Biochimica et Biophysica Acta, 1149 (1993) 249–259

BBAMEM 76027

Localization and functional role of the calmodulin-binding domain of phospholamban in cardiac sarcoplasmic reticulum vesicles

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(Received 9 December 1992)
(Revised manuscript received 26 March 1993)

Key words: Calmodulin; Phospholamban; Phosphorylation; Sarcoplasmic reticulum; Affinity labeling; Limited proteolysis

Limited proteolysis and affinity-labeling techniques have been used to localize the calmodulin-binding domain of phospholamban, the major substrate for both cAMP- and calmodulin-dependent protein kinases in cardiac sarcoplasmic reticulum (SR). SR vesicles, treated with increasing concentrations of trypsin (likely hydrolyzing at Arg-25 in the cytoplasmic region of phospholamban), exhibited a subsequent loss of both cAMP- and calmodulin-dependent phosphorylation, as well as calmodulin affinity-labeling of phospholamban. When SR vesicles were treated with increasing concentrations of chymotrypsin (which likely cleaves at Tyr-6 of phospholamban) there was no effect on the cAMP-dependent phosphorylation of phospholamban. However, similar concentrations of chymotrypsin resulted in a loss of both calmodulin affinity-labeling and calmodulin-dependent phosphorylation of phospholamban (at Thr-17). When SR vesicles were treated with increasing concentrations of Endoproteinase Lys-C (which hydrolyzes phospholamban at Lys-3) both the calmodulin affinity-labeling and the calmodulin-dependent, but not the cAMP-dependent, phosphorylation of phospholamban were inhibited. These data were complemented by ^{1}H-NMR studies on the complex formed by calmodulin and a phospholamban peptide. These data suggest that binding of calmodulin to phospholamban may be an essential intermediate step in the calmodulin-dependent phosphorylation of phospholamban.

Introduction

Phospholamban, an integral membrane protein present in canine cardiac SR, is a substrate for various protein kinases. The sequence of this 52-amino-acid protein has been determined and a model of its structure has been proposed in which five identical 6080-Da subunits [1,2] form a pentamer. Each subunit possesses a single hydrophobic membrane-spanning region and a hydrophilic 30-amino-acid N-terminal cytoplasmic domain. cAMP-PK catalyzes the phosphorylation of Ser-16, while the endogenous Cam-PK catalyzes the phosphorylation of the adjacent Thr-17 residue on this cytoplasmic domain [2].

In studies to define the mechanism by which calmodulin regulates calcium uptake by cardiac SR, we have shown previously that phospholamban is the major calmodulin-affinity-labeled product in free cardiac SR that surrounds the myofibrils [3,4]. Both the covalent cross-linking reagent dithiobis (succinimidyl propionate) plus [^{125}I]calmodulin [3], as well as the photoactivatable radioactive calmodulin derivative, Bz-Cam [4] demonstrated the formation of a 1:1 complex in free cardiac SR of calmodulin (17 kDa)/phospholamban (23 kDa) resulting in a 40-kDa covalent adduct*. In contrast, in junctional cardiac SR which is attached to

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Abbreviations: SR, sarcoplasmic reticulum; cAMP-PK, cAMP-dependent protein kinase; Cam-PK, calmodulin-dependent protein kinase; Bz-Cam, benzophenone-[^{125}I]calmodulin; DSS, disuccinimidyl suberate; PLB(1–25), synthetic peptide comprising phospholamban residues 1–25, TEMPO-IA, *N*-(4-(iodoacetyl)amino)-2,2,6,6-tetramethyl piperidine-1-oxyl.

the transverse tubular network, the > 300 kDa foot protein is the major calmodulin binding component [5]. While a protein of approx. 56 kDa that can phosphorylate phospholamban, designated the Ca^{2+}/calmodulin-dependent protein kinase, has been identified in cardiac cytosolic fractions [6,7] and may be one of the proteins labeled by calmodulin using the gel overlay procedure [8], its presence was not detected in cardiac SR vesicles with either of the more physiological calmodulin-affinity-labeling procedures used in our laboratory. Since phospholamban is the major calmodulin-affinity-labeled product in free cardiac SR, this suggests that calmodulin binds to phospholamban and that this interaction plays a role in the activation of the endogenous Cam-PK and the subsequent phosphorylation of the phospholamban substrate.

The aim of the present study was to define the region of phospholamban that interacts with calmodulin in order to further characterize the calmodulin-dependent phosphorylation of this protein. This has been achieved using limited proteolysis of SR vesicles followed by calmodulin-affinity-labeling. Removal of the N-terminal 3 or 6 residues of phospholamban resulted in a decrease in both calmodulin-affinity-labeling and calmodulin-dependent phosphorylation of phospholamban. Correlating the loss in calmodulin affinity-labeling with the loss of the ability of the endogenous Cam-PK to phosphorylate phospholamban indicates that the binding of calmodulin to phospholamban could be a necessary step in the activation of the endogenous Cam-PK.

Materials and Methods

Materials

Free SR vesicles isolated from canine or porcine ventricular muscle were prepared as described previously [9] and stored at −70°C in 10% sucrose at a protein concentration of 20–30 mg/ml. [γ-^{32}P]ATP was prepared by the method of Walseth and Johnson [10] using [^{32}P]P_i purchased from Du Pont New England Nuclear (Boston, MA, USA). Calmodulin, prepared from beef testes (Pel-Freeze Biologicals, Rogers, AR, USA), by the method of Gopalakrishna and Anderson [11], was radioiodinated in the presence of $Na^{125}I$ (Du Pont-New England Nuclear) and the Enzymobead glucose oxidase-lactoperoxidase system (Bio-Rad, Richmond, CA, USA) according to the procedure of Strasburg et al. [4]. Wheat germ calmodulin was prepared from untoasted wheat germ (a generous gift of Dr. George Decelles, International Multifoods, Minneapolis, MN, USA) based on the procedure of Yoshida et al. [12] as modified by Strasburg et al. [4]. This protein was iodinated and derivatized with benzophenone-4-maleimide (Molecular Probes, Junction City, OR, USA) as described previously [4]. For NMR experiments, wheat germ CaM was labeled at Cys-27 with the paramagnetic probe, TEMPO-IA (Aldrich, Milwaukee, WI, USA). Triton X-100, trypsin, α-chymotrypsin and bovine cardiac cAMP-dependent protein kinase were purchased from Sigma (St. Louis, MO, USA). DSP (dithiobis(succinimidyl propionate)) and DSS (disuccinimidyl suberate) were purchased from Pierce (Rockford, IL, USA). Bio-Beads SM-2 were purchased from Bio-Rad. Endoproteinase Lys-C was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). Water was de-ionized and redistilled from glass. All other reagents were of analytical grade. A phospholamban peptide corresponding to residues 1–25 was synthesized by the Michigan State University Macromolecular Structure Facility. The peptide was characterized by sequencing and mass spectrometry.

