Cardiac sarcoplasmic-reticulum calmodulin-binding proteins

Modulation of calmodulin binding to phospholamban by phosphorylation

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The gel-overlay technique with ^{125}I -labelled calmodulin allowed the detection of several calmodulin-binding proteins of M_r 280 000, 150 000, 97 000, 56 000, 35 000 and 24 000 in canine cardiac sarcoplasmic reticulum. Only two calmodulin-binding proteins could be identified unambiguously. Among them, the 97 000- M_r protein that undergoes phosphorylation in the presence of Ca^{2+} and calmodulin, is likely to be glycogen phosphorylase. In contrast, the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -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 24 000. The dimeric as well as the monomeric form of phospholamban was found to bind calmodulin. Phospholamban shifts the apparent K_d of erythrocyte $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -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 24 000, 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 $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -activated ATPase activation (Kranias *et al.*, 1980; Tada *et al.*, 1975).

In addition to the indirect involvement of calmodulin in the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -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.

Abbreviations used: SR, sarcoplasmic reticulum; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol.

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Experimental

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Na^{125}I were purchased from Amersham International. Calmodulin was isolated from ram 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 M_r markers being used. Samples for electrophoresis were denatured in 0.25M-DTT/2.5M-urea/2.5% (w/v) sodium dodecyl sulphate/20mM-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-wave-length 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 10mM-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/12M-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 (0.15M-NaCl/1mM-magnesium acetate/1mM- CaCl_2 /1mM-DTT/50mM-Tris/HCl, pH 7.5)

supplemented with bovine serum albumin (10mg/ml), then soaked in buffer A containing ^{125}I -calmodulin (240 nM, 10^4 – 10^5 c.p.m./77pmol, 100ml/slab). Unbound labelled calmodulin was removed by washing the gel in buffer A or in buffer A containing 2mM-EGTA instead of CaCl_2 and magnesium acetate. The whole procedure was performed at room temperature.

Phosphorylations and dephosphorylation of SR vesicles. SR vesicle phosphorylation was carried out at 30°C in 0.1mM-DTT/10mM-magnesium acetate/0.5mM-ATP/10mM-NaF/30mM-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 ^{32}P phosphate incorporated/min per mg of enzyme) or 0.5 μM -calmodulin in the presence of 0.1mM- CaCl_2 . 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 0.2M-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) were then counted for ^{32}P radioactivity.

Red-cell ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-activated ATPase activity. The calmodulin-dependent ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-activated ATPase activity was measured by the amount of ^{32}P released from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described by Katz & Blostein (1975). The assay mixture contained 1.2mM-magnesium acetate, 240 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (100 c.p.m./pmol), 144mM-KCl, 120 μM -ouabain, 100 μM - CaCl_2 in 48mM-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 5mM-ATP and 2mM- KH_2PO_4 . A charcoal suspension [Fischer Norit A; 1.5g/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 3000g. 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_r 30000 binds calmodulin in both the presence and absence of Ca^{2+} . Another category comprises two proteins of M_r 35000 and 24000, whose calmodulin binding is only partly abolished in the presence of EGTA.

Finally, most of the target peptides (M_r 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_r protein co-migrated with authentic 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_r 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_r target proteins were not identified. The lower- M_r labelled peptide (M_r 24000) is presumably phospholamban, since calmodulin is found to bind purified phospholamban. The dimeric (24000 M_r) as well as the monomeric (12000 M_r) 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

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_r markers were rabbit filamin and proteins in the Pharmacia low- M_r kit.

Fig. 2. Effect of phosphorylation on the affinity of phospholamban for calmodulin

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 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and unlabelled ATP. All these samples were submitted 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- M_r protein decreased when SR vesicles were phosphorylated by either the cyclic AMP-dependent protein kinase or the Ca^{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^{2+} /calmodulin-dependent-protein kinase (see Fig. 3). Whereas the peak area ratio of 150000- M_r protein to the 280000- M_r protein was constant (around 3.3), the peak area ratio of phospholamban to the 280000- M_r protein decreased drastically when phospholamban was phosphorylated.

Fig. 3. ^{125}I -calmodulin binding to phospho- and dephospho-phospholamban 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^{2+} /calmodulin-dependent protein kinase (●) as described under 'Methods', and the samples were submitted to electrophoresis. After ^{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- M_r protein to the area of the 280000- M_r 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- M_r protein is represented by either ○ or ●.

