steady-state level of phosphoenzyme was unaffected, implying that phosphorylation and dephosphorylation were equally accelerated, but direct evidence was not obtained. Rega and Garrahan [9] have reported experiments from which they inferred that calmodulin stimulates ATP hydrolysis by increasing only the hydrolysis of phosphoenzyme. In continuation of the previous work we describe here experiments to look directly at effects of calmodulin on rates of dephosphorylation, and also to compare the effects of the calmodulin on the phosphorylation and dephosphorylation reactions and on steady-state ATP hydrolysis in the same conditions.

Another aspect of the interaction between calmodulin and the Ca^{2+} pump concerns regulation by ATP. In resealed red cell ghosts (Ca^{2+} + Mg^{2+})-ATPase and ATP-dependent Ca^{2+} pumping are activated by ATP with both high and low apparent affinities (K_m 1–2 μM and 200–300 μM, respectively) [10], and the same phenomenon is observable for ATPase activity in broken membranes [11]. The high affinity site is probably involved in phosphorylation but ATP bound at the low affinity site has a regulatory role [8–11]. The biphasic activation by ATP is observed only in membranes bound with calmodulin [8]. In our experiments with resealed ghosts [10] high concentrations of Ca^{2+} in the cell inhibited the rates of ATP hydrolysis and Ca^{2+} pumping, and also converted the biphasic ATP activation to an approximately hyperbolic activation. It was therefore of interst to look further at the relationship between the nucleotide regulatory effect and inhibition by calcium, on calmodulin-activated Ca^{2+} pumps. This has taken the form of a systematic study of the effects on (Ca^{2+} + Mg^{2+})-ATPase activity of the various ligands, free ATP, MgATP and CaATP, Mg^{2+} and Ca^{2+}, in order to establish which of the nucleotide species act as substrate and regulatory activator, and which of the species Ca^{2+} or CaATP inhibits enzyme activity.

The combination of information from phosphorylation and ATP hydrolysis experiments has been used to build up a picture of the effects of calmodulin in terms of the following kinetic scheme (which is useful also for understanding the experimental design).

$$\mathrm{ATP} + 2\,Ca^{2+}_{in} \cdot E_1 \underset{\mathrm{ADP}}{\overset{\mathrm{ATP}}{\rightleftharpoons}} (2\,Ca)E_1P \overset{2Ca^{2+}_{out}}{\rightleftharpoons} E_2P \underset{P_i}{\overset{}{\rightleftharpoons}} E_2 \rightleftharpoons E_1$$

E_1 is the species thought to bind ATP and Ca^{2+} at the inner surface with high affinity. It undergoes phosphorylation to the high-energy phosphoenzyme E_1P to which Ca^{2+} is still bound. E_1P can transfer its phosphate group to ADP. It is thought that the conformational transition E_1P to the low-energy phosphoenzyme E_2P is associated with Ca^{2+} transport and Ca^{2+} dissociates from low affinity binding sites oriented to the exterior. The conformational transition $E_1P \rightarrow E_2P$ and the hydrolysis step seem to require Mg^{2+} [12]. Dephosphorylation is accelerated by ATP with a low affinity [13] a finding which may account for the nucleotide regulation. Finally the conformational transition $E_2 \rightarrow E_1$, resets the mechanism for a new cycle.

Materials and Methods

1. Preparation of membranes. Calmodulin-stripped membranes from human erythrocytes were prepared as described previously [8] except where otherwise indicated. When necessary 'tightly bound Mg^{2+}' was removed from these membranes by first incubating the membranes in a solution containing 2 mM Hepes-Tris (pH 7.4 at 0°C) and 10 mM CDTA (Tris) for 10 min and then washing twice with the same solution, and three times with a solution of 2 mM Hepes-Tris (pH 7.4 at 0°C). The final pellet was resuspended at a protein concentration of about 4 mg/ml and used immediately.

2. Purification of calmodulin. The calmodulin used in these experiments was prepared according to our reported procedure [14].

3. Assays. The amount of the Ca^{2+}-dependent phosphoenzyme was estimated essentially as described before (8). Membranes were incubated in a medium of volume 0.5 ml containing 150 mM Hepes-Tris buffer (pH 7.4 at 0°C); 10 mM KCl and either 2 mM $MgCl_2$ plus 1 mM EGTA (Tris) or 2 mM $MgCl_2$ plus 0.05 mM $CaCl_2$. The phosphorylation reaction was initiated by the addition of ATP (containing about $5 \cdot 10^5$ cpm of [γ-^{32}P]ATP) to a final concen-

tration of 2 μM. The reaction was terminated by the quick addition of 5 ml of a solution containing 5% perchloric acid; 1 mM ATP and 10 mM potassium phosphate (stopping solution). For the dephosphorylation experiments, the phosphorylation reaction was allowed to proceed for 20 s at 0°C and was then interrupted by the quick addition of 50 μl of a solution containing ATP, Ca^{2+} chelators and or other ligands, as described in detail in the figure legends. After various times the dephosphorylation reaction was terminated with the stopping solution, and after 20 min at 0°C, the precipitated membranes were collected by centrifugation. The membranes were washed three times in an ice-cold solution of 5% perchloric acid containing 10 mM $MgCl_2$ and were then dissolved in 4% SDS solution. The tubes were incubated for 10 min in a boiling water bath, and 0.5-ml aliquots of their contents were transferred into counting vials and the amount of ^{32}P was measured. 5 to 10-μl aliquots from each tube were used for protein determination by the method of Lowry et al. [15]. The amount of the incorporated phosphate was calculated as a fraction of the added ATP, and was expressed as pmol per mg protein. The Ca^{2+}- or in certain cases the (Ca^{2+} + La^{3+})-dependent phosphoenzyme level was calculated by substracting the ^{32}P incorporation in the EGTA medium from that in Ca^{2+}- or (Ca^{2+} + La^{3+})-containing media.

The measurement of the (Ca^{2+} + Mg^{2+})-dependent ATPase activity of the calmodulin-stripped and -reactivated membranes was done as in Ref. 8. In the experiments of Figs. 8 and 9 the total concentrations of ATP, $CaCl_2$ and $MgCl_2$ were varied so as to produce the desired concentrations of MgATP, CaATP and Ca^{2+} calculated by using the following equations and equilibrium constants [16] (see Tables II and III).

$$[MgATP] = K_1 [Mg^{2+}] [ATP^{4-}] \quad K_1 = 10^{4.65} \qquad (1)$$

$$[CaATP] = K_2 [Ca^{2+}] [ATP^{4-}] \quad K_2 = 10^{4.32} \qquad (2)$$

$$[ATP^{3-}] = K_3 [H^+] [ATP^{4-}] \quad K_3 = 10^{7.02} \qquad (3)$$

$$[ATP]_{total} = [ATP]_{free} + [MgATP] + [CaATP] \qquad (4)$$

$$[ATP]_{free} = [ATP^{3-}] + [ATP^{4-}] \qquad (5)$$

$$[Ca]_{total} = [Ca^{2+}] + [CaATP] \qquad (6)$$

$$[Mg]_{total} = [Mg^{2+}] + [MgATP] \qquad (7)$$

combining Eqns. 1, 2, and 7 gives rise to:

$$[Mg]_{total} = \frac{[MgATP]\,[Ca^{2+}]\,K_2}{K_1\,[CaATP]} + [MgATP] \qquad (8)$$

By inserting the desired concentration of MgATP, CaATP and Ca^{2+} into Eqn. 8 the $[Mg]_{total}$ is calculated and hence from Eqn. 7 $[Mg^{2+}]$ is calculated. From Eqns. 1 and 3 $[ATP^{4-}]$ and $[ATP^{3-}]$ are calculated and from Eqns. 4 and 5 the $[ATP]_{total}$ to be added is obtained. The $[Ca]_{total}$ is obtained from Eqn. 6.

