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Cardiac Sarcalumenin: Phosphorylation, Comparison with the Skeletal Muscle Sarcalumenin and Modulation of Ryanodine Receptor

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Abstract. Cardiac sarcoplasmic reticulum (SR) contains an endogenous phosphorylation system that under specific conditions phosphorylates two proteins with apparent molecular masses of 150 and 130 kDa. The conditions for their phosphorylation are as for the skeletal muscle sarcalumenin and the histidine-rich Ca^{2+} binding protein (HCP) with respect to: (i) Ca^{2+} and high concentrations of NaF are required; (ii) phosphorylation is obtained with no added Mg^{2+} and shows a similar time course and ATP concentration dependence; (iii) inhibition by similar concentrations of La^{3+}; (iv) phosphorylation is obtained with [γ-^{32}P]GTP; (v) ryanodine binding is inhibited parallel to the phosphorylation of the two proteins. The endogenous kinase is identified as casein kinase II (CK II) based on its ability to use GTP as effectively as ATP, and its inhibition by La^{3+}. The association of CK II with the cardiac SR, even after EGTA extraction at alkaline pH, is demonstrated using antibodies against CK II. The cardiac 130 kDa protein is identified as sarcalumenin based on its partial amino acid sequence and its blue staining with Stains-All. Cardiac sarcalumenin is different from the skeletal muscle protein based on electrophoretic mobilities, immunological analysis, peptide and phosphopeptide maps, as well as amino acid sequencing. Preincubation of SR with NaF and ATP, but not with NaF and AMP-PNP caused strong inhibition of ryanodine binding. This is due to decrease in Ca^{2+}- and ryanodine-binding affinities of the ryanodine receptor (RyR) by about 6.6 and 18-fold, respectively.

These results suggest that cardiac sarcalumenin is an isoform of the skeletal muscle protein. An endogenous CK II can phosphorylate sarcalumenin, and in parallel to its phosphorylation the properties of the ryanodine receptor are modified.

Key words: SR — Ryanodine receptor — Sarcalumenin — Phosphorylation — Cardiac muscle

Introduction

In both cardiac and skeletal muscle, the function of the sarcoplasmic reticulum (SR) is the regulation of intracellular Ca^{2+} concentration [17]. Comparisons between the SR from cardiac and skeletal muscle indicate several similarities and differences in their structure, protein composition and regulation. Both membranes are enriched with the Ca^{2+}-pump, the Ca^{2+}-binding protein, calsequestrin, and the ryanodine receptor (RyR) [30, 35, 43]. The Ca^{2+}-pump [3], calsequestrin [7, 20, 40], and RyR [25, 38, 53] from cardiac and skeletal muscle SR have been found to be the products of different genes. RyR from skeletal muscle (RyR-1) and cardiac muscle (RyR-2) show many similarities [25] such as nearly, identical Ca^{2+} conductance [19], morphological structure [42, 43, 53], and modulation by effector molecules such as FK binding protein [2, 50, 51], Ca^{2+}, Mg^{2+}, ATP and calmodulin [13, 35, 46]. The differences, however, are not only in their primary amino acid sequences [25, 53],

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Abbreviations: EGTA, ethylene glycol bis [β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid; Tricine, N-[2-hydroxy-1, 1-bis (hydroxymethy)]-ethyl]-glycine; Mops, 3-(N-morpholino) propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; HSR, heavy SR; CHAPS, 3-[(3-cholamidopropyl)dimentyl-ammonio]-1-propane-sulfonate; PMSF, phenylmethylsulfonyl fluoride; CS, calsequestin; RyR, ryanodine receptor; ATPase, Ca^{2+}-ATPase; Sarca, sarcalumenin, HCP, histidine-rich Ca^{2+} binding protein; CK II, casein kinase II.

but also in their sensitivity to various ligands, such as Ca^{2+} [11, 35], Mg^{2+} [35], and dantrolene [21], and in their differential phosphorylation by protein kinases [15, 23, 47, 57].

Calsequestrin as well as sarcalumenin and the histidine-rich Ca^{2+} binding protein (HCP) were shown to stain selectively blue with the carbocyanine cationic dye, Stains-All [41]. Phosphorylation of skeletal muscle calsequestrin has been reported [48, 54, 55], as well as the phosphorylation of cardiac and skeletal muscle calsequestrin isoforms by casein kinase II [4]. The function these luminal proteins from cardiac and skeletal muscle SR have different mobility in SDS-polyacrylamide gel electrophoresis [41]. It has been suggested that the function of calsequestrin is the binding of intraluminal Ca^{2+} [34] and the modulation of RyR activity [12, 27]. Recently, calsequestrin was shown to regulate selectively the RyR activity: in presence of 1 mM luminal Ca^{2+}, *only* the dephosphorylated calsequestrin induced Ca^{2+} release from SR vesicles, whereas the phosphorylated one had no effect [48]. The function of the other luminally located Ca^{2+} binding proteins HCP and sarcalumenin is as yet unknown.

