The effect of calmodulin on the phosphoprotein intermediate of Mg^{2+} -dependent Ca^{2+} -stimulated adenosine triphosphatase in human erythrocyte membranes

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(Received 14 July 1980/Accepted 10 October 1980)

The effect of calmodulin on the formation and decomposition of the Ca^{2+} -dependent phosphoprotein intermediate of the $(Mg^{2+} + Ca^{2+})$ -dependent ATPase in erythrocyte membranes was investigated. In the presence of $60\mu M$ - Ca^{2+} and $25\mu M$ - $MgCl_2$, calmodulin (0.5 – $1.5\mu g$) did not alter the steady-state concentration of the phosphoprotein, but increased its rate of decomposition. Higher calmodulin concentrations significantly decreased the steady-state concentration of phosphoprotein. Calmodulin (0.5 – $1.7\mu g$) increased Ca^{2+} -transport ATPase activity by increasing the turnover rate of its phosphoprotein intermediate. Increasing the $MgCl_2$ concentration from $25\mu M$ to $250\mu M$ increased the $(Mg^{2+} + Ca^{2+})$ -dependent ATPase activity, but decreased the concentration of the phosphoprotein intermediate. Similarly to calmodulin, $MgCl_2$ increased the turnover rate of the Ca^{2+} -transport ATPase complex (about 3-fold). At the higher $MgCl_2$ concentration calmodulin did not further affect the decomposition of the phosphoprotein intermediate. It was concluded that both calmodulin and $MgCl_2$ increase the turnover of the Ca^{2+} -pump by enhancing the decomposition of the Ca^{2+} -dependent phosphoprotein intermediate.

Calmodulin, a Ca^{2+} -binding protein present in all eukaryotic cells examined (Cheung, 1980), has been found to stimulate a number of enzymes, including Ca^{2+} -dependent phosphodiesterase and adenylate cyclase. Calmodulin also stimulates $(Mg^{2+} + Ca^{2+})$ -dependent ATPase activity and Ca^{2+} transport in erythrocyte membranes (Luthra *et al.*, 1976; Jarrett & Penniston, 1977; Gopinath & Vincenzi, 1977; Scharff & Foder, 1978; Katz *et al.*, 1979; Larsen & Vincenzi, 1979), by increasing both the maximum velocity and the affinity of the enzyme system for Ca^{2+} (Quist & Roufogalis, 1977; Hanahan *et al.*, 1978; Roufogalis, 1979). The mechanism by which calmodulin increases the activity of Ca^{2+} -dependent enzymes is not generally understood. In the present study the effects of calmodulin on the steady-state level of $(Mg^{2+} + Ca^{2+})$ -dependent ATPase phosphoprotein formation and its decomposition have been investigated with erythrocyte membranes.

Phosphoprotein formation and decomposition in the presence of Ca^{2+} was studied under conditions previously used to characterize the phosphorylated intermediate of the Ca^{2+} -transport system in erythrocyte membranes (Katz & Blostein, 1975).

Under these conditions (low ATP concentrations, short incubation times and low temperatures) only one phosphorylated peak was found on sodium dodecyl sulphate/polyacrylamide gels. This phosphoprotein of mol.wt. 150 000 was hydroxylamine sensitive and readily decomposed by EGTA. The amount of phosphoprotein present in this region correlated well with the Ca^{2+} -dependent ATPase activity observed. Therefore similar incubation conditions were used to study the effects of calmodulin on the phosphorylated intermediate of the Ca^{2+} -transport system.

Experimental

Preparation of membranes

Erythrocyte 'ghosts' were prepared by the procedure of Dodge *et al.* (1963). These 'ghost' preparations have been observed to contain no measurable indigenous calmodulin (Mauldin & Roufogalis, 1980). The 'ghosts' were kept frozen overnight and were thawed and refrozen three times before use, to maximally expose the enzyme sites.

