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REGULATORY INTERACTION BETWEEN CALMODULIN AND ATP ON THE RED CELL Ca^{2+} PUMP

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Summary

The interactions between calmodulin, ATP and Ca^{2+} on the red cell Ca^{2+} pump have been studied in membranes stripped of native calmodulin or rebound with purified red cell calmodulin. Calmodulin stimulates the maximal rate of (Ca^{2+}+Mg^{2+})-ATPase by 5—10-fold and the rate of Ca^{2+}-dependent phosphorylation by at least 10-fold. In calmodulin-bound membranes ATP activates (Ca^{2+}+Mg^{2+})-ATPase along a biphasic concentration curve ($K_{m1} \approx 1.4$ μM, $K_{m2} \approx 330$ μM), but in stripped membranes the curve is essentially hyperbolic ($K_m \approx 7$ μM). In calmodulin-bound membranes Ca^{2+} activates (Ca^{2+}+Mg^{2+})-ATPase at low concentrations ($K_m < 0.28$ μM) in stripped membranes the apparent Ca^{2+} affinities are at least 10-fold lower.

The results suggest that calmodulin (and perhaps ATP) affect a conformational equilibrium between E_2 and E_1 forms of the Ca^{2+} pump protein.

Plasma membrane Ca^{2+} pumps driven by ATP are becoming recognized as the means whereby many excitable and non-excitable cells maintain their low cytoplasmic Ca^{2+} concentration [1—4]. The red cell Ca^{2+} pump has been characterized in most detail due to both the simplicity of the cell and the relative ease with which active Ca^{2+} transport and (Ca^{2+}+Mg^{2+})-ATPase can be studied in whole cells, resealed ghosts, inside-out vesicles or broken membranes [5]. We have reported previously [6] that, in resealed ghosts, ATP activates active Ca^{2+} transport and (Ca^{2+}+Mg^{2+})-ATPase along a biphasic curve and similar effects have also been seen in broken membranes [7], suggesting a regulatory role for ATP at physiological concentrations. Much attention

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

has been paid recently to activation of the red cell membrane Ca^{2+} pump by the Ca^{2+}-dependent regulator protein, calmodulin [8, 9]. Since many membrane preparations do not show the biphasic ATP activation pattern [10–12] it seemed necessary to inquire whether this was the result of damage or dissociation of endogenous calmodulin during the conventional membrane preparations.

In this report we show that the biphasic activation pattern of ATP requires calmodulin to be bound. Activation of the $(Ca^{2+}+Mg^{2+})$-ATPase by calmodulin involves a large stimulation on the rate of Ca^{2+}-dependent phosphorylation of the Ca^{2+} pump protein. This may be due to an effect on a conformational transition between E_2 and E_1 forms of the pump.

In order to look clearly at the calmodulin effects it was necessary to prepare membranes stripped completely of their natural calmodulin. This involved freeze-thawing in an EGTA-containing medium and washing the hemoglobin-free membranes until there was no further reduction of $(Ca^{2+}+Mg^{2+})$-ATPase. Reactivation of full $(Ca^{2+}+Mg^{2+})$-ATPase was brought about by addition of calmodulin purified from red cell hemolysates by a variation of the method of Jarret and Penniston [13].

Like endogenous calmodulin, externally added calmodulin was bound very tightly to the stripped membranes, for washing in EGTA-free media did not reduce the activity of the newly reactivated ATPase. However, such calmodulin-bound membranes could be stripped and reactivated again by the standard procedure used with native membranes.

The dependence of $(Ca^{2+}+Mg^{2+})$-ATPase activity on ATP concentration for stripped and reactivated membranes (Fig. 1) makes it quite clear that the biphasic activation requires calmodulin to be bound to the membranes. From the hyperbolic curve of the stripped membranes one obtains a K_m value of approx. 7 μM and a maximal rate of 2.0 mmol/l cells per h. From the activated membranes the high-affinity component was estimated from reciprocal plots to have a K_m value of 1.4 μM and a V value of 3.3 mmol/l cells per h whilst the low-affinity region gives a K_m value of approx. 330 μM and a V value of 6.6 mmol/l cells per h (overall maximal rate of 9.9 mmol/l cells per h). The calmodulin thus produces a large activation in both high- and low-concentration regions. At very low ATP concentrations of less than 1 μM the degree of activation was reduced. This was not the result of reduced binding of calmodulin to the membranes, since washing the membranes activated in this medium did not produce a reduction in $(Ca^{2+}+Mg^{2+})$-ATPase activity.

