Cardiac sarcoplasmic-reticulum calmodulin-binding proteins

Modulation of calmodulin binding to phospholamban by phosphorylation

Annie MOLLA,* Jean Paul CAPONY and Jacques G. DEMAILLE
Centre de Recherche de Biochimie macromoléculaire du C.N.R.S. and I.N.S.E.R.M. U. 249, B.P. 5051, 34033 Montpellier Cedex, France

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The gel-overlay technique with $^{125}$I-labelled calmodulin allowed the detection of several calmodulin-binding proteins of $M_r$ 280000, 150000, 97000, 56000, 35000 and 24000 in canine cardiac sarcoplasmic reticulum. Only two calmodulin-binding proteins could be identified unambiguously. Among them, the 97000-$M_r$ protein that undergoes phosphorylation in the presence of Ca$^{2+}$ and calmodulin, is likely to be glycogen phosphorylase. In contrast, the (Ca$^{2+}$+Mg$^{2+}$)-activated ATPase did not appear to bind calmodulin under our experimental conditions. The second known calmodulin target is dephosphophospholamban, which migrates with an apparent $M_r$ of 24000. The dimeric as well as the monomeric form of phospholamban was found to bind calmodulin. Phospholamban shifts the apparent $K_d$ of erythrocyte (Ca$^{2+}$+Mg$^{2+}$)-activated ATPase for calmodulin, suggesting thus a tight binding of calmodulin to the proteolipid. Interestingly enough, phospholamban phosphorylation by either the catalytic subunit of cyclic AMP-dependent protein kinase or the Ca$^{2+}$/calmodulin-dependent phospholamban kinase was found to inhibit calmodulin binding.

Ca$^{2+}$ uptake by cardiac SR is stimulated in response to either the catecholamine-triggered cyclic AMP pathway or an increase in cytosolic free Ca$^{2+}$. This stimulation is associated with phosphorylation of phospholamban, a membrane-bound proteolipid of $M_r$ 24000, by cyclic AMP-dependent protein kinase (Wray et al., 1973; Kirchberger et al., 1974; Planck et al., 1983), and Ca$^{2+}$/calmodulin-dependent phospholamban kinase (Le Peuch et al., 1979). Stimulation of Ca$^{2+}$ uptake by phosphorylation is correlated with (Ca$^{2+}$+Mg$^{2+}$)-activated ATPase activation (Kranias et al., 1980; Tada et al., 1975).

In addition to the indirect involvement of calmodulin in the (Ca$^{2+}$+Mg$^{2+}$)-activated ATPase activation via the calmodulin-dependent phosphorylation of phospholamban, a direct interaction between the Ca$^{2+}$ pump and calmodulin has been suggested (Lopaschuk et al., 1980; Mas Oliva et al., 1983). Furthermore, whereas Louis & Jarvis (1982) have reported that calmodulin binds to phospholamban, Seiler et al. (1984) have not succeeded in detecting such binding. Since calmodulin seems to play a pivotal role in the regulation of Ca$^{2+}$ uptake by cardiac SR, identification of calmodulin-binding proteins in cardiac SR vesicles is a first step in the understanding of the mechanism of the Ca$^{2+}$-uptake regulation.

We report here the identification of calmodulin target proteins in cardiac SR vesicles and confirm that phospholamban does bind calmodulin. In addition, its affinity for calmodulin is shown to be modulated by phosphorylation by either the cyclic AMP-dependent protein kinase or the Ca$^{2+}$/calmodulin-dependent phospholamban kinase. The hypothesis that phospholamban might be either the Ca$^{2+}$/calmodulin-dependent protein kinase or at least its calmodulin-binding subunit, as suggested by Louis & Jarvis (1982), was ruled out. No modification of the apparent $K_d$ of calmodulin for phospholamban kinase was indeed observed after phosphorylation of phospholamban, which results in poor binding of calmodulin to phospholamban.
Experimental

Materials

[γ-32P]ATP and Na125I were purchased from Amersham International. Calmodulin was isolated from rabbit testis by the method of Autric et al. (1980) and was iodinated to the extent of 1 mol/mol by the Iodogen technique (Salacinski et al., 1981).