Materials

All materials used were reagent grade. Tris/ATP and EGTA were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [γ - 32 P]ATP (sp. radioactivity 20 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and [2,8- 3 H]ATP (sp. radioactivity 27 Ci/mmol) was obtained from New England Nuclear, Lachine, Que., Canada. Charcoal (Norit A) was obtained from Fisher Scientific Co., Montreal, Que., Canada.

Measurement of phosphoprotein formation

The procedure of membrane-ATPase phosphorylation was essentially that described previously (Katz & Blostein, 1975). 'Ghost' preparations (275 μ g/200 μ l final incubation volume) were incubated for 15 s at 10°C in the presence of 25 μ M-MgCl₂, 52.5 μ M-ouabain, 40 mM-Tris/HCl, pH 7.4, 2 μ M-ATP containing [γ - 32 P]ATP (sp. radioactivity 1.62 Ci/mmol) and [2,8- 3 H]ATP (sp. radioactivity 1.5 Ci/mmol) and 500 μ M-EGTA. When Ca²⁺ was added it was present at a final concentration of 60 μ M (562 μ M added Ca²⁺), the free Ca²⁺ concentration determined by the equations described by Katz *et al.* (1970), taking into consideration the Mg²⁺, ATP and EGTA concentrations at the pH and temperature of the reaction. The reaction was started by the addition of the membranes to the reaction medium. After 15 s the reaction was terminated by the addition of 5% trichloroacetic acid containing 2.5 mM-ATP (disodium salt) and 5 mM-KH₂PO₄. The samples were centrifuged at 3000 g at 4°C for 10 min and the pellets washed in the same way four times. The pellets were then resuspended in 0.1 M-NaOH and a portion was taken for scintillation counting using double-label counting procedures. The protein content of each pellet was determined by the method of Lowry *et al.* (1951).

In the present studies, extensive phosphoprotein formation was observed in the absence of added Ca²⁺ (in the presence of 500 μ M-EGTA). This phosphoprotein formation was found to significantly increase when the Mg²⁺ concentration of the incubation medium was increased and therefore may be due to an Mg²⁺-dependent phosphoprotein component that has been observed previously (Katz & Blostein, 1975; Garrahan & Rega, 1978). The functional significance of this phosphorylation has not been determined. It is also possible that under the assay conditions utilized some free Ca²⁺ may be present in the membranes and incubation media. Despite this, the EGTA concentrations were not further increased to avoid the direct effects of EGTA on ATPase activity observed by others (Sarkadi *et al.*, 1979; A. Al-Jobore & B. D. Roufogalis, unpublished work).

Decomposition of phosphoenzyme intermediate

Dephosphorylation of the intermediate was investigated by adding either 2 mM-Tris/HCl (control) or 5 μ M-Tris/ATP ('chase') with or without 0.5–1.7 μ g of calmodulin (final concentration in each case), after the 15 s incubation period. After a 10 s 'chase' the reaction was stopped with trichloroacetic acid, as described above.

Phosphorylation refers to that amount of 32 P bound to the membrane after subtraction of the [3 H]ATP-bound. The amount of [3 H]ATP bound represented approx. 30% of the total amount of 32 P bound. Phosphorylation observed in the presence of 500 μ M-EGTA (without added Ca²⁺) was subtracted from each determination.

Measurement of (Mg²⁺ + Ca²⁺)-dependent ATPase

(Mg²⁺ + Ca²⁺)-dependent ATPase activity was determined in duplicate in assays identical with those described above. After incubation for 3 min a charcoal suspension (0.3 ml of a 1.5 g/10 ml solution in 5% trichloroacetic acid) was added to each tube. After 1 h incubation at 0°C with intermittent mixing, the tubes were centrifuged for 4 min at 3000 g and the [32 P]P_i present in a portion of the clear supernatant was determined by liquid-scintillation counting (Katz *et al.*, 1979).