4. Reagents. Vanadate-free ATP was obtained from Boehringer Mannheim GmbH, F.R.G. and [γ-^{32}P]ATP was obtained from the Radiochemical Centre, Amersham, U.K. All other reagents were of analytical grade.

Results

Phosphorylation experiments

Fig. 1 shows the level of Ca^{2+}-dependent phosphoenzyme at 0°C and at different times in calmodulin-stripped or -reactivated membranes, and in the presence of La^{3+}. La^{3+} is thought to inhibit the dephosphorylation stage in the reaction mechanism and is used to estimate the maximal level of the phosphoenzyme [17]. As shown before [8] the rate of phosphorylation in the absence of calmodulin is relatively slow, following an exponential time course with an apparent rate constant, k_{obs} of 0.092 s^{-1}. In calmodulin-reactivated membranes the rate of phosphorylation is greatly accelerated with little or no change in the final steady-state level of EP, which is about 70% of the maximal level measured with La^{3+}. In order to compare directly effects of calmodulin on phosphorylation with those on ATPase we have also looked at Ca^{2+}-dependent phosphorylation at 37°C (Table I). The rates of phosphorylation and dephosphorylation are too fast to be measured but one can estimate easily the steady-state EP levels, which is constant between 2 and 4 s of incubation (Expt. 231080). Again, the steady-state EP is about 70% of the value observed with La^{3+}, and it is not affected by calmodulin.

The results of Fig. 1 and Table I imply that the net phosphorylation rate is 3–4-fold faster than the net dephosphorylation rate. Dephosphorylation of the phosphoenzyme can be revealed by addition of high

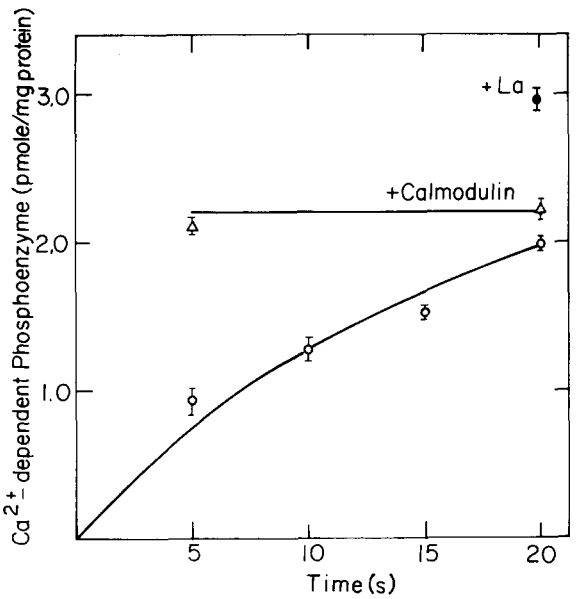


Fig. 1. Stimulation of Ca^{2+}-dependent phosphorylation by calmodulin. Calmodulin-stripped and -reactivated membranes [8] were incubated in the standard phosphorylation medium and ^{32}P incorporation was measured for the times indicated as described in Materials and Methods. The 'maximal level' of the phosphoenzyme was estimated by including 0.1 mM $La(Ac)_3$ in the reaction medium. Each tube contained 2 mg of membrane protein with or without 75 μg of purified calmodulin : −calmodulin (○); +calmodulin (△); +calmodulin + $La(Ac)_3$ (●).

concentration of nonradioactive ATP which displace the radioactive substrate. The rate is fast and any stimulation by binding of calmodulin cannot easily be detected [8]. In addition to displacing the [γ-^{32}P]-ATP, high concentrations of ATP have been shown to accelerate the hydrolysis of the phosphoenzyme [13]. In order to look at the effect of calmodulin on the unaccelerated dephosphorylation rate, it was necessary to interrupt phosphorylation, by adding Ca^{2+} chelators to quickly reduce the Ca^{2+} concentration below that required for phosphorylation (Fig. 2). With calmodulin-stripped membranes, addition of 1 mM CDTA and 1 mM EGTA, sufficient to reduce the Ca^{2+} concentration to less than 10^{-9} M, lead to a very slow fall in the level of phosphoenzyme with a half-time of about 25 s, and it eventually reached zero. There was some doubt whether the chelators reduce the Ca^{2+} concentration quickly

TABLE I

Ca^{2+}-DEPENDENT PHOSPHOENZYME MEASURED AT 37°C

Calmodulin-stripped membranes were prepared essentially as described before [8] except that (a) the cells were lysed and the membranes were washed three times with a solution of 5 mM potassium phosphate, pH 8.2 and (b) after the 'freeze-thaw' stage in the same solution containing also 5 mM EGTA, the membranes were washed twice with 0.5 mM potassium phosphate pH 8.2 and one with 10 mM Hepes-Tris (pH 7.4 at 37°C). The membrane suspension was preincubated at 37°C in the standard medium with or without calmodulin and La^{3+} as indicated. Phosphorylation was initiated by addition of [γ-^{32}P]ATP, and after 2 s (or 4 s in Expt. 231080), the stopping solution was added.

Expt. No.	Conditions	Ca^{2+}-dependent EP (pmol per mg protein)	Fraction of maximal EP level
221080	Ca^{2+}, 50 μM	2.66 ± 0.07	0.70
	Ca^{2+}, 50 μM + calmodulin	2.59 ± 0.15	0.68
	Ca^{2+}, 50 μM + La^{3+}, 100 μM	3.83 ± 0.09	1.00
	Ca^{2+}, 50 μM + La^{3+}, 100 μM, 0°C	3.81 ± 0.09	1.00
231080	Ca^{2+}, 50 μM, 2 s	4.88	
	Ca^{2+}, 50 μM, 4 s	5.04	
	Ca^{2+}, 50 μM + calmodulin 2 s	5.11	
	Ca^{2+}, 50 μM + calmodulin 4 s	5.19	
51180	Ca^{2+}, 50 μM	2.43 ± 0.13	0.72
	Ca^{2+}, 50 μM + calmodulin	2.26 ± 0.17	0.67
	Ca^{2+}, 50 μM + La^{3+}, 100 μM	3.37 ± 0.1	1.00
	Ca^{2+}, 50 μM + La^{3+}, 100 μM, 0°C	3.42 ± 0.1	1.00