Methods

Miscellaneous methods. Protein concentrations were determined by the Coomassie Blue technique using γ-globulin as standard (Spector, 1978). Sodium dodecyl sulphate/polyacrylamide [5-20% (w/v) gradient]-gel electrophoresis was performed as described by Laemmli (1970). Pharmacia Mr, markers being used. Samples for electrophoresis were denatured in 0.25 M-DTT/2.5 M-urea/2.5% (w/v) sodium dodecyl sulphate/20 mM-Tris/HCl, pH 6.8, at 4°C. Gels were dried and autoradiographed at −70°C by using an intensifying screen and Kodak Min-R films.

Densitometric scans of either Coomassie Blue-stained slab gels or autoradiograms were performed by using a CS 930 Shimadzu dual-wavelength scanner.

Membrane preparations. Canine cardiac SR vesicles were prepared from dog hearts arrested in diastole by pentobarbital injection essentially as previously described (Molla et al., 1983a). In order to remove remaining membrane-bound calmodulin, a supplementary wash in Hepes buffer, pH 7.5, containing 10 mM-EDTA, was performed.

EDTA-washed red-cell membranes were prepared as described by Katz & Blostein (1975).

Phospholamban purification. Phospholamban was extracted from freeze-dried vesicles with chloroform/methanol/12 M-HCl (800:400:3, by vol.), the procedure previously described (Capony et al., 1983) being followed, except that the phosphorylation step was omitted. The extract was then purified by h.p.l.c. on a silica column packed with Spherisorb S5W obtained from Chrompack. Phospholamban was eluted by chloroform/methanol/water/trifluoroacetic acid (3000:3000:1000:7, by vol.).

Gel-overlay technique. Calmodulin binding to cardiac SR proteins was studied by the gel-overlay technique, performed essentially as described by Molla et al. (1983b). Briefly, 300 µg of protein were separated by dodecyl sulphate/polyacrylamide-slab-gel electrophoresis as described above. The gel was washed in propan-2-ol/acetic acid/water, (5:2:13, by vol.) to remove dodecyl sulphate. Proteins were then submitted to denaturation before a renaturing step. The gel was overlaid with buffer A (0.15 M-NaCl/1 mM-magnesium acetate/1 mM-CaCl₂/10 mM-DTT/50 mM-Tris/HCl, pH 7.5) supplemented with bovine serum albumin (10 mg/ml), then soaked in buffer A containing 125I-calmodulin (240 nM, 10⁻⁴·10⁻⁵ c.p.m./7 pmol, 100 µl/slab). Unbound labelled calmodulin was removed by washing the gel in buffer A or in buffer A containing 2 mM-EGTA instead of CaCl₂ and magnesium acetate. The whole procedure was performed at room temperature.

Phosphorylations and dephosphorylations of SR vesicles. SR vesicle phosphorylation was carried out at 30°C in 0.1 M-DTT/10 mM-magnesium acetate/0.5 mM-ATP/10 mM-NaF/30 mM-sodium phosphate buffer, pH 7.0, and either pure catalytic subunit of cyclic AMP-dependent protein kinase (enzyme/substrate ratio 1:100; specific activity 1.16 µmol of [32P]phosphate incorporated/min per mg of enzyme) or 0.5 µM-calmodulin in the presence of 0.1 mM-CaCl₂. The reaction initiated by the addition of ATP was terminated by cold trichloroacetic acid (10%, w/v) precipitation on Whatman 3MM filter papers as previously described (Molla et al., 1983a).

Enzymic removal of bound phosphate was achieved, as described by Le Peuch et al. (1979), by incubation at 30°C of a SR-vesicle suspension in 0.2 M-sodium acetate buffer, pH 5.5, with 10 µg of potato acid phosphatase (specific activity 60 units/mg). In order to monitor the extent of dephosphorylation, samples were withdrawn and pipetted on to Whatman 3MM filter papers. The filters were washed as described by Molla et al. (1983a) and then counted for 32P radioactivity.

Red-cell (Ca²⁺ + Mg²⁺)-activated ATPase activity. The calmodulin-dependent (Ca²⁺ + Mg²⁺)-activated ATPase activity was measured by the amount of [32P]Pi, released from [γ-32P]ATP as described by Katz & Blostein (1975). The assay mixture contained 1.2 mM-magnesium acetate, 240 µM-[γ-32P]ATP (100 c.p.m./pmol), 144 mM-KCl, 120 mM-ouabain, 100 µM-CaCl₂ in 48 mM-Hepes buffer, pH 7.4, in a total volume of 600 µl.