Preparation of calmodulin

Calmodulin was prepared from erythrocytes by the method of Penniston and his co-workers (Jarrett & Penniston, 1978). Erythrocytes were haemolysed in a hypo-osmotic medium and the haemolysate was applied to a DEAE-Sephadex column (2.6 cm $\times$ 35 cm). The active fractions were then eluted with 0.6 M-NaCl and concentrated. The calmodulin was desalted on Sephadex G-15 or G-25 columns and stored at -80°C for up to 3 weeks without a significant loss in activity. Purified bovine brain calmodulin, kindly supplied by Dr. John Penniston, Mayo Foundation, Rochester, MN, U.S.A., was also used in some of the studies. Calmodulin from these two sources yielded similar results, both quantitatively and qualitatively.

Results

The effect of varying the calmodulin concentration on the steady-state concentration of phosphoprotein (EP) and the (Mg²⁺ + Ca²⁺)-dependent ATPase activity is shown in Fig. 1. At 60 μ M-Ca²⁺ and 25 μ M-MgCl₂, calmodulin produced little change in Ca²⁺-dependent EP up to 1.2 μ g, but a significant decrease in EP at 1.7 μ g was observed ($P < 0.05$). Increasing calmodulin concentrations progressively increased (Mg²⁺ + Ca²⁺)-dependent ATPase activity ($P < 0.01$ at 1.2 μ g of calmodulin). Calmodulin had no effect on (Mg²⁺ + Ca²⁺)-dependent ATPase activity

at 500 μM -EGTA. In the following Tables experiments were pooled over a range of calmodulin concentrations.

The decomposition of EP was determined after further formation of the ³²P-labelled phosphoprotein was prevented by the addition of 5 μM -Tris/ATP for

10s. The effect of this 'chase' solution in the presence or absence of calmodulin was compared with a control in which 2mM-Tris/HCl was added for the same time instead of the ATP. The presence of calmodulin in the ATP 'chase' medium increased EP decomposition about 80% ($P < 0.05$) (Table 1).

Fig. 1. Effect of calmodulin on phosphoprotein formation (a) and (Mg²⁺ + Ca²⁺)-dependent ATPase activity (b) in erythrocyte-membranes

Erythrocyte membranes were incubated for 15s at 10°C in the presence of 40mM-Tris/HCl, pH 7.4, 25 μM -MgCl₂, [2,8-³H, γ -³²P]ATP and either 500 μM -EGTA (· · ·) or 60 μM -Ca²⁺ (—) in the presence and absence of calmodulin at the value (per 0.2 ml assay) indicated. Calmodulin was prepared from erythrocytes. The results shown are means $\pm$ S.E.M. for at least eight determinations. * $P < 0.05$, compared with the control in the absence of calmodulin; † $P < 0.001$, compared with the control in the absence of calmodulin.

Table 1. The effect of calmodulin on the formation and decomposition of the Ca²⁺-dependent phosphorylated intermediate in erythrocyte-membrane preparations

Erythrocyte-membrane (Mg²⁺ + Ca²⁺)-dependent ATPase was phosphorylated at 25 μM -MgCl₂ in the presence of 60 μM -Ca²⁺ or 60 μM -Ca²⁺ plus calmodulin. After 15s of incubation at 10°C, either Tris/HCl (2mM), Tris/HCl + calmodulin, Tris/ATP (5 μM) or Tris/ATP (5 μM) + calmodulin were added to the incubation media and the reaction continued for a further 10s and terminated as described in the text. In each case, the phosphorylation in the absence of added Ca²⁺ (in the presence of 500 μM -EGTA) was subtracted (approximately 1.1 pmol/mg). The results are means $\pm$ S.E.M. for at least 25 determinations.