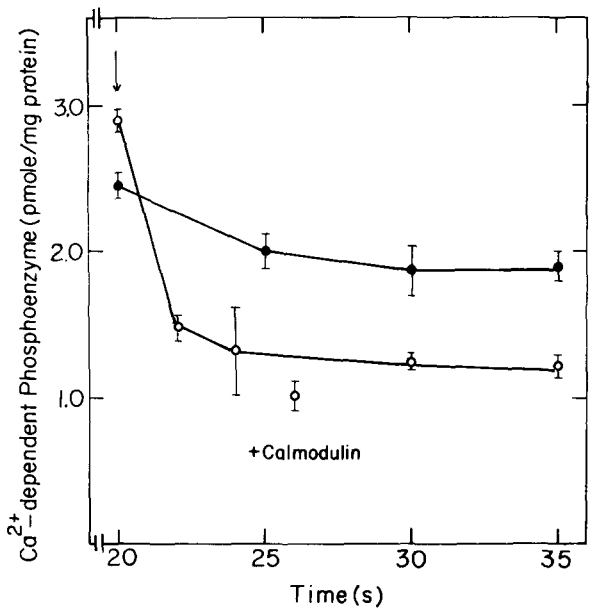


Fig. 2. Dephosphorylation of the Ca^{2+}-dependent phosphoenzyme. The effect of calmodulin. The phosphorylation reaction of calmodulin-stripped and -reactivated membranes (2 mg) was carried out as in Fig. 1 except that the $MgCl_2$ concentration was 3 mM. After 20 s of incubation at 0°C, CDTA and EGTA were added at a final concentration of 1 mM of each chelator, and then at the indicated times the stopping solution was added quickly. The level of phosphoenzyme was determined as in Fig. 1. –calmodulin (•); +calmodulin (○).

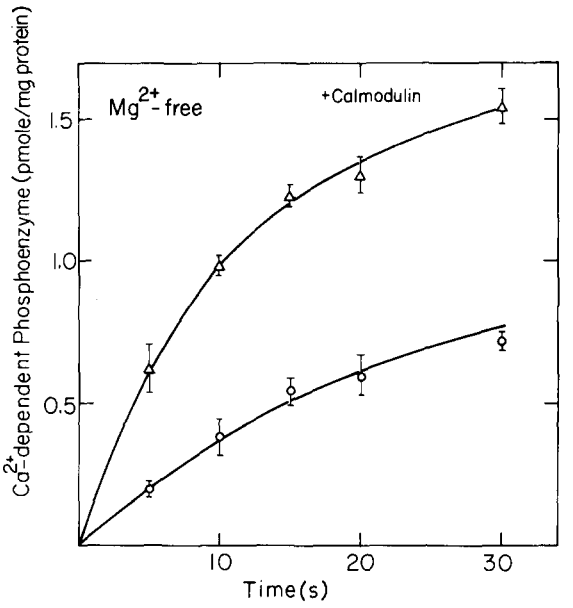


Fig. 3. Stimulation by calmodulin of Ca^{2+}-dependent phosphoenzyme formation in Mg^{2+}-free conditions. Membranes were stripped of Mg^{2+} as described in Materials and Methods. Phosphorylation conditions were as in Fig. 1 except that $MgCl_2$ was omitted from the reaction medium. –calmodulin (○); +calmodulin (△).

enough, and whether therefore the observed decrease in phosphoenzyme is a true indication of the dephosphorylation rate. If we depict the reversible phosphorylation reaction formally at a fixed ATP concentration, as $E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EP$, the apparent first-order rate constant for phosphorylation is equal to $k_1 + k_2$, the rate-constant for dephosphorylation is k_2, and in the steady-state the fraction of phosphoenzyme EP/E_{total} is $k_1/(k_1 + k_2)$. The observed fraction 0.72 compares very favorably with that calculated from the observed rates of phosphorylation ($k_1 + k_2 = 0.092\ s^{-1}$) and dephosphorylation ($k_2 = 0.028\ s^{-1}$) in the calmodulin-stripped membranes, i.e. 0.70. Therefore, the chelators worked fast enough. With the calmodulin-reactivated membranes we have repeatedly observed a rapid initial drop in the level of phosphoenzyme followed by a slow decay similar in rate to that in the absence of calmodulin (Fig. 2). In different experiments the amplitude of the rapid phase varied between about 40 to 80% of the phosphoenzyme level. This seemed to indicate a large stimulation of the dephosphorylation rate (roughly 10-fold) followed by dissociation of calmodulin due to chelation of the Ca^{2+}.

In order to test this interpretation, phosphorylation and dephosphorylation experiments were performed also in media in which Ca^{2+} was always present. Garrahan and Rega [13] have shown that the accelerating effect of ATP on dephosphorylation requires the presence of low concentrations of Mg^{2+}. Therefore interruption of phosphorylation in 'Mg^{2+}-free' media by nonradioactive ATP should reveal the unaccelerated rate of dephosphorylation. For 'Mg^{2+}-free experiments' we have found it necessary to thoroughly remove apparently 'tightly bound Mg^{2+}' by washing the membranes with a high concentration of CDTA, in addition to using Mg^{2+}-free solutions (see Methods). The phosphorylation rate and steady-state level of the phosphoenzyme are much reduced compared to that in the presence of Mg^{2+} (compare Figs. 1 and 3). Calmodulin increased significantly the

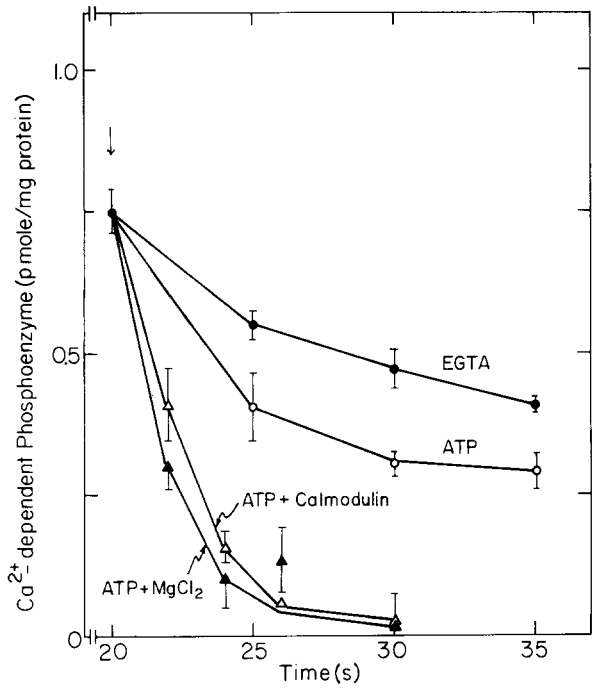


Fig. 4. Stimulation by calmodulin of phosphoenzyme hydrolysis. The phosphorylation of both Mg^{2+}- and calmodulin-stripped membranes was done as in Fig. 3. After 20 s of incubation at 0°C, 50 μl of different solutions were added to produce the following final concentrations: 5 mM EGTA (•); 2 mM ATP-Tris (pH 7.4 at 0°C) (○); 2 mM ATP and 75 μg calmodulin (△); 2 mM ATP + 2 mM $MgCl_2$ (▲). At the indicated times the dephosphorylation reaction was terminated and the phosphoenzyme was estimated as in Fig. 1.