The reaction, initiated by the addition of 10 µg of membranes, was carried out at 30°C for 30 min. The reaction was quenched by the addition of 0.1 ml of cold trichloroacetic acid (40%, w/v) containing 5 mM-ATP and 2 mM-K₂H₃PO₄. A charcoal suspension [Fischer Norit A; 1.5 g/ml in the presence of 5% (w/v) trichloroacetic acid] was added, the mixture was then shaken for 5 min and then centrifuged for 5 min at 3000 g. The radioactivity present in the clear supernatant was determined by liquid-scintillation counting.

Results

Catalogue of cardiac SR calmodulin-binding proteins.

A list of cardiac SR calmodulin-binding proteins was obtained by using gel overlay with labelled
calmodulin (Fig. 1). Calmodulin targets fall into three categories. One of them, migrating with an apparent $M_t$ 30000 binds calmodulin in both the presence and absence of Ca$^{2+}$. Another category comprises two proteins of $M_t$ 35000 and 24000, whose calmodulin binding is only partly abolished in the presence of EGTA.

Finally, most of the target peptides ($M_t$, 280000, 150000, 97000 and 56000) bind calmodulin in a Ca$^{2+}$-dependent manner. Most of these calmodulin proteins were also detected by using a calmodulin azido derivative (results not shown).

Among the above targets, the 97000-$M_t$ protein co-migrated with authentically glycogen phosphorylase. The latter enzyme was shown to bind calmodulin in a Ca$^{2+}$-dependent manner (Vilar Palasi et al., 1983). It is therefore likely that the SR calmodulin-binding protein is phosphorylase b. The 56000-$M_t$ calmodulin-binding undergoes phosphorylation in the presence of Ca$^{2+}$ and calmodulin (as shown in Fig. 2, lane 1) and co-migrates with an ATP-binding protein (results not shown). The higher-$M_t$ target proteins were not identified. The lower-$M_t$ labelled peptide ($M_t$, 24000) is presumably phospholamban, since calmodulin is found to bind purified phospholamban. The dimeric (24000 $M_t$) as well as the monomeric (12000 $M_t$) form of phospholamban is detected by the calmodulin-overlay technique (Fig. 1, lane 3, and Fig. 2, lane 1). Under our gel-electrophoresis conditions about 5–10% of the phospholamban appears to be present as the monomeric species.

![Fig. 1. Labelling of calmodulin-binding proteins from cardiac SR vesicles](image)

Cardiac SR vesicles (300 μg/lane) and purified phospholamban (50 μg) were subjected to slab-gel electrophoresis. After $^{125}$I-calmodulin overlay, the gel was dried and autoradiographed. Lanes 1–3 were washed in the presence of 1 mM-Ca$^{2+}$ and lane 2 was washed in the presence of 2 mM-EGTA. Lanes 1 and 2, cardiac SR vesicles; lane 3, phospholamban. $M_t$ markers were rabbit filamin and proteins in the Pharmacia low-$M_t$ kit.

![Fig. 2. Effect of phosphorylation on the affinity of phospholamban for calmodulin](image)

Cardiac SR vesicles were phosphorylated for 4 min by either the cyclic AMP-dependent protein kinase or the Ca$^{2+}$/calmodulin-dependent protein kinase, as described under ‘Methods’. Experiments were performed in parallel with $[^{32}P]ATP$ and unlabelled ATP. All these samples were subjected to electrophoresis. The $^{32}$P-labelled part of the gel was submitted to autoradiography in order to monitor the extent of phosphorylation; the corresponding autoradiogram is shown in (a). The gel-overlay technique was performed on the non-radioactive part of the gel. The autoradiogram of $^{125}$I-calmodulin binding to target proteins is shown in (b). Non-phosphorylated SR vesicles were loaded in sample lane 2, whereas SR vesicles phosphorylated by the Ca$^{2+}$/calmodulin-dependent protein kinase or by the cyclic AMP-dependent protein kinase were loaded in lanes 1 and 3 respectively.
Effect of phosphorylation on calmodulin binding to phospholamban

Phospholamban is the major phosphate acceptor in cardiac SR vesicles. Modifications of calmodulin-binding properties after phosphorylation were therefore examined by calmodulin overlay. The overlay pattern (Fig. 2) indicated that the labelling of the 24000-Mr protein decreased when SR vesicles were phosphorylated by either the cyclic AMP-dependent protein kinase or the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase.