Composition of the reaction medium	Composition of the 'chase' medium	...	³² P bound (pmol/mg)			
			Tris/HCl (2.0mM)	Tris/HCl + calmodulin	Tris/ATP (5 μM)	Tris/ATP (5 μM) + calmodulin
Ca ²⁺ (60 μM)			1.51 $\pm$ 0.14 (100%)	1.19 $\pm$ 0.10 (79%)*	1.05 $\pm$ 0.10† (70%)	0.69 $\pm$ 0.10†,‡ (46%)
Ca ²⁺ (60 μM) + calmodulin			1.35 $\pm$ 0.14 (89%)	—	0.80 $\pm$ 0.12† (53%)	—

* % of mean value noted in the presence of Ca²⁺ (60 μM).
† $P < 0.01$ compared with Tris/HCl 'chase'.
‡ $P < 0.05$ compared with Tris/ATP 'chase'.

Table 2. *Effect of MgCl₂ on the steady-state formation and decomposition of phosphoprotein and (Mg²⁺ + Ca²⁺)-dependent ATPase in the presence and absence of calmodulin*

Erythrocyte-membrane (Mg²⁺ + Ca²⁺)-dependent ATPase was phosphorylated in the presence of 60 μ M-Ca²⁺ or 60 μ M-Ca²⁺ plus calmodulin and either 25 μ M-MgCl₂ or 250 μ M-MgCl₂. After 15 s of incubation at 10°C, 2 mM-Tris/HCl was added to the incubation medium and the reaction continued for a further 10 s and terminated as described in the text. In each case, phosphorylation in the absence of added Ca²⁺ (in the presence of 500 μ M-EGTA) was subtracted (approx. 1.1 pmol/mg at 25 μ M-MgCl₂ and 2.7 pmol/mg at 250 μ M-MgCl₂). The results are means $\pm$ S.E.M. for at least nine determinations.

Composition of the reaction medium	[³² P-bound EP] (pmol/mg)	ATP hydrolysis (pmol/mg per min) (<i>v</i>)	Turnover (<i>v</i> /[³² P-bound EP])
Ca ²⁺ (60 μ M) + 25 μ M-MgCl ₂	1.51 $\pm$ 0.14 (100%)	100 $\pm$ 14 (100%)	66 (100%)
Ca ²⁺ (60 μ M) + 25 μ M-MgCl ₂ + calmodulin	1.35 $\pm$ 0.14 (89%)*	142 $\pm$ 9.6† (142%)*	141 (213%)*
Ca ²⁺ (60 μ M) + 250 μ M-MgCl ₂	0.78 $\pm$ 0.22† (52%)	154 $\pm$ 32 (154%)	197 (299%)
Ca ²⁺ (60 μ M) + 250 μ M-MgCl ₂ + calmodulin	0.73 $\pm$ 0.26† (48%)	249 $\pm$ 18†,‡ (249%)	341 (517%)

* % of mean value noted in the presence of 25 μ M-MgCl₂ and 60 μ M-Ca²⁺.

† *P* < 0.05 compared with the value for 60 μ M-Ca²⁺ + 25 μ M-MgCl₂.

‡ *P* < 0.05 compared with the value for 60 μ M-Ca²⁺ + 250 μ M-MgCl₂.

This effect was also noted when calmodulin was present throughout the incubation period (Table 1). Calmodulin also tended to decrease the steady-state [EP] when it was added along with Tris/HCl (2 mM) after EP formation, but this was not statistically significant. Thus, calmodulin stimulated the dephosphorylation of the Ca²⁺-dependent phosphoprotein intermediate.

Influence of MgCl₂ on the effect of calmodulin on the steady-state concentration and decomposition of EP

Increasing the MgCl₂ concentration from 25 μ M to 250 μ M significantly altered the steady-state concentration of phosphoprotein and the effects of calmodulin on dephosphorylation. In the presence of 250 μ M-MgCl₂ (at 60 μ M-Ca²⁺), the steady-state concentration of EP was markedly decreased over that noted in the presence of 25 μ M-MgCl₂ (Table 2). Under these conditions, 250 μ M-MgCl₂ increased the turnover rate (*v*/[EP]) of the reaction. In the presence of 25 μ M-MgCl₂ calmodulin slightly decreased the steady-state [EP], whereas a significant increase in (Mg²⁺ + Ca²⁺)-dependent ATPase activity was produced by calmodulin under these conditions. Whereas at 250 μ M-MgCl₂ calmodulin did not further decrease the steady-state [EP], it stimulated (Mg²⁺ + Ca²⁺)-dependent ATPase activity to a significant extent and thus increased the turnover rate over that observed in the presence of 250 μ M-MgCl₂ alone.

