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EFFECTS OF CALMODULIN ON THE PHOSPHOENZYME OF THE Ca^{2+}-ATPase OF HUMAN RED CELL MEMBRANES

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Summary

Comparison of the effects of calmodulin on the Ca^{2+}-ATPase activity and on the steady-state level of the phosphoenzyme, indicates that activation of the Ca^{2+}-ATPase is mainly due to an increase in the turnover of the phosphoenzyme and does not require occupation of the regulatory site of the Ca^{2+}-ATPase by ATP.

A small acidic protein that stimulates the Ca^{2+} pump of red cell membranes has been isolated and purified from red blood cell hemolysates. The activator protein has been identified with calmodulin, an ubiquitous Ca^{2+}-binding protein [1 and 2].

Although the effect of calmodulin on the overall reaction of ATP hydrolysis by the Ca^{2+} pump of red cells is well documented, little is known about the mechanism of the activation process. To get further in the understanding of this, we have looked at the effects of calmodulin on the phosphoenzyme of the Ca^{2+}-ATPase and have compared them with the effects of calmodulin on Ca^{2+}-ATPase activity measured at the same temperature and substrate concentration as those used during phosphorylation. Results allow one to conclude that the main effect of calmodulin is to increase the turnover of the phosphoenzyme of the Ca^{2+}-ATPase.

Purified membranes from human red cells were obtained by hypotonic hemolysis in EDTA-containing media, following the procedure already described [3]. Calmodulin, prepared from beef red hemolysates by a procedure

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slightly modified from that of Jung [4], was a kind gift from Dr. Frank F. Vincenzi (University of Washington). Phosphorylation was performed as described previously [5] in media containing (mM): [γ-^{32}P]ATP, 0.015; $CaCl_2$, 0.150; ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 0.100; imidazole-HCl (pH 7.25), 60; $MgCl_2$, 0 or 0.5. Ca^{2+}-dependent phosphoenzyme is the difference between the amount of ^{32}P bound to the membrane after 20 s at 0°C in the above mentioned media and in a medium without $CaCl_2$ Dephosphorylation was estimated from the amount of Ca^{2+}-dependent phosphoenzyme remaining after the addition of an excess of unlabelled ATP [5]. The rate constants for dephosphorylation were calculated assuming first order kinetics. Ca^{2+}-ATPase activity was estimated from the release of [^{32}P]-orthophosphate from [γ-^{32}P]ATP [6] after incubation at 0°C during 30 min in media having the same composition as those used for phosphorylation. To compensate for the low ATPase activity in the cold, the amount of membrane protein in the reaction media was 8 mg/ml. Protein was measured by the procedure of Lowry et al. [7].

Results in Table I show that in media containing 15 μM ATP with and without 0.5 mM $MgCl_2$, Ca^{2+}-ATPase activity from red cell membranes that had been preincubated with calmodulin is increased 170 to 270%. We have shown elsewhere [6], that when the concentration of ATP is 15 μM the nucleotide occupies the catalytic site leaving almost empty the low-affinity regulatory site of the Ca^{2+}-ATPase for ATP. Thus, occupation by ATP of the regulatory site is not required for calmodulin to stimulate the Ca^{2+}-ATPase. Activation seems to be larger in media containing Mg^{2+} but it persists in media without $MgCl_2$.

Although Mg^{2+} is not essential for activation it modifies the response of the Ca^{2+}-ATPase to calmodulin. Results in Table I show that in the absence of Mg^{2+} the steady-state level of Ca^{2+}-dependent phosphoenzyme increases 40% in membranes treated with calmodulin. Under these conditions the rate constant for the dephosphorylation reaction increases 124% and the ratio: Ca^{2+}-ATPase activity/phosphoenzyme level rises from 34 to 67 h^{-1}. Therefore, in

TABLE I

THE EFFECT OF PREINCUBATION WITH CALMODULIN ON Ca^{2+}-ATPase ACTIVITY AND ON Ca^{2+}-DEPENDENT PHOSPHOENZYME

Before the assay the red cell membranes (15 mg protein/ml) were incubated during 30 min at 37°C in (mM): imidazole-HCl (pH 7.25), 120; $CaCl_2$, 0.010 with or without 6 μg/ml of calmodulin. Paired determinations of the effects of calmodulin were performed. The mean percent change is the mean of the percent change calculated for each pair of experimental values and it will differ from the percent change of the mean values given in the table. *P* values were calculated using Student's "*t*" test for paired samples (not significant $P \geq 0.05$).

	Preincubation		Percent change (mean ± S.E.M.)	*P* value
	Without calmodulin	With calmodulin		
Ca^{2+}-ATPase activity (pmol/mg protein per h)				
in 0 mM $MgCl_2$ (n = 2)	15.5	41.5	170 ± 11	< 0.05
in 0.5 mM $MgCl_2$ (n = 3)	28.3	108.6	271 ± 47	< 0.05
Phosphoenzyme (pmol/mg protein)				
in 0 mM $MgCl_2$ (n = 5)	0.45	0.62	40 ± 5.6	< 0.01
in 0.5 mM $MgCl_2$ (n = 4)	1.09	0.71	34 ± 3.1	< 0.01
Rate constant for dephosphorylation (h^{-1})				
in 0 mM $MgCl_2$ (n = 3)	0.055	0.123	124 ± 21	< 0.05

the absence of Mg^{2+}, the increase in Ca^{2+}-ATPase activity caused by the activator results from both an increase in the level and in the turnover of the phosphoenzyme. When the effect of calmodulin on the phosphoenzyme is tested in media containing 0.5 mM $MgCl_2$ the level of the phosphoenzyme decreases and the ratio: Ca^{2+}-ATPase activity/phosphoenzyme level rises almost 6 times. In the presence of Mg^{2+} therefore the effects of calmodulin on ATP hydrolysis can be fully accounted for by the increase in the turnover of the phosphoenzyme. The higher effect on turnover together with the decrease in the level of the phosphoenzyme, indicates that in the presence of Mg^{2+} calmodulin increases the rate of dephosphorylation to a larger extent than in the absence of the cation. No direct measurement of this effect was attempted because: (i) when high concentrations of ATP are added to chase the phosphoenzyme in the presence of Mg^{2+}, dephosphorylation is so fast that increments in its rate will not be detectable with the techniques used for these studies [8]; and (ii) the use of Ca^{2+}-chelating agents is precluded because they would dissociate the enzyme-calmodulin complex.

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