Calmodulin binding to phospholamban was quantified by monitoring the kinetics of phosphorylation of SR vesicles by either the catalytic subunit of cyclic AMP-dependent protein kinase or the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (see Fig. 3). Whereas the peak area ratio of 150000-Mr protein to the 280000-Mr protein was constant (around 3.3), the peak area ratio of phospholamban to the 280000-Mr protein decreased drastically when phospholamban was phosphorylated.

This ratio fell from 4.5 for dephosphophospholamban to 0.12 and 0.45 within 1 min of phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase and the Ca\textsuperscript{2+}-dependent endogenous kinase respectively. Furthermore, the dephosphorylation of phospholamban by potato acid phosphatase was found to increase calmodulin binding to the 24000-Mr protein (see Fig. 4). When SR vesicles were incubated in the presence of potato acid phosphatase, 60% of protein-bound phosphate were removed within 20 min, whereas calmodulin binding to phospholamban increased and reached 58% of the maximal value.

Fig. 3. \textsuperscript{125}I-calmodulin binding to phospho- and dephosphophospholamban monitored by densitometry
Cardiac SR vesicles were phosphorylated with unlabelled ATP for 0, 1, 4 and 10 min by either the cyclic AMP-dependent protein kinase (○) or the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (●) as described under 'Methods', and the samples were submitted to electrophoresis. After \textsuperscript{125}I-calmodulin overlay, the gel was dried and autoradiographed. The corresponding autoradiogram was scanned and the areas of the peaks were determined by computer analysis. The ratio of the area of the 150000-Mr protein to the area of the 280000-Mr protein is represented by ○; the values are the means of two determinations. The ratio of the area of phospholamban to the area of the 280000-Mr protein is represented by either ○ or ●.

Fig. 4. Effect of dephosphorylation on the affinity of phospholamban for calmodulin
Cardiac SR vesicles were phosphorylated for 1 min by the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase as described under 'Methods'. The vesicles were centrifuged at 100000 \textit{g} for 15 min and the pellet was then suspended in 0.2 M-sodium acetate buffer, pH 5.5. Removal of bound phosphate was achieved through incubation of SR vesicles in the presence of 10 \textmu g of potato acid phosphatase (specific activity 60 units/mg). Experiments were performed in parallel with [\gamma\textsuperscript{32}P]ATP and unlabelled ATP. All these samples were submitted to electrophoresis. The \textsuperscript{32}P-labelled part of the gel was autoradiographed; the corresponding autoradiogram is shown in (a). The gel-overlay technique was performed on the non-radioactive part of the gel (b). Non-phosphorylated SR vesicles were loaded in sample lane 1, whereas phosphorylated SR vesicles were in lane 2. SR vesicles incubated for 10 and 20 min in the presence of phosphatase were loaded in lanes 3 and 4 respectively. The peak area ratio of phospholamban to the 280000-Mr protein was determined to be 1.2, 0.2, 0.4 and 0.7 for lanes 1, 2, 3 and 4 respectively.
Effect of the presence of phospholamban on the activation of red-cell (Ca\(^{2+}\) + Mg\(^{2+}\))-activated ATPase by calmodulin

The apparent \(K_d\) of calmodulin for the ATPase was determined to be 12 nM. As shown in Fig. 5, the apparent \(K_d\) increased to 35 nM when phospholamban was present in the assay mixture. Because this experiment was performed in a polyphasic medium, it did not allow quantification of calmodulin binding to phospholamban, but it did provide evidence for a competition for the calmodulin ligand between the ATPase and phospholamban.

Effect of phosphorylation on the affinity of phospholamban kinase for calmodulin

As shown in Fig. 6, phosphorylation of cardiac SR vesicles by the cyclic AMP-dependent protein kinase did not modify the apparent \(K_d\) of the kinase for calmodulin. Half-maximal activation of the SR Ca\(^{2+}\)/calmodulin-dependent kinase was 8 nM, regardless of the phosphorylation state of the vesicles.