In our previous studies [22, 36] we have characterized an endogenous, Ca^{2+}-dependent phosphorylation system which phosphorylates two SR polypeptides migrating on SDS-PAGE with an apparent molecular mass of 150 kDa and 160 kDa. Recently [37, 45], we have identified these proteins as sarcalumenin and HCP, and also found that their phosphorylation resulted in inhibition of ryanodine binding due to alteration in the Ca^{2+}- and ryanodine-binding affinities of the RyR. It has been suggested that ATP transport into the SR lumen, necessary for the phosphorylation of the luminally located proteins, occurs through the voltage dependent anion channel/VDAC located to SR membranes [44].

In this study we have characterized a luminally located phosphorylation system in cardiac SR, and we have elucidated the differences between the cardiac and the skeletal muscle sarcalumenin.

Materials and Methods

Materials

ATP, EGTA, $LaCl_3$, Tris, Mops, GTP, NaF and AMP-PNP, CHAPS, spermine, spermine-agarose, and acid phosphatase from potato type III were obtained from Sigma Chemical [^{3}H]ryanodine was purchased from New England Nuclear, and unlabelled ryanodine was obtained from Calbiochem. [γ-^{32}P]ATP and [γ-^{32}P]GTP were obtained from Amersham. Sephadex G-50 (fine) was obtained from Pharmacia. Casein kinase II was purchased from Boehringer.

Membrane Preparations

SR membranes were prepared from rabbit fast twitch skeletal muscle as described by Saito et al. [39], and cardiac SR membranes were isolated from dog heart [9]. In these SR preparations, the protease inhibitors; PMSF (0.2 mM), benzamidine (0.8 mM), and leupeptin (0.5 μg/ml) were included in all solutions. The membranes were suspended in a final concentration of about 25 mg protein/ml in a buffer containing 0.2 M sucrose, 10 mM Tricine, pH 8.0, and 1 mM histidine, and stored at −70°C. Protein concentration was determined by the method of Lowry et al. [33].

Protein Phosphorylation

Phosphorylation of SR membrane (1 mg/ml) was performed at 30°C in 50 μl of a solution containing 20 mM Tricine, pH 7.2, 100 mM NaCl, 200 or 400 μM ATP or [γ-^{32}P]ATP (0.5 to 1.0 μCi/nmol), 40–60 mM NaF, and other reagents, as indicated in the legends of the tables and figures. After incubation (0.5 to 4 min), the reaction was terminated by dilution with 1/3 volume of buffer containing 260 mM Tris, pH 6.8, 40% (vol/vol) glycerol, 8% (mass/vol) SDS, 4% (vol/vol) β-mercaptoethanol, and then immediately incubated for 3 min at 100°C.

[^{3}H]Ryanodine Binding

Unless otherwise indicated, phosphorylated or nonphosphorylated SR membranes (final concentration of 0.5 mg/ml) were incubated with 20 nM [^{3}H]ryanodine in a standard binding solution containing 50 μM $CaCl_2$, 1 M NaCl, and 20 mM Mops, pH 7.4, and assayed for 20 min at 37°C (because of possible dephosphorylation). The unbound ryanodine was separated from the protein-bound ryanodine by filtration of protein aliquots (50 μg) through Whatman GF/C filters, followed by washing three times with 5 ml of ice-cold buffer containing 0.2 M NaCl, 5 mM Mops, pH 7.4, and 50 μM $CaCl_2$. The filters were dried, and the retained radioactivity was determined by liquid scintillation counting technique. Nonspecific binding was determined in the presence of a 1,250-fold excess of unlabelled ryanodine.

Gel Electrophoresis and Autoradiography

Analysis of the protein profile and of the phosphoproteins by SDS-polyacrylamide slab gel electrophoresis (PAGE) was performed using the discontinuous buffer system of Laemmli [29] in 1.5 mm thick slab gels with 6% or 3–13% acrylamide, using 3% stacking gel. Gels were stained with Coomassie Brilliant Blue. Molecular weight standards were: myosin heavy chain 200,000: β-galactosidase, 116,000; phosphorylase b, 97,400; bovine serum albumin, 66,200; and ovalbumin, 42,700 (BioRad). Autoradiography of dried gels was carried out using Kodak X-omat film. Quantitative analysis of the protein bands and of the phosphorylated protein bands was carried out by densitometric scanning of the gels or autoradiograms, using a Molecular Dynamics computing densitometer. Analysis was performed using Image Quant software provided by the manufacturer. Also, the labeled bands were cut from the gels and counted in a liquid scintillation counter.