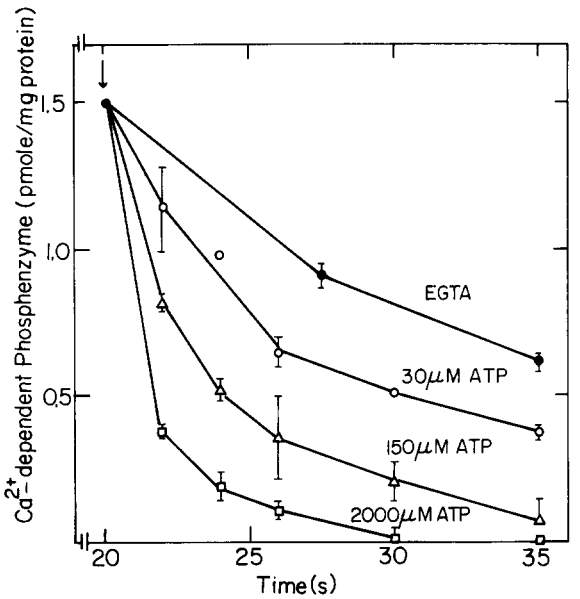


Fig. 5. Acceleration of dephosphorylation by ATP, in calmodulin-stripped membranes. After 20 s of incubation in the phosphorylation reaction medium dephosphorylation was initiated by the quick addition of 50 μl of different solutions which produce the following concentrations of ligands: 1 mM EGTA, 1 mM $MgCl_2$ (•); 0.03 mM ATP, 0.03 mM $MgCl_2$, (○); 0.15 mM ATP, 0.15 mM $MgCl_2$ (△); 2 mM ATP, 2 mM $MgCl_2$ (□). The phosphoenzyme level was estimated at the indicated times.

phosphorylation rate (although less than in the conditions of Fig. 1) and the level of phosphoenzyme, at 30 s (close to the steady-state value) rose from about 33 to 66% of the maximal. The dephosphorylation rate measured after addition of EGTA was slow, k_2 = 0.015 s^{-1} (Fig. 4). Addition of unlabelled ATP produced only a small stimulation of the phosphoenzyme decay rate. However, a rapid rate of dephosphorylation was observed when ATP was added together with calmodulin and all the phosphoenzyme was sensitive to ATP and calmodulin. Also the calmodulin must have bound to the Ca^{2+} pump at a rate equal, at least, to the observed dephosphorylation rate, i.e. with a half-time of at most 2 s. As expected, in the presence of both Mg^{2+} and ATP a fast dephosphorylation rate was observed.

Fig. 5 shows that increasing the concentration of MgATP from 30–2 000 μM lead to a progressive rise in the rate of dephosphorylation of calmodulin-stripped membranes. In this experiment ATP was present as MgATP, but other experiments showed that only micromolar concentrations of Mg^{2+} were necessary (not shown), confirming the report [13] that uncomplexed ATP must also be effective.

Measurements of (Ca^{2+} + Mg^{2+})-ATPase activity

It has been reported frequently that calmodulin decreases the concentration of Ca^{2+} required for activation of ATPase [3,4,18]. We have looked at Ca^{2+} activation at 0°C, in conditions identical to those of the phosphorylation experiments. The results are presented in Fig. 6 in the form of free Ca^{2+} activation curves for (Ca^{2+} + Mg^{2+})-ATPase with 2 μM ATP, for both calmodulin-stripped and -reactivated membranes. The reaction medium, containing 2.5 mM $MgCl_2$ and 10 mM KCl may be contaminated with as

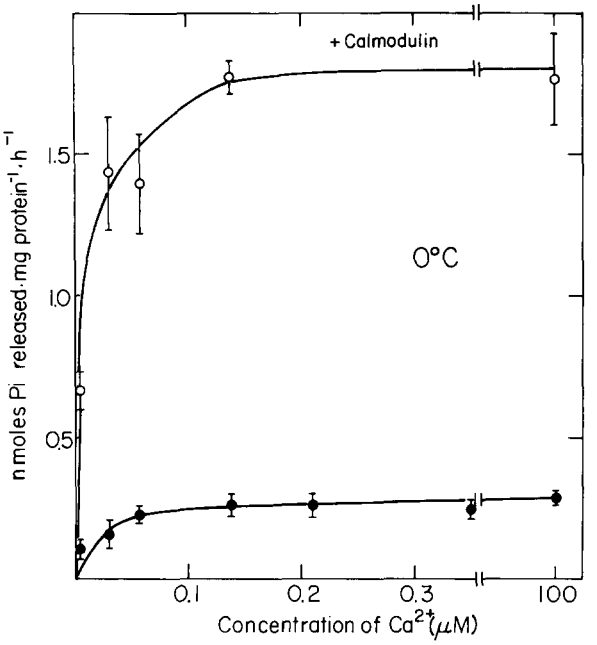


Fig. 6. The Ca^{2+}-dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity at 0°C, in the presence or absence of calmodulin. Membranes were prepared as in Table I. Calmodulin-stripped membranes (about 2 mg protein) were incubated with or without calmodulin in the following reaction medium: 150 mM Hepes-Tris (pH 7.4 at 0°C); 10 mM KCl; 2.5 mM $MgCl_2$; 0.025 mM EGTA; 0.002 mM ATP (containing $6 \cdot 10^4$ cpm [γ-^{32}P]ATP) and different concentrations of $CaCl_2$ calculated to give the indicated Ca^{2+}. The calmodulin-stripped or reactivated membranes were incubated at 0°C for appropriate times (10 min or 1 min, respectively). The proteins were precipitated by the addition of 0.1 ml of 30% perchloric acid solution, and after 20 min the protein were removed by centrifugation in the Eppendorf centrifuge 3200. 0.2 ml of the supernatant was transferred into fresh Eppendorf tubes for the extraction of $^{32}P_i$. The ATP hydrolysis in the presence of 1 mM EGTA and the Ca^{2+}-free reaction medium (i,e, Mg^{2+}-dependent ATPase activity) was subtracted from the total ATPase activity observed in the presence of both Ca^{2+} and Mg^{2+}. –calmodulin (•); +calmodulin (○).

much as 2 μM of total calcium. In order to control precisely the free Ca^{2+} concentration it is necessary to added chelators such as EGTA. Millimolar concentrations of EGTA and Ca^{2+} mixtures provide an optimal buffer system, but we have several indications that the Ca-EGTA complex can interfere with the Ca^{2+}-pump functions (unpublished observations, see also Ref. 19) and EGTA has therefore been included at a minimal but still effective concentration of 25 μM. It turns out that even in calmodulin-stripped membranes the apparent Ca^{2+} affinity for activating (Ca^{2+} + Mg^{2+})-ATPase at 0°C is very high, $K_{0.5} \simeq 3\text{–}4 \cdot 10^{-8}$ M, and although the calmodulin produced a large increase in the rate (about 7-fold), little or no increase in apparent Ca^{2+} affinity is detectable.