Methods

Proteolytic digestion of SR vesicles and phospholamban peptide. Cardiac SR vesicles (1 mg/ml) or PLB(1–25) (1 mg/ml) were treated with various concentrations of trypsin or α-chymotrypsin in a 120 mM NaCl, 40 mM histidine-HCl buffer (pH 6.8) for 10 min at 25°C. Proteolysis with Endoproteinase Lys-C was performed in the same buffer adjusted to pH 8.0. The free Ca^{2+} concentration, measured with a Ca-specific electrode, was 6–8 μM. For both enzymes, proteolysis was terminated by the addition of phenylmethylsulfonyl fluoride to a final concentration of 0.1 mM. All proteinase-treated samples were then centrifuged at 165 000 × g for 20 min at 4°C prior to phosphorylation or calmodulin-affinity labeling of the resuspended SR vesicles.

Phosphorylation of SR vesicles and PLB(1–25) with protein kinases. SR vesicles (0.1 mg) were phosphorylated in 0.1 ml of 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 20 mM Hepes buffer (pH 7.0) at 20°C in the presence of either bovine calmodulin (0.25 μM) or exogenous cAMP-PK (25 μg) plus 10 μM cAMP. Unless indicated, reactions were initiated by the addition of 0.1 mM [γ-^{32}P]ATP and terminated after 1 min by the addition of sodium dodecyl sulfate (1% (w/v)). Samples were then electrophoretically fractionated on 5–20% polyacrylamide gradient slab gels in the presence of SDS [13]. Gels were stained with Coomassie blue, dried and exposed to Kodak X-AR5 X-ray film using Du Pont High Plus intensifying screens. The ^{32}P-containing phospholamban band was identified in autoradiograms, excised from the corresponding gels and

* A similar sized adduct is also formed when purified phospholamban is affinity labeled with Bz-Cam (Jones, L.R., personal communication).

its radioactivity determined by liquid scintillation counting.

For calmodulin-dependent phosphorylation of PLB(1–25), 10 μg of peptide was included in the SR-containing phosphorylation medium. Reactions were stopped after 1 min by addition of EGTA to 1 mM and chilling to 0°C with an ice/salt-water bath. Samples were then centrifuged for 20 min at 50000 rpm in a Beckman TL 100 ultracentrifuge to separate PLB(1–25) from the membranes. The supernatants and resuspended pellets were separately fractionated on 20% polyacrylamide gels. The ^{32}P-containing PLB(1–25) bands were identified in autoradiograms, excised from the corresponding gels and the radioactivity determined by scintillation counting.

Affinity labeling of cardiac SR with calmodulin. Two protocols were adopted. In the first, Bz-Cam (0.25 μM) was incubated with SR vesicles (1 mg/ml), in 20 mM Hepes buffer (pH 7.0), 10 mM $MgCl_2$ and 0.1 mM $CaCl_2$ at 0°C under a sodium lamp. This mixture was then photoactivated in a Rayonet photoreactor (λ_{max} = 350 nm) for 5 min at 0°C. The mixture was centrifuged for 20 min at 165000 × g, when the resuspended SR vesicles were electrophoretically fractionated as described above. The radioactive bands were identified in autoradiograms, cut out of the corresponding gels and their radioactivity determined by liquid scintillation counting.

In the second procedure a medium similar to that for Bz-Cam was used except that benzophenone-calmodulin was replaced by 0.25 μM bovine [^{125}I]calmodulin and either 0.2 mM disuccinimidyl suberate or dithiobis(succinimidyl propionate) was added to initiate affinity labeling. Crosslinking for 30 min at 20°C was terminated by the addition of 0.15 M glycine (pH 8.0). The mixture was centrifuged at 165000 × g for 20 min, and the vesicles were analyzed electrophoretically as described above.

^{1}H-NMR experiments. ^{1}H-NMR spectra were recorded at 500 MHz on a Bruker AMX spectrometer. The proton resonances of PLB(1–25) were assigned using standard two-dimensional NMR procedures described elsewhere [14]. PLB(1–25) was titrated with small aliquots of spin-labeled calmodulin in 25 mM deuterated Tris-HCl buffer (pH 7.2). Interaction between PLB(1–25) and calmodulin was detected by difference spectroscopy.

Protein determinations. Protein concentrations were measured using either the method of Lowry [15], or the bicichoninic acid protein assay of Smith et al. [16]. Bovine serum albumin was used as the standard.

Results

The goal of this study was to identify the calmodulin-binding domain of phospholamban in SR vesicles.

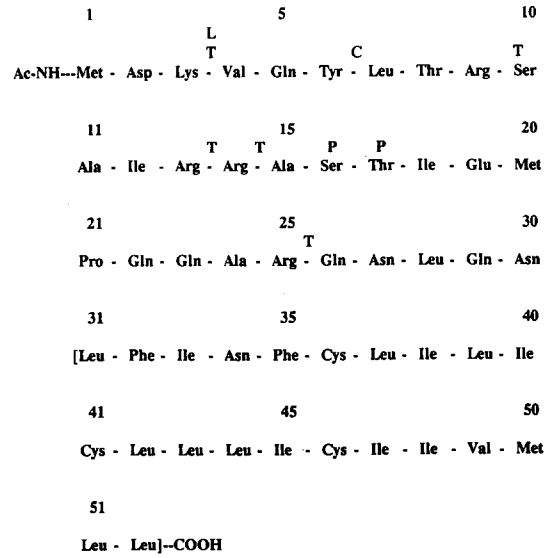


Fig. 1. The amino-acid sequence of phospholamban from canine cardiac SR. The sequence is that derived by Fujii et al. [2] from cDNA clones from a canine cardiac cDNA library. The proposed sites of hydrolysis for the proteinases used in this study: T, trypsin; C, chymotrypsin and L, Endoproteinase Lys-C. The brackets designate the probable membrane spanning region of phospholamban previously concluded from hydropathy calculations [2]. The P on Ser-16 and Thr-17 residues reflects the sites of phosphorylation by cAMP-PK and Cam-PK, respectively.

The approach adopted was to selectively remove different length N-terminal-derived peptides of phospholamban, and subsequently examine the ability of the remaining membrane-bound fragment to both interact with calmodulin and be phosphorylated by either Cam-PK or cAMP-PK. The selection of appropriate proteinases for this study has been based on the previously published amino-acid sequence of phospholamban [2] (Fig. 1). Trypsin was chosen because of the success shown by other laboratories in utilizing this proteinase to hydrolyze phospholamban [17,18]. Chymotrypsin and Endoproteinase Lys-C were chosen because of their specificity for amino acids present in the N-terminal region of phospholamban.