Immunoblotting

Western blot analysis was carried out as described previously [52]. The separated proteins from SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk and 0.1% Tween-20 in Tris buffered saline, incubated with the antibodies (1:1000), then with alkaline-phosphatase conjugated to anti-mouse IgG antibodies or to protein A. The color was then developed (5 min) with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

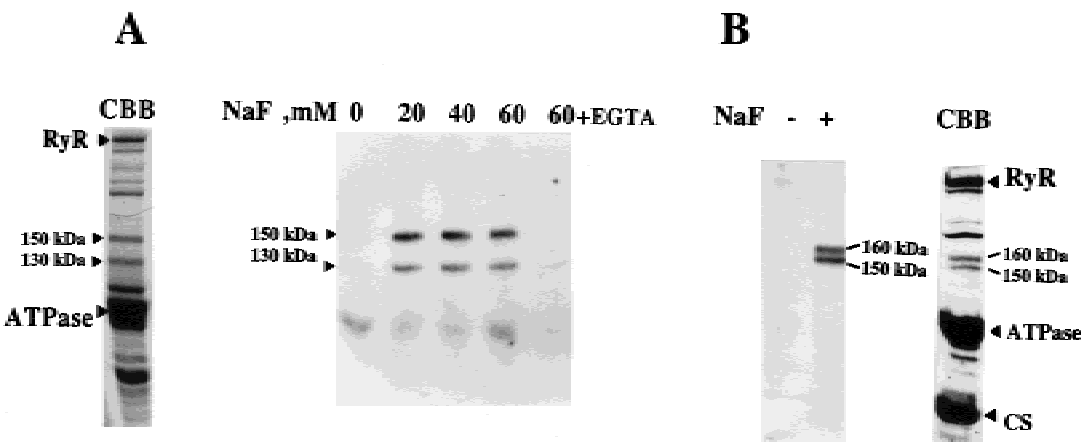

Fig. 1. Ca^{2+}- and NaF-dependent phosphorylation of the cardiac 130/150 kDa polypeptides. Cardiac (*A*) or skeletal (*B*) muscle SR (1 mg/ml) were phosphorylated for 2 min with 200 μM [γ-^{32}P]ATP at pH 7.2, in the absence or in the presence of the indicated concentration of NaF or 0.5 mM EGTA, and 50 μg of protein was subjected to SDS-PAGE (7% acrylamide) and to autoradiography, as described in Materials and Methods. RyR; ryanodine receptor; ATPase, (Ca^{2+} + Mg^{2+})ATPase; CS, calsequestrin.

Partial Purification of 130 kDa (Sarcalumenin) and the 105 kDa Proteins

The proteins were partially purified on spermine-agarose column. Cardiac SR membranes (40 mg) were sedimented at 100,000 × *g* for 30 min and resuspended at a final protein concentration of 5 mg/ml in a solution containing 10 mM Tris/HCl, pH 8.5, 0.1 mM PMSF, 0.5 μg/ml leupeptin, and 1 mM EGTA. The vesicles were gently stirred at 4°C for 30 min, and then centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant (EGTA extract) was collected, and the pellet was resuspended in the above EGTA solution, and the extraction was repeated. The two EGTA-extracts were combined, and NaCl and $CaCl_2$ (from 4 M and 0.1 M stock solutions, respectively) were added to the final concentration of 0.12 M and 0.8 mM, respectively. This extract was loaded onto a small spermine-agarose column (0.8/5.7 cm) pre-equilibrated with 10 mM Tris/HCl pH 8.5, 0.12 M NaCl, and 0.1 mM PMSF (buffer A). The column was washed with 10 ml cold (~8°C) buffer A, which elutes different proteins. Sarcalumenin (130 kDa protein) was eluted (with 105 kDa protein), with buffer A (10 ml) containing 5 mM of spermine. The 150 kDa protein and calsequestrin were eluted from the column by 0.5 M NaCl. The rate of loading and washing was about 1 ml/3 min. Fractions (0.6 ml) were collected and 40 μl samples were subjected to SDS-PAGE followed by Coomassie staining or electroblotting [52]. The purified proteins were stored at −20°C.