This result contrasted dramatically with the behaviour at 37°C (Fig. 7) in which the activation by calcium were studied at 1 mM ATP. The results are presented as effects of total added Ca, and the sole Ca^{2+} buffer in the reaction mixture is ATP. Here, both activating and inhibitory effects are observed, with an apparent Ca^{2+} affinity which depends greatly on the presence or absence of calmodulin. In calmodulin-stripped membranes about 50 μM total calcium was required for half maximal stimulation of ATP hydrolysis and this corresponds to about 10 μM free Ca^{2+}, at the prevailing ATP and Mg^{2+} concentrations. In the calmodulin-activated membranes (Ca^{2+} + Mg^{2+})-ATPase was stimulated maximally at the lowest total Ca concentrations added, i.e. 1 μM. However, the NaCl containing medium could be contaminated with as much as 5 μM calcium giving a total of 6 μM, and free Ca^{2+} as high as 1.12 μM. Hence the $K_{0.5}$ for Ca^{2+} must be lower than or equal to 0.55 μM and calmodulin reduces the $K_{0.5}$ for Ca^{2+} by at least 20-fold. The maximal degree of activation of calmodulin was about 7-fold. In other measurements of (Ca^{2+} + Mg^{2+})-ATPase activity at 37°C, with 2 μM ATP, a large calmodulin-dependent increase in apparent Ca^{2+} affinity was also detected, but there was little or no inhibition at high calcium concentrations, (a feature seen also in Fig. 6 for the experiment at 0°C). Thus effective inhibition by calcium of ATPase activity was observed only when millimolar concentrations of ATP were used, and in membranes bound with calmodulin. Other experiments showed that if the calmodulin-activated membranes were incubated with high Ca^{2+}, centrifuged and were then washed in a low Ca^{2+} medium (10 μM), the usual high (Ca^{2+} + Mg^{2+})-ATPase activity was restored (not shown). Therefore inhibition cannot be due to dissociation of the calmodulin and must be a more direct effect on the calcium pump itself.

The inhibitory species in the experiment of Fig. 7 could be either free Ca^{2+} or CaATP. The identity of the substrate and activating or inhibitory ligands of

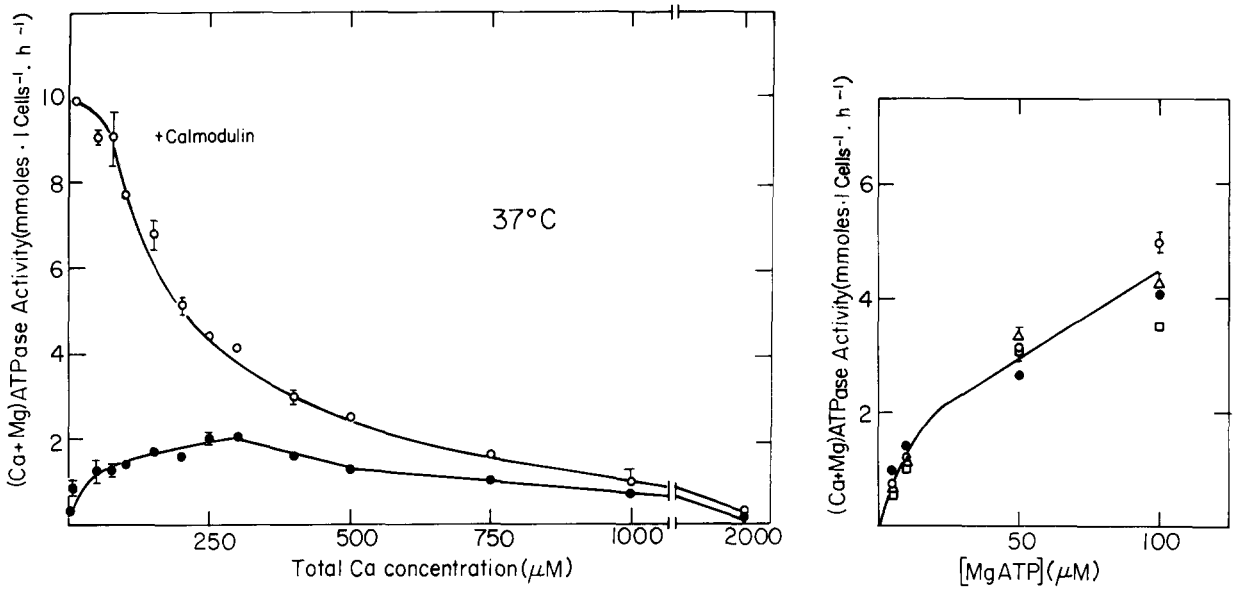


Fig. 7. Effects of calcium on the $(Ca^{2+} + Mg^{2+})$-ATPase activity at 37°C in calmodulin-stripped and -reactivated membranes. 10 μl of membranes suspension (about 60 μg protein) with and without 2.5 μg of purified calmodulin was added to 0.2 ml of the following reaction medium: 100 mM NaCl; 10 mM KCl; 10 mM Hepes-Tris (pH 7.4 at 37°C); 1 mM $MgCl_2$; 1 mM ATP (containing $4 \cdot 10^4$ cpm [γ-^{32}P]ATP) and different $CaCl_2$ concentrations as indicated in the figure. The tubes were incubated for 30 min at 37°C and the $(Ca^{2+} + Mg^{2+})$-ATPase activity was estimated. –calmodulin (•); +calmodulin (○).

Fig. 8. Dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase activity on MgATP concentrations. Lack of inhibition by free Ca^{2+}. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of calmodulin-reactivated membranes was measured essentially as in Fig. 7 except that different concentrations of $MgCl_2$, $CaCl_2$ and ATP were used. The media contained a constant CaATP concentration of 1 μM, the indicated MgATP concentrations and free Ca^{2+} concentrations of: 5 μM (•); 50 μM (○); 250 μM (△) and 500 μM (□). See also Table II.

the $(Ca^{2+} + Mg^{2+})$-ATPase has been investigated by measuring ATP hydrolysis at predetermined concentrations of Ca^{2+}, CaATP and MgATP, in a medium in which the sole chelator of divalent metal cations is ATP. Fig. 8 shows results of an experiment in which MgATP was varied at four different free Ca^{2+} concentrations between 5 and 500 μM, while CaATP was maintained constant at 1 μM. ATPase activity is stimulated along the characteristic biphasic curve with respect to MgATP concentration (see also Ref. 10) but there is no systematic inhibitory effect of free Ca^{2+} at any MgATP level. Table II shows the concentrations of all ligands present. The converse experiment in which we have tested the effects on ATPase activity in calmodulin-activated membranes of different CaATP concentration at varying [MgATP] and a fixed Ca^{2+} of 5 μM, is shown in Fig. 9. Table III gives details of the concentrations of all the ligands present. The rate of $(Ca^{2+} + Mg^{2+})$-ATPase varies in a systematic way with respect to MgATP concentration in the range 5–1 000 μM. CaATP inhibits activity at high MgATP concentrations. At low concentrations of MgATP the rate is independent of the CaATP concentration. With 5 μM MgATP and 1, 10, 100 or 500 μM CaATP the rates of $(Ca^{2+} + Mg^{2+})$-ATPase, in the experiment of Fig. 9 were 0.63, 0.73, 0.8 and 0.62 $mmol \cdot l^{-1} \cdot h^{-1}$, respectively. In the experiments of Figs. 8 and 9 it was of course not possible to control also the concentration of Mg^{2+} and free ATP, for their concentrations are determined by the chosen Ca^{2+}, MgATP and CaATP concentrations. From Fig. 8 and Table II it can be seen that the rate of ATP hydrolysis at any MgATP concentration is quite independent of the Mg^{2+} (see Table II) down to the minimal concentration present of 11.7 μM. In the experiment of Fig. 9 (and see Table III) the rate of ATP hydrolysis at 5 μM MgATP was independent of both CaATP concentra-