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^{2+} -dependent endogenous kinase respectively. Furthermore, the dephosphorylation of phospholamban by potato acid phosphatase was found to increase calmodulin binding to the 24000- M_r 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. 4. Effect of dephosphorylation on the affinity of phospholamban for calmodulin

Cardiac SR vesicles were phosphorylated for 1 min by the Ca^{2+} /calmodulin-dependent protein kinase as described under 'Methods'. The vesicles were centrifuged at 100000g for 15 min and the pellet was then suspended in 0.2M-sodium acetate buffer, pH 5.5. Removal of bound phosphate was achieved through incubation of SR vesicles in the presence of 10 μg of potato acid phosphatase (specific activity 60 units/mg). Experiments were performed in parallel with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and unlabelled ATP. All these samples were submitted to electrophoresis. The ^{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- M_r 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 ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-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.

Fig. 5. Effect of the presence of phospholamban on the activation of erythrocyte ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-activated ATPase by calmodulin

ATPase assays were performed in the presence (●) or the absence (○) of phospholamban. Freeze-dried phospholamban was suspended in chloroform/methanol (2:1, v/v) and distributed in the assay tubes. Approx. 1 nmol of phospholamban was added in each assay. The organic solvent was then evaporated and the ATPase assay was performed as described under 'Methods' in the presence of various amounts of calmodulin. The specific activity of the enzyme was 3 $\mu\text{mol/h}$ per mg.

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-

Fig. 6. Cardiac SR phospholamban kinase activation by calmodulin

Assays were performed on phosphorylated (●) and non-phosphorylated (○) SR vesicles. SR vesicles (1.2 mg) were phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase (2 μg in the assay) as described under 'Methods'. The reaction was stopped by addition of 1 μg of pure protein kinase inhibitor; vesicles were then diluted 1:10 with water. Non-phosphorylated SR vesicles were incubated under the same conditions, except that the catalytic subunit and protein kinase inhibitor were added simultaneously after the dilution step. SR phospholamban kinase activity was measured by the amount of [^{32}P]phosphate incorporated into endogenous phospholamban and into exogenous phosphorylase *b* (by the method of Le Peuch *et al.*, 1979). The assay mixture contained 1 mM-DTT, 10 mM-magnesium acetate, 0.1 mM- CaCl_2 , 1 mM-[$\gamma\text{-}^{32}\text{P}$]ATP (200 c.p.m./pmol), 10 mM-NaF, 30 mM-sodium phosphate buffer, pH 7.0, and calmodulin at the concentration indicated. The assay mixture was supplemented with phosphorylase *b* (0.5 mg). The incubation was initiated by addition of 56 μg of phosphorylated or control SR vesicles. After 5 min of incubation at 30°C, samples (50 μl out of a total volume of 65 μl) were withdrawn and pipetted on to Whatman 3MM filter papers.

binding proteins previously described by Louis & Jarvis (1982), who used dithiobis(succinimidyl propionate) to cross-link calmodulin to target proteins. The 97000- M_r protein was postulated by the latter authors to be the $(Ca^{2+} + Mg^{2+})$ -activated ATPase, but in fact it migrates slightly faster than the ATPase. The 97000- M_r 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 M_r 97000 present in cardiac SR is likely to be phosphorylase *b*. The $(Ca^{2+} + Mg^{2+})$ -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- M_r calmodulin target protein is perhaps identical with the high- M_r calmodulin-binding protein detected by Seiler *et al.* (1984) in junctional SR vesicles.

The possibility that the 30000- M_r protein which binds calmodulin, whether or not Ca^{2+} 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- M_r calmodulin-binding protein undergoes phosphorylation in the presence of Ca^{2+} 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- M_r target protein is assumed to be phospholamban, the SR proteolipid that was described as a 24000- M_r dimer and a 12000- M_r 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 Ca^{2+} /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 Ca^{2+} /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 Ca^{2+} . Conversely, the Ca^{2+} /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 Ca^{2+} level is low enough for calmodulin to be free. Increase in the cytosolic Ca^{2+} 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 Ca^{2+} , might be mutually exclusive in time.

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