Internal Amino Acid Sequence Analysis

This was carried out essentially as described previously [1]. Briefly, partially purified cardiac sarcalumenin was separated by SDS-PAGE and the separated polypeptides were electroblotted in a Hoefer transblot system onto PVDF membranes using 50 mM Tris/borate buffer. After transfer, the protein bands were stained with Ponceau S, cut out and transferred to Eppendorf tube. The four cut bands were treated with polyvinylpyrrolidone (PVP-40) to prevent protein absorption to the PVDF membrane, and subjected to *in situ* cleavage of the electroblotted protein by specific grade of trypsin (Promega) as described previously [1]. The tryptic fragments were separated on a C_{18} reverse-phase column and eluted with a linear gradient of 0–84% acetonitrile in 0.1% trifluoroacetic acid at 80 μl/min. Several HPLC-separated peptides were subjected to amino acid sequence using amino acid sequencer (Applied Biosystem, model 476).

Results

The phosphorylation of canine cardiac SR incubated with [γ-^{32}P]ATP in the absence and or presence of NaF and in the absence of exogenously added divalent cations is shown in Fig. 1. Among the several polypeptides stained by Coomassie Blue, the endogenous kinase catalyzes the incorporation of the [γ-^{32}P] of ATP into three polypeptides with apparent molecular masses of 150 kDa, 130 kDa and 105 kDa (Fig. 1*A*). The phosphorylation of the 150 kDa and 130 kDa proteins, but not of the 105 kDa protein, requires the presence of the phosphoprotein phosphatase inhibitor NaF. Addition of EGTA completely prevents the phosphorylation, indicating an absolute requirement for Ca^{2+} (Fig. 1*A*). In some SR preparations, the phosphorylation of 260 and 105 kDa proteins (Figs. 2 and 4), and of a 32 kDa protein (*not shown*) has been observed, but their phosphorylation can also be obtained in absence of NaF. These results are identical to those obtained with the endogenous phosphorylation of skeletal muscle SR proteins; the 150 and 160 kDa (Ref. 36 and Fig. 1*B*), which have been identified as sarcalumenin and the histidine-rich Ca^{2+} binding protein (HCP), respectively [45].

Identification of the Endogenous Kinase

The phosphorylation of the cardiac and skeletal muscle SR proteins shows similar ATP concentration dependence (Fig. 2), similar time course and inhibition pattern by La^{3+} (Fig. 3). The results (Figs. 1 to 3) suggest that

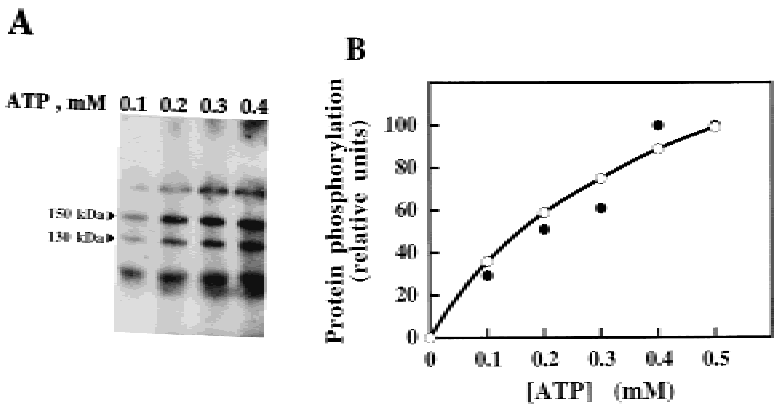

Fig. 2. Phosphorylation of cardiac and skeletal muscle proteins as a function of ATP concentration. Cardiac or skeletal muscle SR (1 mg/ml) were incubated for 2 min at 30°C in 20 mM Tricine, pH 7.2, 100 mM NaCl and 60 mM NaF with varying concentrations of [γ-^{32}P]ATP (1 μCi/nmol) and 50 μg of protein was subjected to SDS-PAGE (7% acrylamide), Coomassie staining, autoradiography and quantitative analysis, as described in Materials and Methods. In *A*, the autoradiogram of the SDS-PAGE of the phosphorylated cardiac SR is shown, and in *B*, the quantitation of the phosphorylated cardiac 130 kDa protein (●) and skeletal muscle sarcalumenin (○) are presented as a function of ATP concentration.