α-Chymotrypsin digestion of cardiac SR

α-Chymotrypsin at low concentrations is specific for aromatic amino-acid residues [19], so it would be expected to hydrolyze PLB at the single tyrosine at position 6 of the proposed cytoplasmic region of phospholamban (Fig. 1).

Because of the presence of many different proteins in the SR membrane and the limited abundance of PLB, isolation and characterization of the products of limited proteolysis was not possible. The specificity of chymotryptic hydrolysis of phospholamban in SR was determined by digestion of a synthetic peptide, PLB(1–25), which corresponds to the cytoplasmic domain of phospholamban. Digestion of this peptide by chymotrypsin yielded three peaks in the HPLC elution profile

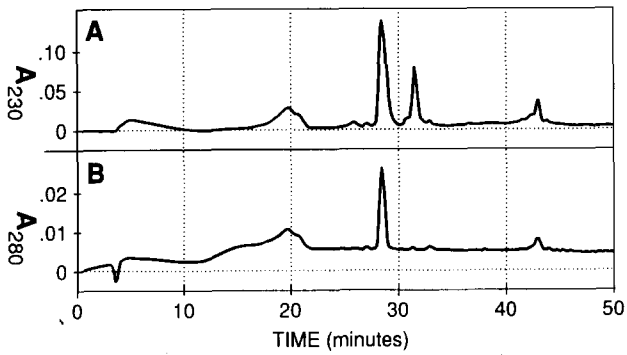


Fig. 2. HPLC elution profile of limited chymotrypsin digestion of PLB(1–25) in 1 ml of 120 mM NaCl, 40 mM histidine-HCl (pH 6.8). 1 mg of synthetic peptide PLB(1–25) was incubated for 10 min with 20 μg/ml of chymotrypsin. The reaction was stopped by addition of PMSF to 0.1 mM. Aliquots (100 μg) of this digest were applied to a Vydac C_{18} reverse-phase HPLC column equilibrated with 0.1% trifluoroacetic acid (Buffer A). The peptides were eluted using a gradient generated from 0.1% trifluoroacetic acid and acetonitrile/0.1% trifluoroacetic acid (Buffer B). Gradient conditions: (1) 5 min of Buffer A; (2) 20 min of 0–20% Buffer B; (3) 25 min of 20% to 30% Buffer B. Flow rate, 1 ml/min. Absorbances were monitored at 280, 254, 230 and 214 nm and fractions were manually collected for each peak (absorbances at 254 and 214 nm not shown).

(Fig. 2). One peptide eluting at 28 min was identified as intact PLB(1–25), because it co-eluted with undigested PLB(1–25) (data not shown) and it contained the sole tyrosine residue (based on absorbance at 280 nm). The peptide eluting at 31 min which lacked tyrosine (based on lack of absorbance at 280 nm) was sequenced by Edman degradation and was identified as PLB residues 7–25. The peak at 43 min comprised residues 1–6 of the original peptide. Thus, based on the digestion of the PLB(1–25) peptide as a model for phospholamban in SR, chymotryptic proteolysis of phospholamban in intact SR vesicles would likely occur at the C-terminal side of Tyr-6.

When cardiac SR was incubated with increasing concentrations of chymotrypsin (Fig. 3), there was a decrease in both the Bz-Cam affinity-labeling and the endogenous Cam-PK-catalyzed phosphorylation of PLB. The IC_{50} value, designated as the concentration of proteinase at which phosphorylation or calmodulin-affinity-labeling of PLB is decreased 50%, was 7.4 ± 2.0 μg chymotrypsin/ml for calmodulin affinity-labeling and 3.7 ± 0.7 μg chymotrypsin/ml for the Cam-PK-catalyzed phosphorylation of the membrane-bound phospholamban fragment (Table I). Similarly, when PLB(1–25) was incubated with increasing concentrations of chymotrypsin (Fig. 4), the phosphorylation of the 7–25 fragment by the Cam-PK was decreased (IC_{50} = 4.4 μg/ml). In contrast, the cAMP-PK-catalyzed phosphorylation of phospholamban was unaltered over the range of chymotrypsin concentrations examined (Fig. 3B).

Trypsin digestion of cardiac SR

Trypsin, which has a specificity for lysine and arginine residues [20], and has previously been demonstrated to hydrolyze phospholamban in intact SR [17,18], could potentially cleave at four sites in the cytoplasmic portion of phospholamban (Fig. 1). Notably, this proteinase could hydrolyze phospholamban at Arg-25 which is on the C-terminal side of the phosphorylation sites close to the proposed trans-membrane segment [2]. When cardiac SR vesicles were incubated with increasing concentrations of trypsin (Fig. 5), the remaining membrane-bound phospholamban-derived was now neither a substrate for either cAMP-PK or Cam-PK, nor was it affinity-labeled by Bz-Cam (IC_{50} = 3.8 ± 0.4 μg trypsin/ml in all three cases).