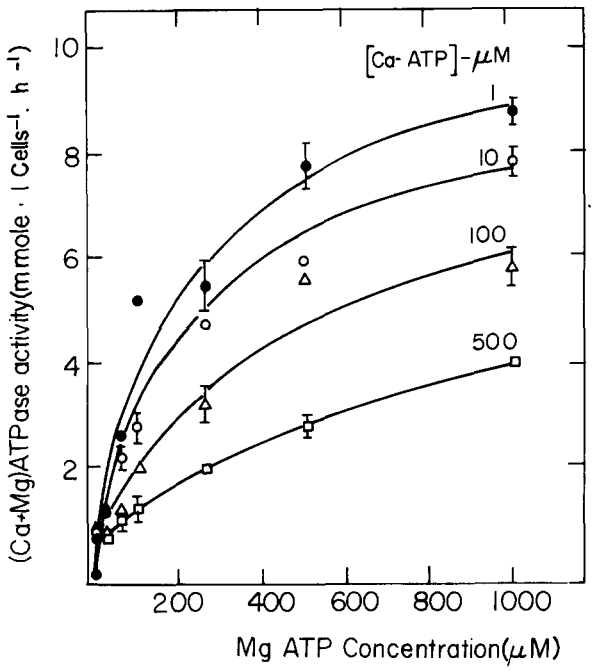


Fig. 9. Dependence of $(Ca^{2+} + Mg^{2+})$-ATPase activity on MgATP concentration. Inhibition by CaATP. The $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured as in Fig. 8. The CaATP concentrations were as follows: 1 μM (•); 10 μM (○); 100 μM (△) and 500 μM (□). The free Ca^{2+} concentration was kept constant (5 μM) at all CaATP concentrations. The curves have been calculated to give the best fit to the experimental points (see legend to Table IV).

tion and Mg^{2+} between 0.02 and 11.7 μM. This lack of requirement for Mg^{2+} does not of course exclude a necessity for 'tightly bound Mg^{2+}' (see Discussion). In the same experiment, Fig. 9 (and see Table III), the

TABLE II

CONCENTRATIONS OF LIGANDS IN μM FOR THE EXPERIMENTS OF FIG. 8

The calculations of the ligand concentrations were done using the equations and the parameters described in Materials and Methods.

MgATP	CaATP	Ca^{2+}	Mg^{2+}	ATP free	ATP_{total}
5	1	5	11.69	13.69	19.56
10			23.39		30.56
50			116.93		64.56
100			233.87		114.56
5	1	50	116.93	1.06	7.36
10			233.87		12.36
50			1 169.34		52.36
100			2 338.68		102.36
5	1	250	584.67	0.27	6.27
10			1 169.34		11.27
50			5 846.69		51.27
100			11 693.38		101.27
5	1	500	1 169.34	0.14	6.14
10			2 338.68		11.14
50			11 693.38		51.14
100			23 386.76		101.14

TABLE III

CONCENTRATION OF LIGANDS IN μM FOR THE EXPERIMENTS OF FIG. 9 AND TABLE IV

The calculation of the ligand concentrations were done as in Table II.

MgATP	CaATP	Ca^{2+}	Mg^{2+}	ATP free	ATP_{total}
5	1	5	11.69	13.56	19.56
10			23.39		30.56
50			116.93		64.56
100			233.87		114.56
250			584.67		264.56
500			1 169.34		514.56
1 000			2 338.68		1 014.56
5	10	5	1.17	135.64	150.64
10			2.34		155.64
50			11.69		195.64
100			23.39		245.64
250			58.47		345.64
500			116.93		645.64
1 000			233.87		1 145.64
5	100	5	0.12	1 356.31	1 461.31
10			0.23		1 466.31
50			5.17		1 506.31
100			2.34		1 556.31
250			5.85		1 706.31
500			11.69		1 956.31
1 000			23.39		2 456.31
5	500	5	0.02	6 781	7 286
10			0.05		7 291
50			0.23		7 331
100			0.47		7 381
250			1.17		7 531
500			2.34		7 781
1 000			4.68		8 281

TABLE IV

CALCULATED KINETIC PARAMETERS FOR THE CURVES IN FIG. 9

The best-fit kinetic parameters were computed from the minimum deviation of the experimental points from the theoretical curves $v = V_1 S/(K_1 + S) + V_2 S/(K_2 + S)$, using a standard least squares fitting programme, statistical analysis system (SAS) NLIN, obtained from Whitlow Computer Systems, Inc., Carry, NC.

Pararmeters	CaATP concentration (μM)			
	1	10	100	500
V_1 (mmol · l^{-1} · h^{-1})	1.6	1.6	0.6	0.64
V_2 (mmol · l^{-1} · h^{-1})	9.8	8.5	8.5	7.0
K_1 (μM)	5.0	5.0	4.0	0.95
K_2 (μM)	325.0	370.0	520.0	1 048.0

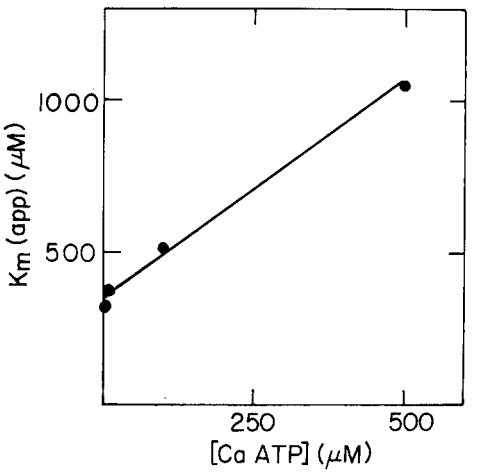


Fig. 10. The dependence of the apparent K_m for MgATP on CaATP concentrations.

concentration of free ATP rose in parallel with that of CaATP. Free ATP clearly cannot activate at the low affinity nucleotide site, for its concentration is constant at any level of CaATP, whilt the rate of ATP hydrolysis rises in response to the MgATP concentration. Furthermore, free ATP cannot be an efficient inhibitor of ATP hydrolysis if at all, for the strong Ca-dependent inhibition must be attributed to CaATP.

The MgATP activation curves drawn in Fig. 9 have been calculated on the assumption that the relationship between the rate and the substrate concentration is of the form

$$v = \frac{V_1 S}{K_1 + S} + \frac{V_2 S}{K_2 + S}$$

Calculated kinetic parameters V_1, V_2 and K_1, K_2 at different CaATP concentration are given in Table IV. Experimental points are of course few at the low ATP concentrations and the parameters of most reliability and interest are V_2 and K_2. The major effect of CaATP is to reduce the apparent affinity for MgATP. The measured values of K_2, are proportional to the CaATP concentration (Fig. 10) suggesting a relationship of the form

$$K_{2\,obs} = K_2 \left[1 + \frac{[CaATP]}{K_i} \right]$$

The calculated K_2 for MgATP is 351 μM and K_i for CaATP is 165 μM.