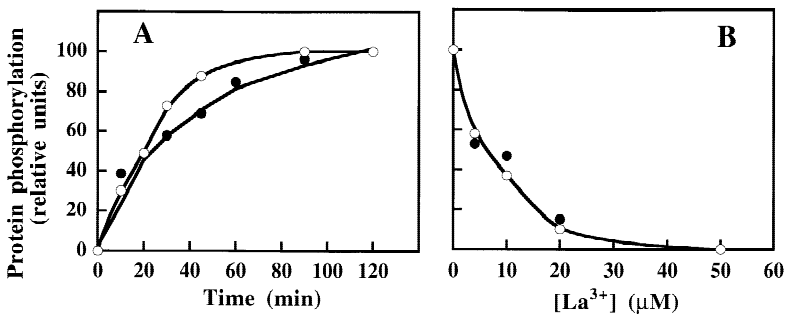

Fig. 3. Time course and inhibition by La^{3+} of the endogenous phosphorylation of cardiac and skeletal muscle proteins. In *A*, cardiac (●) and skeletal (○) muscle SR membranes were phosphorylated for the indicated time (*A*) with 0.4 mM [γ-^{32}P]ATP or in (*B*) for 2 min, and in the presence of the indicated La^{3+} concentrations, and then subjected to SDS-PAGE, autoradiography and quantitative analysis, as described in Materials and Methods. Phosphorylation is expressed relative to the maximal phosphorylation of sarcalumenin (skeletal SR) or 130 kDa protein (cardiac SR).

most likely the same kinase type is responsible for the phosphorylation of both cardiac and skeletal muscle SR proteins. To test whether the kinase is a casein kinase II type, as in the skeletal muscle SR, the phosphorylation of the cardiac SR has been carried out with [γ-^{32}P]GTP. A unique feature of casein kinase II is its ability to use GTP as phosphate donor almost as effectively as ATP [24]. Figure 4*A* shows that only in presence of NaF the endogenous kinase phosphorylates the 130 kDa and 150 kDa proteins using [γ-^{32}P]GTP. As with [γ-^{32}P]ATP (Fig. 2), 260 kDa and 105 kDa proteins can also be phosphorylated in absence of NaF. These results suggest that the endogenous kinase responsible for the phosphorylation of 150/130 kDa proteins belongs to the casein kinase II type. The presence of CK II in different cardiac SR preparations is demonstrated in Fig. 4*B* by using antibodies against CK II (kindly provided by Dr. Issinger, Hamburg). CK II remains associated with the SR membranes following EGTA treatment that releases luminal and peripheral proteins. CK II, however, can be released from the EGTA-treated SR membranes with 0.3 M NaCl (*data not shown*). Similar results have been obtained with the skeletal muscle protein [45].

Partial Purification of 150 and 130 kDa Proteins and their Phosphorylation with CK II

To test whether the isolated 130 kDa protein can be phosphorylated by CK II, we have partially purified the protein from the EGTA extract of the cardiac SR using a new method (*see* Materials and Methods). The 150 kDa, 130 kDa, and 105 kDa proteins, and calsequestrin have been isolated from the EGTA extract of cardiac SR (Fig. 5). On the spermine-agarose column, under the ionic strength and pH used (0.12 M NaCl, pH 8.5), several unbound proteins can effectively be washed out with 0.12 M NaCl. Addition of 5 mM spermine to the washing buffer elutes two major proteins with apparent molecular weights of about 130,000 and 105,000. Increasing the NaCl concentration to 0.5 M elutes the 150 kDa protein and calsequestrin. The partially purified 150, 130 kDa proteins and calsequestrin were stained blue with Stains-All (Fig. 7).

We have examined the phosphorylation of the partially purified 130 kDa protein using exogeneously added casein kinase II (Fig. 6). Casein kinase II stimulates phosphorylation of the 130 kDa and 105 kDa proteins, and this phosphorylation is inhibited by La^{3+}. Half-maximal inhibition is obtained at about 4 μM for both 130 kDa and 105 kDa proteins (Fig. 6*A*), similar to that obtained for the membrane-associated 130 kDa protein (Fig. 3). The same phosphorylation of the partially purified sarcalumenin was obtained by CK II in the absence and the presence of EGTA (*data not shown*).

The effect of acid phosphatase on the phosphorylated purified proteins is demonstrated in Fig. 6*B*. Dephosphorylation of the proteins phosphorylated by CK II is