Discussion

The various calmodulin-binding proteins of cardiac SR vesicles have been detected by calmodulin gel overlay. We confirmed the existence, in cardiac SR, of 24000- and 97000-\(M_r\) calmodulin-
binding proteins previously described by Louis & Jarvis (1982), who used dithiobis(succinimidyl propionate) to cross-link calmodulin to target proteins. The 97000-M₆ protein was postulated by the latter authors to be the (Ca²⁺ + Mg²⁺)-activated ATPase, but in fact it migrates slightly faster than the ATPase. The 97000-M₆ protein migrates with the same velocity as the skeletal phosphorylase b that was shown to bind calmodulin (Vilar Palasi et al., 1983). Furthermore, early reports on skeletal SR membranes indicate the presence of phosphorylase b in SR preparations (MacLennan & Holland, 1976). Therefore the faint band of M₉ 97000 present in cardiac SR is likely to be phosphorylase b. The (Ca²⁺ + Mg²⁺)-activated ATPase does not appear to bind calmodulin, raising doubts as to the direct activation of the pump by calmodulin.

Most of the target proteins are not identified as yet. The p240 ubiquitous calmodulin-binding protein detected in various vertebrate tissues (Palfrey et al., 1982) and especially in the rat heart particulate fraction does not appear to be present in dog heart SR vesicles. The 280000-M₅ calmodulin target protein is perhaps identical with the high-M₉ calmodulin-binding protein detected by Seiler et al. (1984) in junctional SR vesicles.

The possibility that the 30000-M₅ protein which binds calmodulin, whether or not Ca²⁺ is present, is troponin I (Koops & Barany, 1979) can be dismissed, since this protein is extracted by the acidic organic solvent mixture used in phospholamban purification. The 56000-M₅ calmodulin-binding protein undergoes phosphorylation in the presence of Ca²⁺ and calmodulin and co-migrates with an ATP-binding protein. This protein, which is presumably identical with the intrinsic glycoprotein of cardiac SR described by Campbell & MacLennan (1983), is therefore a possible candidate for being a subunit of phospholamban kinase.

The low-M₉ target protein is assumed to be phospholamban, the SR proteolipid that was described as a 24000-M₅ dimer and a 12000-M₅ monomer (Le Peuch et al., 1980). That membrane phosphorylatable proteolipids bind calmodulin is not surprising, since other substrates of cyclic AMP-dependent protein kinase, such as phosphorylase kinase, smooth-muscle myosin light-chain kinase and troponin I, also bind calmodulin (Picton et al., 1981; Conti & Adelstein, 1981; Amphlett et al., 1976). More recently Malencik & Anderson (1982) have shown that calmodulin-binding peptides contain regions structurally similar to the recognition sequence for cyclic AMP-dependent protein kinase (Malencik et al., 1982). Binding of calmodulin to phospholamban was shown to be inhibited after phosphorylation by either the cyclic AMP-depen-
dent protein kinase or the Ca²⁺/calmodulin-dependent phospholamban kinase. Modulation of calmodulin binding after phosphorylation has also been described for smooth-muscle myosin light-chain kinase, myelin basic protein and glycogen phosphorylase (Conti & Adelstein, 1981; Malencik & Anderson, 1982; Vilar Palasi et al., 1983). Phosphorylation indeed alters the hydrophobicity and charge of the calmodulin-binding site. Conversely, calmodulin binding to the site presumably prevents its phosphorylation. Preincubation of SR vesicles in the presence of calmodulin was indeed found to decrease 1.6-fold the initial velocity of phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase (results not shown).

Calmodulin binding to phospholamban might suggest that the proteolipid is part of the Ca²⁺/calmodulin-dependent protein kinase undergoing autophosphorylation (Louis & Jarvis, 1982). This possibility was dismissed, since there is no modification of the affinity of the kinase for calmodulin after phosphorylation.

It is important to note that the phospholamban phosphorylation by either cyclic AMP- or calmodulin-dependent protein kinases has identical effects, though they catalyse phosphorylation at different sites (Le Peuch et al., 1979; Louis & Jarvis, 1982). If calmodulin does shield the phosphorylatable site(s), the cyclic AMP-dependent phosphorylation is likely to occur only in the absence of Ca²⁺. Conversely, the Ca²⁺/calmodulin-dependent phosphorylation is likely to compete with direct calmodulin-binding to phospholamban. If the above assumptions were true, one would expect the adrenaline induced cyclic AMP-dependent phosphorylation to occur mostly during diastole, when the free Ca²⁺ level is low enough for calmodulin to be free. Increase in the cytosolic Ca²⁺ concentration would allow either calmodulin binding to phospholamban and/or calmodulin-dependent phosphorylation of phospholamban.

This suggests that the two regulatory systems, triggered respectively by cyclic AMP and Ca²⁺, might be mutually exclusive in time.

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References

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