The biphasic ATP activation in calmodulin-bound membranes was similar to that observed previously in resealed ghosts [6]. In an experiment to ascertain the state of calmodulin in resealed ghosts we have estimated the $(Ca^{2+}+Mg^{2+})$-ATPase as described previously [6] in ghosts prepared by lysing red cells in either 10- or 400-fold their own volume of solution. There was no difference in $(Ca^{2+}+Mg^{2+})$-ATPase (approx. 10 mmol/l cells per h) and addition of the purified calmodulin to the lysing solutions did not stimulate activity of the resealed ghosts (not shown). This result suggested strongly that a large dilution of red cells upon lysis did not reduce the endogenous activation by calmodulin, due presumably to its tight binding to the inner face of the resealed ghosts' membranes.

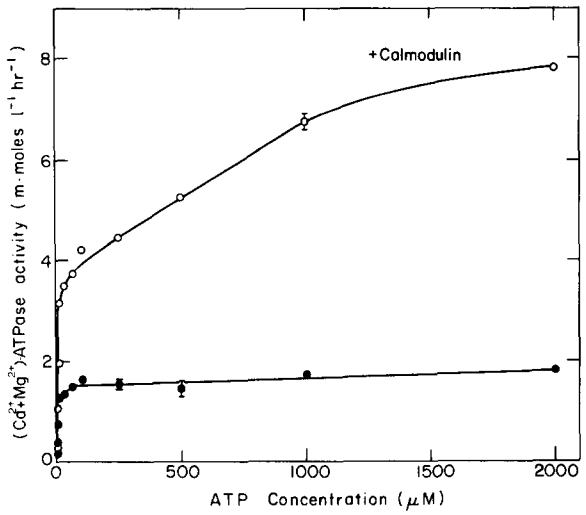

Fig. 1. ATP dependence of $(Ca^{2+}+Mg^{2+})$-ATPase in calmodulin-stripped or reactivated membranes. Preparation of calmodulin-stripped membranes: red blood cells were washed three times in a solution containing 172 mM Tris-HCl (pH 7.6, 20° C) by centrifugation for 10 min at 2000 × *g*. Packed cells were lysed in 10 vols. of ice-cold distilled water with stirring for 5 min. The membranes were pelleted by centrifugation for 10 min at 45 000 × *g*, washed three times in 50 vols. of 1 mM $MgCl_2$ and 2 mM Hepes-Tris (pH 7.4, 22° C) and suspended in a solution containing 2 mM Hepes-Tris (pH 7.4, 22° C) and 5 mM EGTA-Tris. Calmodulin was removed from the membranes by freeze-thawing twice in liquid N_2 followed by three washes in a solution of 1 mM $MgCl_2$ and 2 mM Hepes-Tris. The membranes were stored at −20° C until required and were stable for at least 1 month. Calmodulin was purified from human red cell hemolysates by the method of Jarrett and Penniston [13] modified as follows: (a) protein in pooled fractions from columns was concentrated by ultrafiltration using the Diaflo apparatus with a PM 10 filter, rather than being lyophilized; (b) the salt gradient fractionation was performed with a column of DE 52 (Whatman, 1 × 10 cm) equilibrated with 10 mM imidazole-HCl, pH 6.5. After application of protein the column was washed with 20 ml imidazole (10 mM, pH 6.5) and then 80 ml imidazole (10 mM, pH 6.5) and NaCl (200 mM). Calmodulin was eluted in a salt gradient of NaCl (0.2—0.5 M) in imidazole (10 mM, pH 6.5). The protein peak containing calmodulin activity was pure as judged by SDS gel electrophoresis. ATPase assay (Brown, A.R. and Lew, V.L., personal communication): 0.2 ml of medium containing 100 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes-Tris (pH 7.4, 37° C), 0.1 mM ouabain, 50 μM $CaCl_2$ and ATP (plus [γ-^{32}P]ATP, $5 \cdot 10^4$ cpm) in the concentration range 0.5—1000 μM, was incubated at 37° C for different times, with membranes equivalent to 1 or 10 μl of cells with or without added calmodulin (30 or 300 ng, respectively). Mg^{2+}-ATPase was determined in a similar medium in which 1 mM EGTA replaced the $CaCl_2$. At the end of incubation, tubes were transferred to the 0° C bath and after 5 min, 0.1 ml of a solution containing 4% ammonium molybdate in 4N H_2SO_4, 10% perchloric acid and 2 mM P_i was added. Then 0.8 ml of isobutanol was added. The tubes were shaken for 10—15 s, returned to the ice-bath for 5 min and the shaking procedure repeated twice more, after which tubes were centrifuged at 12 000 × *g* for 30 s in an Eppendorf Microcentrifuge. 0.5 ml of the isobutanol phase was transferred to counting vials and 5 ml of Bray's fluid added. The $(Ca^{2+}+Mg^{2+})$-ATPase is calculated from the fractional breakdown of total ATP per given time, after subtracting Mg^{2+}-ATPase activity. Each point is the average ± 1 S.E. from duplicate estimates.