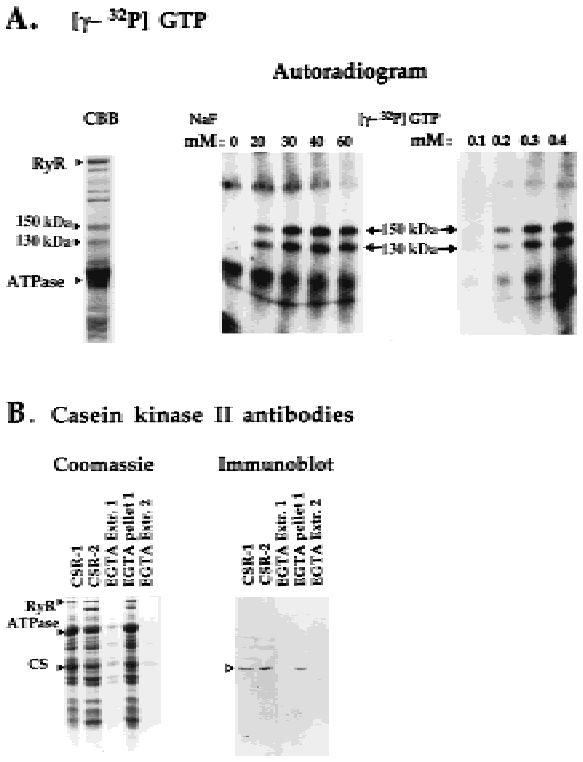

Fig. 4. Endogenous phosphorylation of 130/150 kDa proteins is catalyzed by casein kinase II. In *A*, cardiac SR membranes were phosphorylated with 0.2 mM [γ-^{32}P]GTP in the presence of the indicated NaF concentrations, or were phosphorylated with the indicated concentration of [γ-^{32}P]GTP in the presence of 60 mM NaF, as described in Fig. 1. In *B*, two different cardiac SR preparations (CSR-1, and CSR-2), their EGTA-extracts (an amount equivalent to the pellet from which they were separated) and the corresponding pellet were analyzed by SDS-PAGE (8–20% acrylamide), and either stained with Coomassie Blue or electroblotted and immunostained with polyclonal antibodies against CK II. The EGTA extraction of the SR was carried out as described in Materials and Methods.

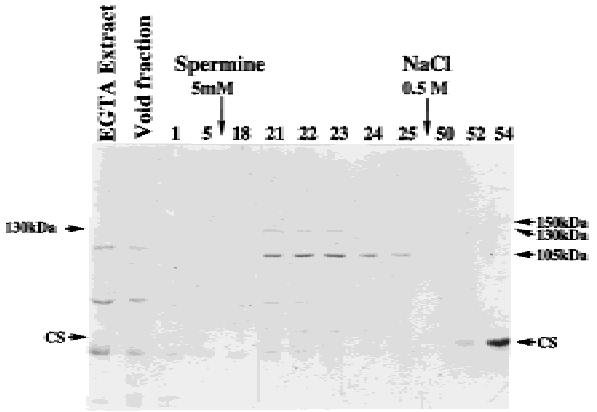

Fig. 5. Purification of 150 kDA, 130 kDa and the 105 kDa proteins, using a spermine-agarose column. EGTA extract of cardiac SR was obtained, and 130 kDa (sarcalumenin), 150 kDa and 105 kDa proteins were purified as described in Materials and Methods. EGTA extract (lane 1), the void fraction of the spermine-agarose column (lane 2), the washed fractions (fractions 1, 5), and the proteins eluted with 5 mM spermine; (fractions 18, 21–25) or 0.5 M NaCl (fractions 50, 52, 54) were subjected to SDS-PAGE (7% acrylamide).

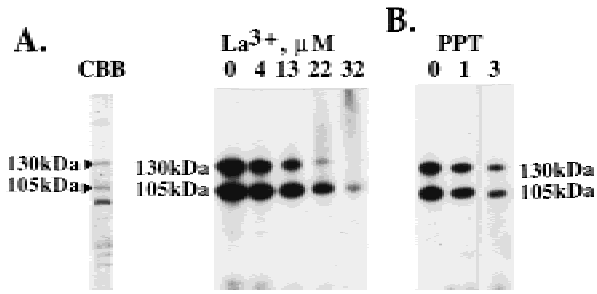

Fig. 6. Phosphorylation of 130 kDa and 105 kDa proteins by casein kinase II; inhibition by La^{3+} and dephosphorylation by phosphoprotein phosphatase. In *A*, 130 and 105 kDa proteins enriched fraction was phosphorylated for 15 min with CKII (0.05 unit) in the absence and in the presence of the indicated concentrations of $LaCl_3$. The reaction mixture contained 2.5 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP (0.4 μCi/nmol), 10 mM Tris/HCl, pH 7.1, 32 mM NaCl, and 1.2 mM DTT. The reaction was initited by the addition of the kinase and was terminated after 15 min of incubation at 37°C by the addition of Laemmli sample buffer. The samples (10 μg protein) were subjected to SDS-PAGE (7% acrylamide) and autoradiography, as described in Materials and Methods. In (*B*) the phosphorylation with CK II, was carried out for 10 min, then 1 or 3 μl of the potato acid phosphatase (PTT) were added and 10 min later the reaction was terminated as above.

obtained by this nonspecific phosphatase. As expected, this phosphatase has no effect on the membrane-associated proteins because of their luminal location (*data not shown*).