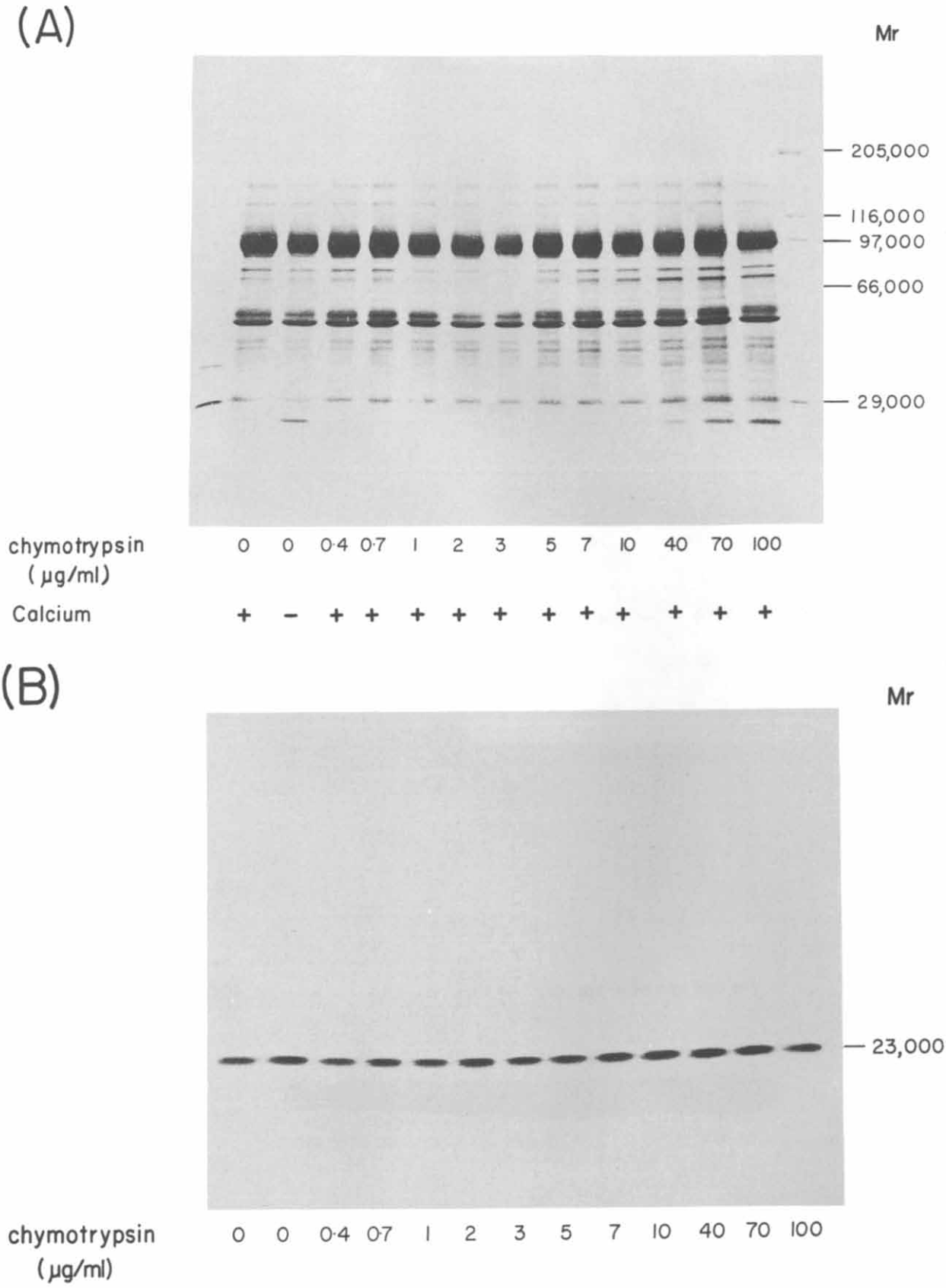
Endoproteinase Lys-C digestion of cardiac SR

Endoproteinase Lys-C is specific for cleavage of peptide bonds at the C-terminal side of lysine residues [30] and would thus hydrolyze phospholamban only at Lys-3 (Fig. 1). When cardiac SR vesicles were incubated with increasing concentrations of Endoproteinase Lys-C (Fig. 6), there was a decrease in both Bz-Cam affinity labeling (IC_{50} = 56 ± 35 μg Endoproteinase Lys-C/ml), as well as the endogenous Cam-PK-catalyzed phosphorylation (IC_{50} = 8.0 ± 1.0 μg Endoproteinase Lys-C/ml) of the membrane-bound phospholamban-derived fragment (Table I). Following Endoproteinase Lys-C treatment of cardiac SR, cAMP-catalyzed phosphorylation of phospholamban again remained unaltered over the range of proteinase concentrations examined.

Cam-PK Phosphorylation of phospholamban following chymotryptic digestion of cardiac SR

There are at least two explanations that could account for the decreased level of Cam-PK-catalyzed phosphorylation of phospholamban following proteoly-

Fig. 3. Effect of limited proteolysis of cardiac SR vesicles with chymotrypsin on the phosphorylation and Bz-Cam-affinity-labeling patterns of phospholamban. 1 mg/ml of cardiac SR was incubated with various concentrations of chymotrypsin as described in Materials and Methods. The samples were subsequently either phosphorylated in the presence of cAMP-PK or calmodulin, or affinity-labeled with Bz-Cam. Samples were electrophoretically fractionated on 5–20% polyacrylamide gels and autoradiograms were prepared as described in Materials and Methods. (Panel A) Coomassie Blue stained gel; (Panel B) autoradiogram following cAMP-PK-catalyzed phosphorylation; (Panel C) autoradiogram following Cam-PK-catalyzed phosphorylation; (Panel D) autoradiogram following Bz-Cam affinity-labeling; (Panel E) the protein kinase-catalyzed incorporation of ^{32}P into the 23-kDa phospholamban component and ^{125}I present in the covalent phospholamban/Bz-calmodulin 40-kDa affinity-labeled complex were determined as described in Materials and Methods.