The influence of MgCl₂ on the decomposition of the Ca²⁺-dependent phosphoprotein intermediate is shown in Table 3. In the presence of 25 μ M-MgCl₂, calmodulin, added either to the Tris/ATP 'chase' or

present throughout the incubation reaction, decreased the steady-state [EP]. At 250 μ M-MgCl₂ this effect of calmodulin on the steady-state [EP] was no longer observed; the already decreased concentration of phosphoprotein present after the addition of Tris/HCl or Tris/ATP was not significantly different whether calmodulin was present or absent. It was also observed that at 250 μ M-MgCl₂ the Tris/ATP 'chase' no longer decreased the [EP] already decreased by the high MgCl₂.

Discussion

The present studies show that calmodulin stimulated (Mg²⁺ + Ca²⁺)-independent ATPase in erythrocyte-membrane preparations by increasing the turnover rate (*v*/[EP]) of the Ca²⁺-transport ATPase. The increased turnover rate was found to be due mainly to an enhancement of the decomposition of the Ca²⁺-dependent phosphorylation intermediate. This observation is not without precedent, as it has been observed that in cardiac sarcoplasmic-reticulum preparations cyclic AMP-dependent protein kinase stimulates Ca²⁺-transport activity by a similar mechanism (Tada *et al.*, 1979). K⁺ was also observed to increase the turnover of the Ca²⁺-dependent phosphoprotein intermediate of the (Mg²⁺ + Ca²⁺)-dependent ATPase of cardiac sarcoplasmic reticulum (Jones *et al.*, 1978). This mechanism may therefore be common to a number of modulators of Ca²⁺ transport.

The effect of calmodulin on the steady-state concentration of Ca²⁺-dependent phosphoprotein and the turnover rate of the reaction sequence was

Table 3. *The effect of MgCl_2 on Ca^{2+} -dependent phosphoprotein decomposition in the presence and absence of calmodulin*
 Erythrocyte membranes were phosphorylated in the presence of $60\text{ }\mu\text{M}$ - Ca^{2+} or $60\text{ }\mu\text{M}$ - Ca^{2+} plus calmodulin and either $25\text{ }\mu\text{M}$ - MgCl_2 or $250\text{ }\mu\text{M}$ - MgCl_2 . After 15 s of incubation at 10°C , either Tris/HCl (2 mM), Tris/HCl (2 mM) + calmodulin, Tris/ATP (5 μM) or Tris/ATP (5 mM) + calmodulin were added to the incubation and the reaction continued for a further 10 s, then terminated as described in the text. In each case, phosphorylation in the absence of added Ca^{2+} (in the presence of $500\text{ }\mu\text{M}$ -EGTA) was subtracted from the total (approximately 1.1 pmol/mg at $25\text{ }\mu\text{M}$ - MgCl_2 and 2.7 pmol/mg at $250\text{ }\mu\text{M}$ - MgCl_2). The results shown are means $\pm$ S.E.M. for at least nine determinations.