Discussion

A. Mechanism of action of calmodulin

The major effects of calmodulin on ATP hydrolysis are: (1) An increase of 6–7-fold in the maximal turnover rate at both 37°C [8] and 0°C (see Fig. 6).

(2) A large increase in apparent Ca^{2+} activation affinity at 37°C (Fig. 7), but little or no effect at 0°C (Fig. 6).

(3) Increase in rate of $(Ca^{2+} + Mg^{2+})$-ATPase and apparent affinity for ATP at the high affinity nucleotide binding site, at 37°C [8].

(4) The appearance of the regulatory effect of MgATP with a low affinity [8].

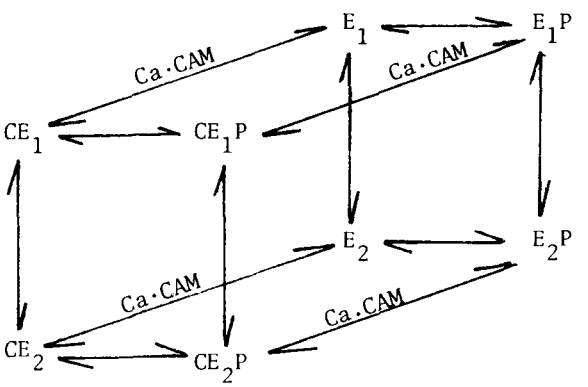
(5) Inhibition of activity by calcium (Fig. 7) which is attributed to CaATP (Fig. 9).

The phosphorylation experiments described here, with Mg^{2+} containing media, point to the following conclusions: (a) The steady-state phosphoenzyme level at 0°C and 37°C is about 0.7 of the maximal level and therefore net dephosphorylation is rate-limiting and roughly 3-times slower than net phosphorylation. This has been verified directly in conditions permitting measurement of the individual rates (i.e. in calmodulin-stripped membranes).

(b) Calmodulin does not affect the steady-state level of phosphoenzyme at 0°C or 37°C in the conditions of our experiments and so it must accelerate phosphorylation and dephosphorylation equally. It is unlikely that the rate-limiting step(s) of the turnover cycle change following binding of calmodulin.

Rega and Garrahan [9] reported a calmodulin-mediated fall in phosphoenzyme level, in Mg^{2+}-containing media, and attribute stimulation of (Ca^{2+} + Mg^{2+})-ATPase activity to increased hydrolysis of phosphoenzyme. This interpretation does not of course exclude acceleration also of phosphorylation by calmodulin. The differences in the experimental findings could be due to the presence of 15 μM ATP in the media of Rega and Garrahan's experiments compared to 2 μM ATP in our conditions. Very recently Enyedi et al. [20] have confirmed, that calmodulin raises the rate of phosphorylation without significantly altering the steady-state level of EP, in a medium containing 1 μM ATP.

The activation of enzymes by calmodulin is thought to involve binding of three to four calcium ions to the calmodulin with a high affinity, a change in calmodulin conformation, combination of the Ca^{2+}-calmodulin complex to the target enzyme, causing finally a change in the enzyme structure and function [2]. Combination of the Ca^{2+}-calmodulin complex with the intermediates of the Ca^{2+}-pump cycle is depicted tentatively in Scheme I. On the basis of this model we now proceed to discuss in turn:



Scheme I. Model of the Ca^{2+}-pump cycle with the Ca^{2+}-calmodulin complex (Ca · CAM). E forms of the pump are stripped of calmodulin. CE forms of the pump are bound with calmodulin.

Reaction rates. An equal stimulation of the net phosphorylation or dephosphorylation rates implies either that all steps in the cycle involving CE forms are equally faster than for the corresponding E forms, or as shown in the Appendix, that pairs of highly rate-limiting steps are equally accelerated by calmodulin. Equal acceleration of all stages of the turnover cycle by calmodulin seems a priori to be a likely possibility. The steady-state rate of ATP hydrolysis should be stimulated by the same factor as are either phosphorylation or dephosphorylation when measured in comparable conditions. A 7-fold increase in the rate of ATPase at 0°C, Fig. 6, does not seem inconsistent with this prediction, and the findings in Figs. 1, 2 and 3.

Calcium binding. If all steps in the turnover cycle are equally stimulated by calmodulin the steady-state level of any enzyme species cannot change, and therefore the apparent Ca^{2+} affinity (K_m) for activating (Ca^{2+} + Mg^{2+})-ATPase should be the same with or without calmodulin. The kinetic arguments in the Appendix demonstrate also that even if pairs of rate-limiting steps are assumed to be stimulated, this cannot produce a decrease in the K_m for Ca^{2+}. The K_m for Ca^{2+} is essentially unchanged at 0°C, Fig. 6, at which temperature E_1 seems to have a high intrinsic Ca^{2+}-binding affinity (or Ca^{2+} may become trapped (occluded) during turnover). By contrast, at 37°C it seems that binding of calmodulin results in combination of Ca^{2+} to CE_1 with an affinity at least 20-fold greater than to E_1. A change in intrinsic Ca^{2+} binding affinity could be due to a calmodulin-induced change in structure of the Ca^{2+}-binding site on the Ca^{2+}-pump protein.

Nucleotide effects. The fact that acceleration of (Ca^{2+} + Mg^{2+})-ATPase by MgATP at the low affinity site is observed convincingly only in calmodulin-activated membranes [8] could be explained in principle if a step(s) accelerated by MgATP became rate-limiting only upon binding of calmodulin. But the same steps appear to be rate-limiting (at 37°C) in both calmodulin-stripped and -activated membranes and so again it seems necessary to postulate a more direct structural role of the calmodulin. Therefore we suggest that at 37°C MgATP is bound to CE_1P or CE_2P in such a way as permits acceleration of turnover, but binding to E_1P or E_2P either does not occur, or if it occurs does not accelerate turnover.

Fig. 5 confirms the report [12,13] that at 0°C, ATP accelerates phosphoenzyme hydrolysis and shows that it occurs in calmodulin-free membranes. This effect can be induced by free ATP, (although Mg^{2+} must also be present as micromolar concentrations [13] and therefore it cannot account simply for acceleration of ATP hydrolysis at 37°C for this is mediated only by MgATP in calmodulin-activated membranes. Thus ATP dependent effects appear to be different in detail at 37°C and 0°C.

B. The substrate of the Ca^{2+} pump

There is confusion as to whether the 'substrate' for the (Ca^{2+} + Mg^{2+})-ATPase is free ATP or the MgATP and CaATP complexes. Wolf has suggested [21] that MgATP is the substrate with a K_m of about 50 μM. In his experiments ATP hydrolysis was activated along a curve hyperbolic with respect to MgATP concentration. Conversely Schatzmann [22] concluded that free ATP is the true substrate with a K_m in the high affinity range of 1–2 μM. With the exception of the two recent reports [8,11], the biphasic ATP activation curves of (Ca^{2+} + Mg^{2+})-ATPase has not generally been observed, indicating, almost certainly, incomplete activation of the Ca^{2+} pump by calmodulin. Results in this paper indicate that one must consider separately the identity and role of the nucleotide substrate at the high K_m (200–400 μM) and low K_m (1–5 μM) range, respectively, and also inhibition by calcium.