(A)
Mr
205,000
116,000
97,000
66,000
29,000
chymotrypsin (μg/ml)
0 0 0·4 0·7 1 2 3 5 7 10 40 70 100
Calcium
+ − + + + + + + + + + + +
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Mr
23,000
chymotrypsin (μg/ml)
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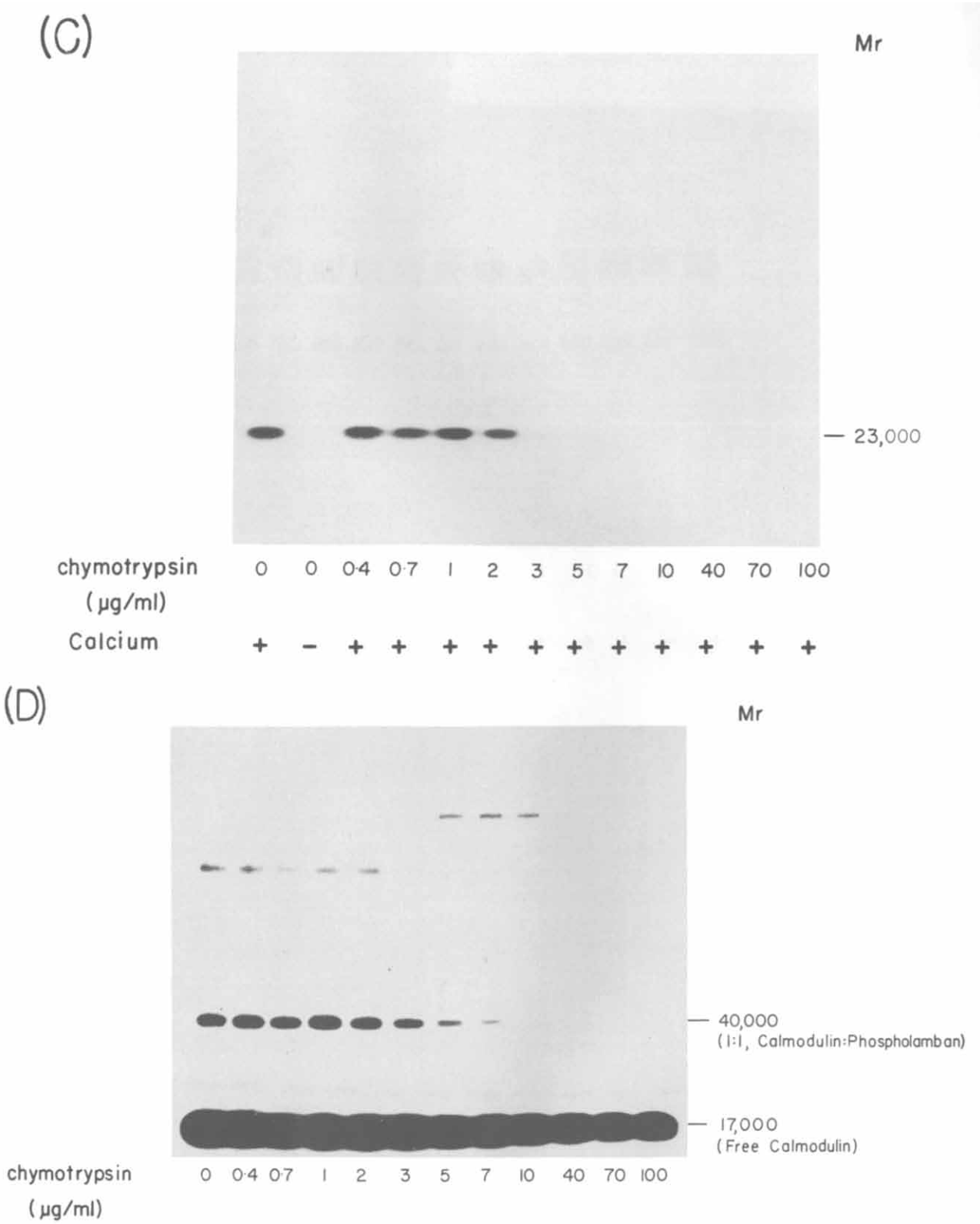

Fig. 3. (continued)

sis of SR vesicles. The first is that proteolysis removed a portion of phospholamban that bound calmodulin, such that a calmodulin/phospholamban complex is unable to form and activate the endogenous Cam-PK. The second explanation is that the decreased Cam-PK-catalyzed phosphorylation of phospholamban could result from the proteolytic digestion (and inactivation) of the endogenous Cam-PK itself. To discriminate be-

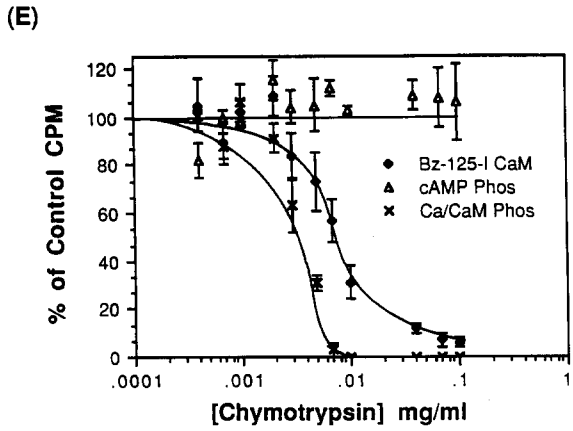

Fig. 3. (continued)

TABLE I

Proteinase treatment of cardiac SR followed by phosphorylation or calmodulin-affinity-labeling

Proteinase treatments were performed as described in Materials and Methods. The apparent IC_{50} values represent the mean ± S.D. of n experiments (in parentheses) and are expressed in μg proteinase/ml derived from Figs. 2, 4, 5, 7 and 8. The Cam-PK phosphorylations and calmodulin-affinity-labeling were performed as described in Materials and Methods.

Labeling	Proteinase pretreatment		
	Chymotrypsin	Trypsin	Endoproteinase Lys-C
Cam-PK phosphorylation	3.7 ± 0.7(3)	3.8 ± 0.4(2)	8.0 ± 1.0(3)
Bz-Cam-affinity label	7.4 ± 2.0(3)	3.8 ± 0.4(2)	56 ± 35(3)
DSS-Cam-affinity label	9.5 ± 2.2(5)	n.d.	9.3 ± 0.4(2)

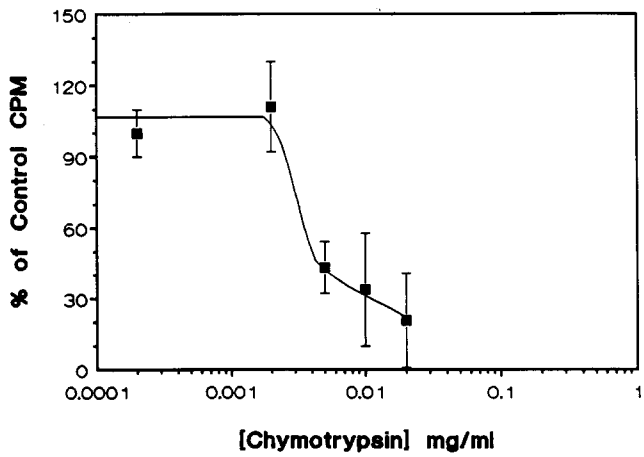

Fig. 4. Calmodulin-dependent incorporation of ^{32}P into synthetic peptide PLB(1–25). 100 μg of PLB(1–25) was digested with various amounts of chymotrypsin for 10 min as described in Materials and Methods. After stopping the reaction, 10 μg of the digest were added to an SR-containing phosphorylation medium and phosphorylation of peptide was determined as described in Materials and Methods.