Composition of incubation mixture	Composition of the 'chase' medium	^{32}P bound (pmol/mg)			
		Tris/HCl	Tris/HCl + calmodulin	Tris/ATP (5 μM)	Tris/ATP (5 μM) + calmodulin
Ca^{2+} (60 μM) + $25\text{ }\mu\text{M}$ - MgCl_2		1.51 ± 0.14 (100%)	1.19 ± 0.10 (79%)*	$1.05 \pm 0.10^\dagger$ (70%)	$0.69 \pm 0.10^\dagger$ (46%)
Ca^{2+} (60 μM) + $25\text{ }\mu\text{M}$ - MgCl_2 + calmodulin		1.34 ± 0.14 (89%)*	—	$0.80 \pm 0.12^\dagger$ (53%)	—
Ca^{2+} (60 μM) + $250\text{ }\mu\text{M}$ - MgCl_2		$0.78 \pm 0.22^\dagger$ (52%)	0.57 ± 0.36 (38%)	$1.02 \pm 0.11^\dagger$ (68%)	$0.82 \pm 0.19^\dagger$ (54%)
Ca^{2+} (60 μM) + $250\text{ }\mu\text{M}$ - MgCl_2 + calmodulin		$0.73 \pm 0.26^\dagger$ (48%)	—	$0.79 \pm 0.19^\dagger$ (52%)	—

* % of mean value noted in the presence of $25\text{ }\mu\text{M}$ - MgCl_2 and $60\text{ }\mu\text{M}$ - Ca^{2+} .

$^\dagger P < 0.05$ compared with those values observed in the presence of $60\text{ }\mu\text{M}$ - Ca^{2+} + $25\text{ }\mu\text{M}$ - MgCl_2 and the Tris/HCl 'chase'.

distinctly altered when $250\text{ }\mu\text{M}$ - MgCl_2 was substituted for $25\text{ }\mu\text{M}$ - MgCl_2 in the incubation media. Under these conditions, the steady-state concentration of EP was decreased by one-half and an enhancement of the rate of decomposition in the presence of the Tris/ATP 'chase', either in the presence or in the absence of calmodulin, were no longer observed. Rega and his co-workers (Richards *et al.*, 1978) have shown that MgCl_2 increases the rate of dephosphorylation of the Ca^{2+} -dependent phosphoprotein. They suggested that MgCl_2 converted the phosphorylated enzyme from the E_1P form into the E_2P form. The E_2P form decomposes at a faster rate than E_1P , probably due to an alteration in the chemical configuration of the intermediate. In the present studies at low MgCl_2 concentrations, the presence of calmodulin, either throughout the reaction procedure or after the formation of EP, was shown to enhance the decomposition of EP. As this effect of calmodulin was similar to that of Mg^{2+} , and was in fact absent at high MgCl_2 concentrations, both calmodulin and $250\text{ }\mu\text{M}$ - MgCl_2 may enhance EP decomposition by effecting the same step of the reaction sequence. At the higher MgCl_2 concentration calmodulin increased (Mg^{2+} + Ca^{2+})-dependent ATPase and hence the turnover rate of the reaction sequence, indicating that under these conditions calmodulin further accelerated E_2P decomposition.

Rega & Garrahan (1980) have reported that calmodulin increases the turnover of the Ca^{2+} pump. Our results corroborate this finding and indicate that these effects are due to an enhancement of the rate of

dephosphorylation of the phosphoprotein intermediate.

Calmodulin thus appears to exert its effect on Ca^{2+} transport by a direct effect on the Ca^{2+} -transport mechanism. Lynch & Cheung (1979) have recently shown that calmodulin binds to a detergent-solubilized preparation of (Mg^{2+} + Ca^{2+})-dependent ATPase, indicating that calmodulin interacts directly with the enzyme. Selective formation of a Ca^{2+} -dependent calmodulin-(Mg^{2+} + Ca^{2+})-dependent ATPase complex has been utilized in the separation and purification of the (Mg^{2+} + Ca^{2+})-dependent ATPase from other erythrocyte-membrane proteins (Niggli *et al.*, 1979).

This work was supported by grants from the Medical Research Council of Canada (B. D. R.) and the Canadian Cystic Fibrosis Foundation (S. K.). We thank Douglas Mauldin, Arda Minocherhomjee and Ameera Al-Jobore for preparing the 'ghosts' used in these studies.

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