The 130 kDa Protein Cardiac Sarcalumenin is Distinct from the Skeletal Muscle Protein

Figure 7 shows the protein profile of skeletal and cardiac muscle SR as revealed by SDS-PAGE and staining with Stains-All. The carbocyanine cationic dye is known to stain Ca^{2+} binding and acidic proteins in blue, while staining the other proteins in pink [7, 41]. In both cardiac and skeletal muscle SR, other proteins in addition to calsequestrin were also stained blue. In the cardiac SR these proteins run with an apparent Mr different from those of the skeletal muscle, and similar to that of the phosphorylated proteins (Fig. 1). The purified skeletal muscle sarcalumenin and 130 kDa protein are also shown.

The following experiments identified the 130 kDa protein band as sarcalumenin: The different mobility of the cardiac sarcalumenin suggests that it is different from the skeletal muscle protein. To support this suggestion

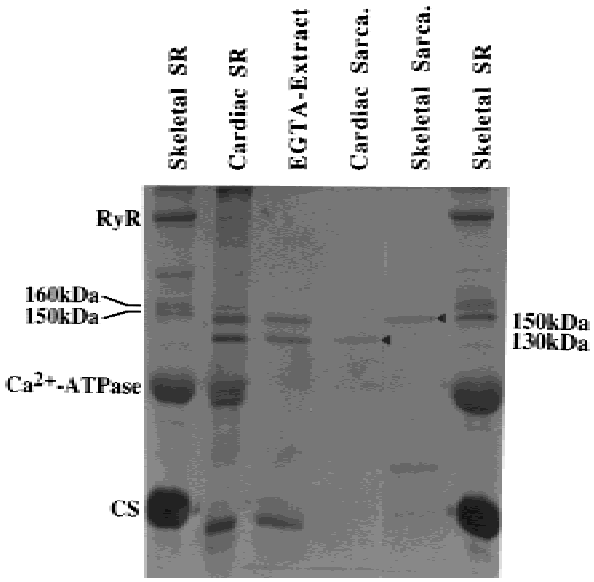

Fig. 7. Stains-All staining of cardiac and skeletal muscle SR and purified sarcalumenin. Cardiac and skeletal muscle SR membranes (50 μg), EGTA extract and purified cardiac and skeletal muscle 150 (sarcalumenin) and 130 kDa proteins were obtained and subjected to SDS-PAGE (7% acrylamide), as described in Materials and Methods. The 160, 150 and 130 kDa proteins as well as CS were stained blue. All other proteins were stained pink. Staining with Stains-All was carried out as described previously [28].

we have carried out several experiments including the use of specific antibodies prepared against the skeletal muscle sarcalumenin, peptide- and phosphopeptid-maps, and protein sequencing of the cardiac protein. We have examined the cross-reactivity of three different monoclonal antibodies against the skeletal muscle sarcalumenin with the cardiac sarcalumenin (Fig. 8). Cross-reactivity was observed with the monoclonal antibody XII C_4, but not with the monoclonal antibodies G_{10} and G_7. These results demonstrate the presence of both common and different epitopes in the two proteins.

Figure 9 shows the peptide and phosphopeptide maps of cardiac and skeletal muscle sarcalumenin. Cleveland peptide maps [10] of endogenously phosphorylated skeletal muscle sarcalumenin and cardiac 130 kDa protein were obtained by digesting the corresponding phosphorylated protein bands with *S. aureus* V_8 proteinase following a preparative SDS-PAGE. The results in Fig. 9 demonstrate that the peptide and phosphopeptide profiles of the two proteins are different. Several peptides (20, 24, 22 and 16 kDa) and phosphopeptides (34, 30 and 18 kDa) are common to the proteins isolated from cardiac and skeletal muscle SR. However, the peptide 48 kDa and the phosphopeptide 48 and 38 kDa are found only in the skeletal muscle protein, while the peptide 40 kDa and phosphopeptide 56 and 21 kDa are found only in the cardiac protein (Fig. 9).

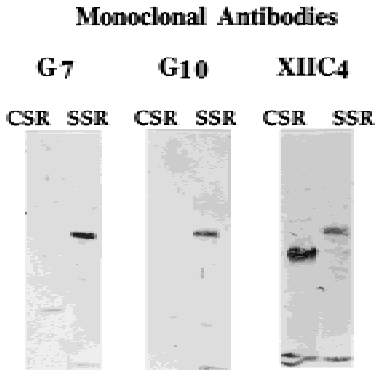

Fig. 8. Cross-reactivity of cardiac sarcalumenin with monoclonal antibodies against the skeletal muscle sarcalumenin. Fifty microgram of cardiac SR (CSR) or skeletal muscle SR (SSR) were subjected to SDS-PAGE (6%), and then the gel was transferred onto nitrocellulose membrane and immunostained with monoclonal antibodies G_7, G_{10} (provided by D.H. MacLennan) or XII C_4 (provided by K.P. Campbell) against skeletal muscle sarcalumenin (1:1000. Alkaline phosphatase conjugated to anti-mouse IgG antibodies was used as secondary antibodies. SDS-PAGE and immunoblot staining were carried out as described in Materials and Methods.