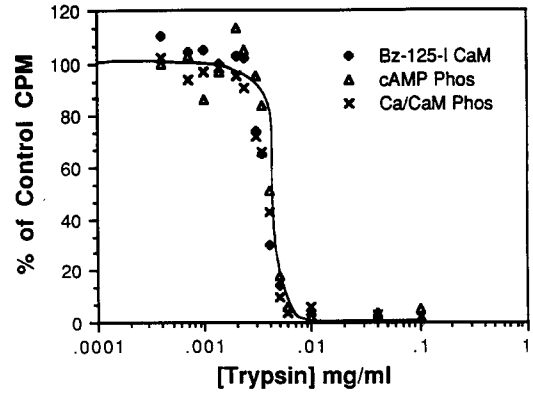

Fig. 5. Effect of limited proteolysis of cardiac SR vesicles with trypsin on the phosphorylation and Bz-Cam affinity labeling of phospholamban. Cardiac SR were treated with various concentrations of trypsin as described in the legend to Fig. 3 and in Materials and Methods. The protein kinase-catalyzed incorporation of ^{32}P into the 23-kDa phospholamban component and ^{125}I incorporated into the covalent phospholamban/Bz-calmodulin 40-kDa affinity-labeled complex were determined as described as in Materials and Methods. Data are the mean of three experiments.

tween these two possibilities, three SR samples were each incubated with different chymotrypsin concentrations for 10 min prior to Cam-PK-catalyzed phosphorylation for periods of up to 10 min with 2 mM [γ-^{32}P]ATP. The increased concentration of ATP was included to maintain the level of phospholamban phosphorylation. The chymotrypsin concentrations were chosen such that following proteinase treatment of cardiac SR for 10 min, the subsequent endogenous Cam-PK-catalyzed phosphorylation of phospholamban in the presence of [γ-^{32}P]ATP for one min would be either 100%, 50%, or 0% of the control values as determined from Fig. 3E.

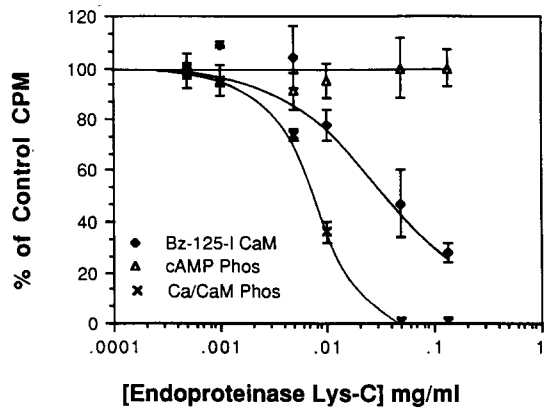

Fig. 6. Effect of limited proteolysis of cardiac SR vesicles with Endoproteinase Lys-C on the phosphorylation and Bz-Cam affinity-labeling of phospholamban. Cardiac SR were treated with various concentrations of Endoproteinase Lys-C as described in the legend to Fig. 3. The Cam-PK-catalyzed incorporation of ^{32}P into the 23-kDa phospholamban component and ^{125}I incorporated into the covalent phospholamban/Bz-calmodulin complex were determined as described in Materials and Methods. Data are the mean of three experiments.

In all cases, the level of phosphorylation achieved after 90 s did not increase further for up to 10 min (Fig. 7). If the decreased Cam-PK-catalyzed phosphorylation of phospholamban resulted from the proteolytic digestion and inactivation of the Cam-PK enzyme (rather than the hydrolysis of a portion of the phospholamban present), then the amount of native phospholamban would be the same for the three different samples in Fig. 7. Thus, for the sample which reached 50% of the control phosphorylation after 1 min, the remaining endogenous Cam-PK (50% of control concentration) would have been able to phosphorylate 100% of the phospholamban, albeit at a slower rate than the sample that had been treated with the lowest concentration of chymotrypsin. Inspection of Fig. 7 reveals this was not the case, as phosphorylation in all three samples was maximal at 90 s and remained essentially constant for up to 10 min. This indicates that the limited proteslysis of SR, which results in the loss of the region of phospholamban that interacts with calmodulin, is correlated with an inability of calmodulin to activate the Cam-PK and thus phosphorylate the phospholamban-derived fragment.

Comparison of different calmodulin-affinity-labeling procedures following proteinase digestion of cardiac SR

We have shown previously that phospholamban in SR vesicles can be affinity-labeled by [^{125}I]calmodulin with the amine-reactive crosslinker dithiobis(succinimidyl suberate) [3]. Pretreatment of cardiac SR with chymotrypsin resulted in the inhibition of subsequent

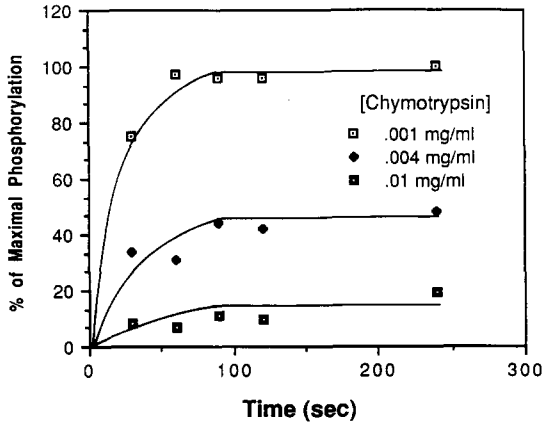

Fig. 7. Time-course of the Cam-PK-catalyzed phosphorylation of chymotrypsin-treated cardiac SR vesicles. Cardiac SR vesicles were treated with three different chymotrypsin concentrations for 10 min at 20°C, such that the subsequent levels of Cam-PK-catalyzed phosphorylation of phospholamban were approx. 100%, 50% and 10%, respectively, of the control values as determined from Fig. 3. Cam-PK-catalyzed phosphorylation in the presence of 2 mM [^{32}P]ATP was stopped at various times by the addition of 1% (w/v) SDS when samples were electrophoretically fractionated on 5–20% polyacrylamide gels. The phosphate incorporated into phospholamban was determined as described in Materials and Methods.

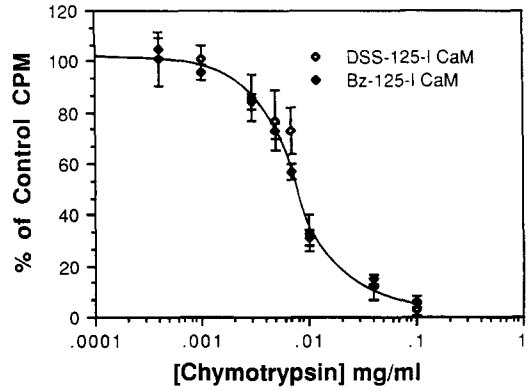

Fig. 8. Effect of limited proteolysis of cardiac SR vesicles with chymotrypsin on the Bz-Cam photoaffinity labeling and DSS crosslinking of calmodulin to phospholamban. Cardiac SR vesicles incubated with various concentrations of chymotrypsin as described in Materials and Methods, were subsequently affinity-labeled with either disuccinimidyl suberate plus [^{125}I]calmodulin, or Bz-Cam. Following electrophoretic fractionation of samples on 5–20% polyacrylamide gels, autoradiograms were obtained and the radioactivity in the 40-kDa calmodulin/phospholamban complex was determined as described in Materials and Methods. Data are the mean of three experiments.

disuccinimidyl suberate plus [^{125}I]calmodulin-affinity-labeling ($IC_{50} = 9.5 \pm 2.2$ μg chymotrypsin/ml), indicating that the peptide containing Lys-3 was removed (Fig. 8). That Bz-Cam affinity labeling of phospholamban was inhibited at similar chymotrypsin concentrations ($IC_{50} = 7.4 \pm 2.0$ μg chymotrypsin/ml) indicates that the six N-terminal residues of phospholamban are required for calmodulin binding (Fig. 8).