Another striking effect of calmodulin was to raise the apparent Ca^{2+} affinity for stimulation of $(Ca^{2+}+Mg^{2+})$-ATPase. Since an effect of this sort has been reported previously [9, 14] it is not described here in detail, although it is clear that the magnitude of the increase in Ca^{2+} affinity in controlled conditions was at least 10-fold (not shown).

The reaction mechanism of the $(Ca^{2+}+Mg^{2+})$-ATPase is usually described on the basis of phosphorylation experiments [15—17] as follows:

$$E_1 \xrightarrow{ATP,\ Ca^{2+}} E_1P \xrightarrow{Mg^{2+}} E_2P \xrightarrow{} E_2 \longrightarrow E_1$$
$$\searrow ADP \qquad\qquad \searrow P_i$$

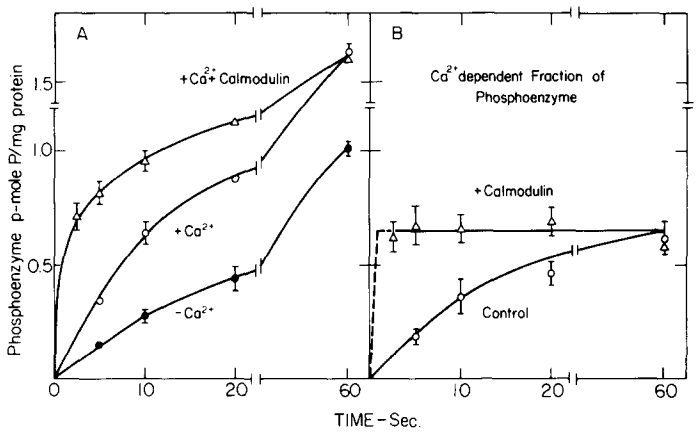

Fig. 2. Calmodulin stimulates the rate of Ca^{2+}-dependent phosphorylation in red blood cell membranes. Stripped and calmodulin-reactivated membranes were prepared as described in the legend to Fig. 1 and the text. For the phosphorylation reaction, membranes (2 mg protein) were incubated at 0° C in 0.5 ml of a medium containing: Hepes-Tris, 150 mM (pH 7.4, 0° C); $MgCl_2$, 1 mM and either EGTA-Tris, 0.5 mM (pH 7.4, 0° C) or $CaCl_2$, 0.05 mM. The reaction was initiated by the addition of ATP (plus [γ-^{32}P] ATP, $2.5 \cdot 10^5$ cpm) to a final concentration of 2 μM. After various times at 0° C, the reaction was terminated by rapid addition of 5 ml of a stopping solution containing 5% trichloroacetic acid, 1 mM ATP and 20 mM phosphate. After 20 min at 0° C the membranes were pelleted by centrifugation for 10 min at 3000 × *g* and washed three times in 5% trichloroacetic acid. The washed protein was dissolved in 0.5 ml of 4% SDS solution and incubated for 10 min in a boiling water bath. 10 μl from each tube were taken for protein determination by the method of Lowry et al. [18] and the remaining solution was transferred to counting vials and 5 ml of Bray's fluid added. The vials were counted for ^{32}P and the amount of phosphate incorporated was calculated from the total counts in the added ATP and expressed as pmol per mg protein. The correction for protein loss was important, for the maximal variation of total protein at the end of the experimental procedure in different samples was as much as 25%. Each point is the average ± 1 S.E. from duplicate estimates.