Rega and Garrahan [12] showed that Ca^{2+}-dependent phosphorylation of red cell membranes (at 2 μM total ATP) occured in the absence of added Mg^{2+} and the predominant nucleotide species CaATP must therefore be able to phosphorylate the Ca^{2+} pump. The experiment in Fig. 8 (and see also Table II) shows that at 5 μM MgATP, the rate of (Ca^{2+} + Mg^{2+})-ATPase is unaffected by a concentration of free ATP up to 13.56 μM. Also the results of Fig. 9 shows that the rate of ATP hydrolysis at 5 μM MgATP is unaffected by a wide variation in the concentrations of both CaATP (1–500 μM) and free ATP (13.56–6 781 μM). These observations could be taken to imply that the substrate at the high affinity site is MgATP and neither CaATP nor free ATP interfere. But considered together with the finding of Rega and Garrahan [12] is seems much more likely that this site does not discriminate between MgATP, CaATP and free ATP and therefore any of the three species serves as substrate for phosphorylation.

The situation at the low affinity regulatory site is quite different. Here the rate of (Ca^{2+} + Mg^{2+})-ATPase is dependent on the MgATP concentration, $K_m \simeq 350$ μM, (Figs. 8 and 9, Table IV). CaATP inhibits activity, and binds even more tightly than MgATP ($K_i \simeq 165$ μM). Inhibition by CaATP is mostly competitive. The modest reduction of V, particularly with 500 μM CaATP (see Table IV) may be due to the fact that in order to achieve the appropriate concentrations of MgATP and CaATP it was necessary to reduce free Mg^{2+} concentrations to less than 5 μM, a concentration which may be suboptimal for activity (see below). Free ATP is not the effective species in the low affinity region and does not appear to interfere seriously with binding and the effects of MgATP and CaATP. Significant inhibition of (Ca^{2+} + Mg^{2+})-ATPase by Ca i.e. by CaATP is only to be expected at high total nucleotide concentrations in which significant concentrations of CaATP are present, and with calmodulin-activated membranes in which acceleration of turnover by MgATP is observed.

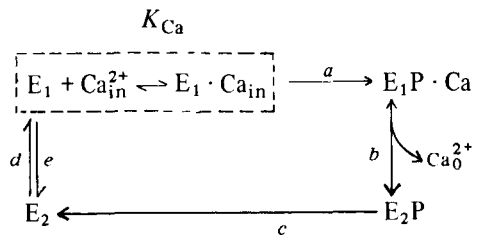
C. The role of Mg^{2+}

Mg^{2+} accelerate both phosphorylation and dephosphorylation (Figs. 3 and 4, Refs. 12 and 23). The Mg^{2+} seem to be tightly bound to the Ca^{2+} pump for it was necessary to wash the membranes several times with high concentrations of CDTA in order to reveal an Mg^{2+} requirement. Micromolar concentrations of added $MgCl_2$ suffice. The experiment of Fig. 8 shows that 11.7 μM free Mg^{2+} was sufficient to sustain full (Ca^{2+} + Mg^{2+})-ATPase activity. Of course, for optimal (Ca^{2+} + Mg^{2+})-ATPase, at millimolar ATP concentrations, millimolar concentrations of total $MgCl_2$ must be present (as in Figs. 8 and 9 and Refs. 11 and 22), in order to form the MgATP complex. Rega and Garrahan [12,13] and Schatzmann and Burgin [23] suggest that binding of Mg^{2+} controls the equilibrium between E_1P and E_2P forms; the same may be true also for the E_2-E_1 equilibrium.

Appendix

The analysis below considers the effect of acceleration of individual rate constants on the total level of

phosphoenzyme, at saturating Ca^{2+} and fixed ATP concentration, and on the apparent affinity for Ca^{2+} in activating (Ca^{2+} + Mg^{2+})-ATPase at a fixed ATP concentration (conditions of Figs. 1 and 6).



Ca^{2+} binding to E_1 at the internal site is assumed to be in effective equilibrium, and binding traps the enzyme as the $E_1 \cdot Ca$ form. For simplicity, a stoichiometry of one Ca^{2+} per pump is assumed. The ATP concentration (of 2 μM) is constant and is included in rate constant, a. Due to the absence of ADP, P_i and high Ca_0^{2+} the phosphorylation, dephosphorylation and conformational change $E_1P \rightarrow E_2P$ are effectively irreversible. K_{Ca} is the equilibrium binding constant for Ca^{2+}. a, b, c, d and e are individual rate constants.

The fraction (F) of total enzyme phosphorylated, in the steady-state, with saturating Ca^{2+} concentrations is:

$$F = \frac{[E_1P] + [E_2P]}{[E]_{total}} = \frac{ad(b+c)}{ad(b+c) + cb(d+a+e)}$$

An equal increase of all constants by calmodulin will not of course change this fraction. There are four possible sets of restrictive conditions in which pairs of rate constants are highly rate-limiting.

$b >> c$ and $d >> a + e$, $\quad F = a/(a+c)$ (1)

$b >> c$ and $a >> d + e$, $\quad F = d/(d+c)$ (2)

$c >> b$ and $d >> a + e$, $\quad F = a/(a+b)$ (3)

$c >> b$ and $a >> d + e$, $\quad F = d/(d+b)$ (4)

In no cases will an equal stimulation of the pairs of slow steps affect the fraction F. Cases (2) and (4) are however unlikely because the phosphorylation experiments suggest that a is accelerated by calmodulin.

The steady-state rate of ATP hydrolysis as a function of Ca^{2+} concentration is:

$$v = \frac{[E]_{total} \dfrac{abcd}{ad(b+c) + cb(d+a)} \cdot [Ca^{2+}]}{[Ca^{2+}] + K_{Ca} \cdot \dfrac{cb(d+e)}{da(b+c) + cb(d+a)}}$$

$$K_{mCa} = K_{Ca} \cdot \frac{cb(d+e)}{da(b+c) + cb(d+a)},$$

$$V = [E]_{total} \cdot \frac{abcd}{ad(b+c) + cb(d+a)}$$

If all the rate constants are accelerated equally no effect is to be expected on K_{Ca}, and V should rise by the acceleration factor for anyone step.

Consider again conditions in which pairs of steps are rate-limiting.

$b >> c, d >> a$; $\quad K_m = K_{Ca} \cdot \dfrac{d+e}{d} \cdot \dfrac{c}{c+a}$;

c and a are accelerated. (1)

$b >> c, a >> d$; $\quad K_m = K_{Ca} \cdot \dfrac{d+e}{a} \cdot \dfrac{c}{d+c}$;

c and d are accelerated. (2)

$c >> b, d >> a$; $\quad K_m = K_{Ca} \cdot \dfrac{d+e}{d} \cdot \dfrac{b}{a+b}$;

b and a are accelerated. (3)

$c >> b, a >> d$; $\quad K_m = K_{Ca} \cdot \dfrac{d+e}{a} \cdot \dfrac{b}{d+b}$;

d and b are accelerated. (4)

In no case will stimulation of the rate-limiting pairs reduce the K_m. In cases (1) and (3) the K_m is unchanged, and in cases (2) and (4) which, as explained before, are considered unlikely, the K_m will rise.

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