To remove just the three N-terminal amino acids of phospholamban, cardiac SR was treated with Endoproteinase Lys-C prior to affinity-labeling (Fig. 9). Disuccinimidyl suberate plus [^{125}I]calmodulin-affinity-labeling was inhibited at significantly lower concentrations ($IC_{50} = 9.3 \pm 0.4$ μg Endoproteinase Lys-C/ml) than was Bz-Cam-affinity-labeling ($IC_{50} = 56 \pm 35$ μg Endoproteinase Lys-C/ml).

Interaction of PLB(1–25) with spin-labeled calmodulin

The calmodulin/phospholamban complex, the primary product formed by both crosslinking methods employed in this study, suggests that these proteins actually interact with each other. NMR spectroscopic methods were employed to define the specificity of calmodulin binding to phospholamban. Assignment of resonances to specific amino acids are described elsewhere [14]. Titration of PLB(1–25) at low [Ca^{2+}] (< 1 mol Ca/mol calmodulin) with spin-labeled calmodulin resulted in no perturbation of the spectrum (data not shown). However, upon addition of increments of spin-labeled calmodulin to the peptide in the presence of saturating [Ca^{2+}] (> 4 mol Ca/mol calmodulin), specific signals from PLB(1–25) were progressively broadened, indicating that the spin label was perturb-

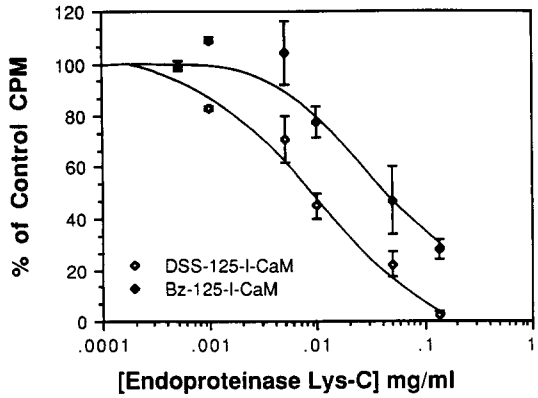

Fig. 9. Effect of limited proteolysis of cardiac SR vesicles with Endoproteinase Lys-C on the Bz-CaM photoaffinity-labeling and DSS crosslinking of calmodulin to phospholamban. Cardiac SR vesicles incubated with various concentrations of Endoproteinase Lys-C as described in Materials and Methods, were subsequently affinity-labeled with either disuccinimidyl suberate plus [^{125}I]calmodulin, or Bz-Cam. Following electrophoretic fractionation of samples on 5–20% polyacrylamide gels, autoradiograms were obtained and the radioactivity in the 40-kDa calmodulin/phospholamban complex was determined as described in Materials and Methods. Data are the mean of two experiments.

ing residues of PLB(1–25) within 1.5 nm of the probe, thus strongly suggesting that a PLB/CaM complex is formed in SR vesicles in the presence of Ca^{2+} (Fig. 10).

Resonances of functional groups within the 1.5 nm sphere of influence of the spin-label on Cys-27 of

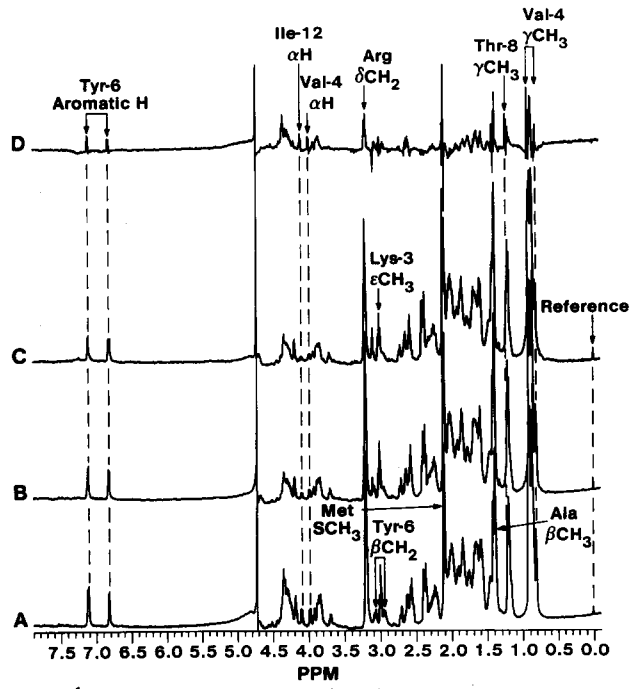

Fig. 10. ^{1}H-NMR spectrum of PLB(1–25) and paramagnetic spectral perturbations induced upon titration with spin-labeled calmodulin. (A) 200 μM PLB(1–25); (B) addition of 14 μM calmodulin spin-labeled at Cys-27; (C) addition of spin-labeled calmodulin to 30 μM; (D) difference spectrum of A − C. Resonance assignments are described by Gao et al. [14].

calmodulin are readily identified by perturbations in the diference spectrum (Fig. 10D). On this basis, several groups are clearly in proximity of the spin label, including Tyr-6, Val-4, Thr-8, and to a lesser extent Ile-12, Ala-15, Lys-3 and Met-1.

Discussion

In this study, limited proteolysis, affinity-labeling, phosphorylation and spectroscopic techniques have been used to localize the calmodulin-binding domain of phospholamban in SR vesicles. Based on studies with the synthetic (PLB(1–25) peptide, proteinases removing short fragments (3 or 6 amino acids) from the N-terminal portion of intact phospholamban caused a loss of both calmodulin affinity-labeling and the endogenous Cam-PK-catalyzed phosphorylation of this protein. In contrast, the cAMP-PK-catalyzed phosphorylation of phospholamban appeared to be insensitive to proteinases which left the substrate Ser-16 in the membrane-bound peptide fragment of this protein. These results suggest that the interaction of calmodulin with phospholamban could reflect a required step in the activation of the endogenous Cam-PK-catalyzed phosphorylation of phospholamban.

To identify the amino-acid residues comprising the calmodulin-binding region of phospholamban, proteinases were used to remove progressively fewer amino acids from the N-terminus of phospholamban. The C-terminal fragment of phospholamban, which is proposed to be membrane-bound [1,2], would be inaccessible to proteinases. Furthermore, the endogenous [Ca^{2+}] in our proteolysis experiment was always 6–8 μM, thus ensuring that PLB and the CaATPase were dissociated as shown by James et al. [31].