E_1 is the state binding ATP and Ca^{2+} with high affinity which becomes phosphorylated to E_1P, a high-energy phosphoenzyme which can transfer its terminal phosphate back to ADP. A conformational transition (affected by Mg^{2+}) results in the appearance of E_2P, a phosphoenzyme thought to release Ca^{2+} into the high-Ca^{2+} exterior and the hydrolysis of which is sensitive to H_2O. Dephosphorylation produces the E_2 form of the dephosphoenzyme which is then reconverted to E_1. We should like to distinguish stimulatory effects of calmodulin or high ATP either on the net phosphorylation rate in the sequence, $E_2 \rightarrow E_1 \rightarrow E_1P$, or on the net dephosphorylation rate in the sequence, $E_1P \rightarrow E_2P \rightarrow E_2$. Fig. 2 shows an experiment to measure the rate of phosphorylation of stripped and calmodulin-reactivated membranes at 0° C. Stripped membranes display a relatively slow rate of Ca^{2+}-independent phosphorylation and an appreciable stimulation by Ca^{2+}. The Ca^{2+}-dependent incorporation of ^{32}P in trichloroacetic acid-stable protein occurs in a polypeptide of molecular weight approx. 150 000 as expected for the (Ca^{2+}+Mg^{2+})-ATPase [19]. The striking feature of Fig. 2 is the large stimulation of the rate of Ca^{2+}-dependent phosphorylation in calmodulin-activated membranes, with little or no change in the steady-state level (Fig. 2b). In other experiments without added Mg^{2+} we have observed both a large calmodulin-dependent increase in the rate of phosphorylation and a 30—40% increase in the level of Ca^{2+}-dependent phosphoenzyme (not shown). The stimulation of the rate in different experiments varied somewhat from approx. 10-fold to a value too

great to be measured by our manual technique as in Fig. 2b. Ca^{2+}-independent phosphorylation of the membranes was not affected by the calmodulin. The experiment in Fig. 2 could indicate activation either of the phosphoryl transfer stage, $E_1 \rightarrow E_1P$, or of the preceding conformational change. If the Ca^{2+} pump was initially largely in state E_2, a calmodulin-dependent shift in the conformational equilibrium towards E_1 would accelerate the observed rate of phosphorylation by making available that form of the ATPase to which ATP and Ca^{2+} must bind prior to phosphorylation. An effect of this sort in the ATP hydrolysis conditions of Fig. 1 could explain economically both activation by calmodulin of ATPase at low ATP concentrations and also the large increase in the apparent activation affinity for Ca^{2+}. Experiments as in Fig. 2 cannot be performed with high 'regulatory' concentrations of ATP and so we cannot say whether, in the range of interest, ATP accelerated the rate of the transition $E_2 \rightarrow E_1$ and thus net phosphorylation, as is the case with $(Na^+ + K^+)$-ATPase [20—22] and probably also for sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase [23, 24]. Dependence of activation by ATP on the presence of bound calmodulin (Fig. 1) implies either that both ligands must act at the same point in the cycle, or that a step stimulated by ATP becomes rate-limiting, only when binding of the calmodulin relieves a rate limitation at a different stage of the turnover cycle.

Turnover of the phosphoenzyme formed under the conditions of Fig. 2 was exposed by addition of cold ATP at high concentrations and at 0°C. The phosphoenzyme was rapidly hydrolyzed under these conditions (half-time approx. 2 s, see Refs. 15 and 17) and there was clearly no large acceleration in the calmodulin-bound membranes, although the accuracy of measurements would make a small stimulation difficult to detect or exclude (Muallem and Karlish, unpublished results).

In considering the regulation of the Ca^{2+} pump in whole red cells, it is remarkable that the Ca^{2+}-activating affinity appears to be much lower than that observed in resealed ghosts or broken membranes [6, 25, 27]. The difference may reflect the interplay in vivo between regulation by ATP, binding of calmodulin, the influence of an inhibitor protein reported recently [28, 29] or other unknown variables. Squid axons [30] and brain synaptosomes [31] have recently been shown to contain ATP-driven Ca^{2+} pumps and we have also found in brain microsomes a red cell-like Ca^{2+} pump activity which responds well to the purified red cell calmodulin (Muallem, S., Karlish, S.J.D. and Lew, V.L., unpublished results).

Thus, we are hopeful that the regulatory phenomena described here for the red cell Ca^{2+} pump will turn out to be general features of active Ca^{2+} transport in other cells.

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