Phospholamban is accessible to proteinases in SR vesicles because digestion with trypsin results in the elimination of Bz-Cam affinity-labeling, cAMP-PK-catalyzed phosphorylation and Cam-PK-catalyzed phosphorylation. Furthermore, treatment of cardiac SR vesicles with chymotrypsin, which removes the six N-terminal amino acids of phospholamban (Fig. 1), resulted in the inhibition of both Bz-Cam (Fig. 3) and the disuccinimidyl suberate plus [^{125}I]calmodulin (Fig. 8) labeling of phospholamban, suggesting these six amino acids may comprise an essential portion of the calmodulin-binding region of phospholamban. This was further substantiated using Endoproteinase Lys-C which removes the three N-terminal amino acids of phospholamban, resulting in the inhibition of both Bz-Cam labeling and Cam-PK-catalyzed phosphorylation of phospholamban (Fig. 6), but not the cAMP-PK-catalyzed phosphorylation of phospholamban. That the three N-terminal amino acids were indeed being removed is indicated by the decrease in disuccinimidyl suberate plus [^{125}I]calmodulin-affinity-labeling that oc-

curred as the concentration of Endoproteinase Lys-C was increased (Fig. 9). The loss of the phospholamban Lys-3 amino-acid residue, as determined by the decline in disuccinimidyl suberate-affinity-labeling, correlated with the loss of the endogenous Cam-PK-catalyzed phosphorylation of phospholamban (Fig. 6).

Differences were noted in the yields of the two calmodulin affinity-labeling procedures following Endoproteinase Lys-C digestion of cardiac SR. Crosslinking of Bz-Cam to phospholamban is possible even when the endogenous Cam-PK-catalyzed phosphorylation of phospholamban is totally inhibited (Fig. 6), indicating a weak complex may be formed which is covalently linked by the benzophenone, which attacks methylenes preferentially. In contrast, disuccinimidyl suberate plus [^{125}I]calmodulin-affinity-labeling of phospholamban (Fig. 9) displays the same Endoproteinase Lys-C sensitivity, as does the Cam-PK-catalyzed phosphorylation of phospholamban (Fig. 6). It is likely that the loss of these three amino acids results in the loss of only a portion of the calmodulin binding domain of phospholamban because [^{125}I]calmodulin-affinity-labeling of phospholamban with disuccinimidyl suberate cannot occur when the lysine (that provides the covalent link) is removed by the proteinase treatment. In the case of the Bz-Cam affinity-labeling, however, the loss of these three amino-acid residues still allows Bz-Cam to interact with phospholamban, albeit with a lower affinity.

In all of these proteinase experiments, the loss of endogenous Cam-PK-catalyzed phosphorylation of phospholamban occurred at the same or lower proteinase concentrations than did the calmodulin affinity-labeling of phospholamban. In no case, was affinity labeling more sensitive to proteinase digestion than was the endogenous Cam-PK-catalyzed phosphorylation of phospholamban; i.e., the Cam-PK phosphorylation of phospholamban was always correlated with calmodulin binding to phospholamban.

These studies, which employ chemical crosslinking, demonstrate that in SR vesicles, phospholamban is in proximity (< 0.1 nm) of Cys 27 of calmodulin. Several lines of evidence indicate that calmodulin indeed binds to phospholamban. First, calmodulin crosslinks to purified phospholamban (see footnote on page 250). Second, spectroscopic studies employing a synthetic peptide fragment of phospholamban indicate that signals attributable to residues in the N-terminal portion of PLB(1–25) are specifically perturbed by the paramagnetic spin label on Cys-27 of calmodulin, suggesting specific binding of the peptide to calmodulin (Fig. 10). Third, Chiesi et al. [21] have demonstrated that a synthetic peptide corresponding to the N-terminal 32 residues of phospholamban binds to dansyl-calmodulin with a K_d of 0.66 μM.

If phospholamban binds to calmodulin in SR vesicles, then it is appropriate to ask how the primary structure of phospholamban and the proposed N-terminal calmodulin-binding region deduced from our proteolysis studies, correlates with other known calmodulin target proteins. The interaction of calmodulin with target proteins has been examined using small peptides that have a high affinity for calmodulin [22,23]. Malencik and Anderson [24] have placed many of these high-affinity target peptides into a group that possesses a basic sequence of 2–3 amino acids and three amino acids that space this basic sequence from two bulky hydrophobic residues. Many of these schemes also outline the need for calmodulin-binding proteins to be basic, and have the potential to form an amphiphilic α-helix [25–28] in which a majority of the polar amino-acid residues are on one side and apolar residues are on the opposite side of the α-helix. Most of the peptides studied so far that bind calmodulin with a $K_d < 100$ nM, contain a stretch of at least 12 amino-acid residues which have the potential to form an amphiphilic α-helix [25].

It has been proposed that the cytoplasmic portion of phospholamban could form an α-helix [2]. From the method of Chou and Fasman [29], it is clear that the first nine residues of phospholamban would be predicted to form an α-helix of a similar amphiphilic nature to the high-affinity calmodulin target peptides, including melittin and mastoparan [25]. However, phospholamban differs from the previously examined high-affinity calmodulin-binding peptides that have a basic tripeptide sequence followed after three amino acids by a pair of hydrophobic amino acids [24]. A phospholamban sequence that approximates this pattern is formed by residues 3–7, consisting of a basic amino acid, separated by two amino acids from two hydrophobic residues (Lys-Val-Gln-Tyr-Leu). Alternatively, for phospholamban to generate a single calmodulin binding site, it is possible that basic and hydrophobic residues are derived from two or more of the five phospholamban subunits.

We conclude that residues 1–6 of PLB comprise an essential portion of the calmodulin binding domain of phospholamban. The decrease in calmodulin-dependent phosphorylation of phospholamban correlates with the decrease in affinity labeling of phospholamban by calmodulin, which suggests that the formation of a calmodulin-phospholamban complex could be a necessary step in the activation of the endogenous SR Cam-PK.

Acknowledgements

We are grateful to Mr. Joseph Leykam of the Michigan State University Macromolecular Structure Facility for his assistance in peptide synthesis and for use of his HPLC. We would also like to thank Dr. Barry Levine,

University of Oxford, for the NMR studies on phospholamban and for invaluable comments. This research was supported by National Institutes of Health Grant HL 26880 (CFL), by Michigan State University Research Excellence Funds and by the Michigan Agricultural Experiment Station.

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