Another approach for testing differences in the primary amino acid sequence of the cardiac protein is to carry out partial amino acid sequencing. The partially purified cardiac sarcalumenin (130 kDa protein) has been separated on SDS-PAGE, electroblotted onto PVDF membranes and the blots were subjected to *in situ* trypsin digestion [1], and the resulting peptides were separated by reverse phase HPLC. Selected peptides have been sequenced, and the obtained sequences are presented in Table 1 together with the corresponding sequences of the skeletal muscle sarcalumenin [31]. Seven of the eight peptide sequences from the cardiac sarcalumenin were identical to sequences from the skeletal muscle sarcalumenin, and in one peptide asparagin and tyrosine in skeletal muscle sarcalumenin are replaced in cardiac sarcalumenin with serine and threonine, respectively.

Effect of Phosphorylation of 150/130 kDa Proteins on RyR Activity

To test whether phosphorylation of the cardiac 150/130 kDa proteins modulate the properties of the RyR like in the skeletal muscle system [37, 45], we have examined the effects of preincubation of cardiac SR membranes with ATP + NaF on the ryanodine binding. Table 2 shows the effect of preincubation of cardiac and skeletal SR membranes with ATP or AMP-PNP on their ryanodine binding activity both in absence and presence of NaF. Preincubation of the membranes with either compound in absence of NaF had no significant effect on ryanodine binding. However, preincubation with ATP, *but not* with AMP-PNP, in the presence of NaF results in inhibition of ryanodine binding. Since the nonhydrolyz-

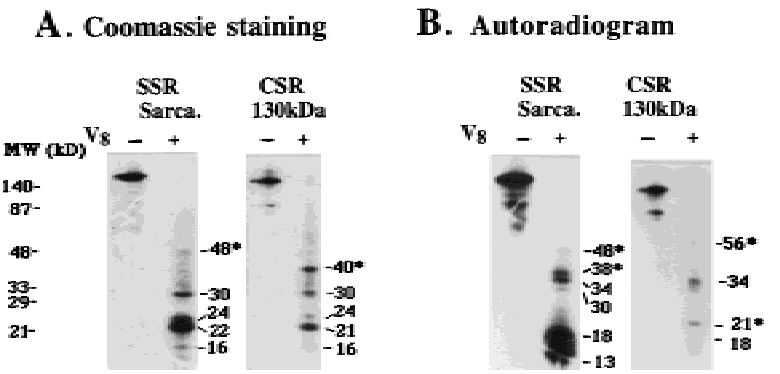

Fig. 9. Comparison of peptides map of phosphorylated cardiac and skeletal muscle sarcalumenin. Cardiac and skeletal muscle SR membranes were endogenously phosphorylated with 0.2 mM [γ-^{32}P]ATP as in Fig. 1 and then subjected to SDS-PAGE. The gel was stained with Coomassie Blue and destained briefly. Bands of cardiac and skeletal muscle SR 130 and 150 kDa (sarcalumenin), respectively, were cut out from the gel, rinsed in H_2O and then with solution containing 0.125 M Tris/HCl, pH 6.8, 0.1% SDS and 1 mM EDTA for 30 min. Proteolysis was carried out as described previously [24]. Briefly, four slices of each protein band were applied to each gel wall (7.5–20% of acrylamide) and exposed to the indicated amount of the protease V_8 in the above solution containing 20% glycerol. Electrophoresis was performed as described by Cleveland et al. [24]. in *A* the Coomassie stained gel is shown, and in *B* its autoradiogram. Sarca indicate sarcalumenin.

Table 1. Amino acid sequences of peptides derived from 130kDa protein (sarcalumenin) and comparison with sequences from skeletal muscle sarcalumenin

Sarcalumenin from:	Peptide sequence
1. Skeletal muscle	62 D E **N** L L L H **Y** P D G R 73
Cardiac muscle	**T** L L L H **S** P D G R
2. Skeletal muscle	355 Q D E G G E A S S 363
Cardiac muscle	Q D E G G E A S S
3. Skeletal muscle	476 T L M L N E D K P T D D F S A V L Q R 494
Cardiac muscle	T L M L N E D K P T D D F S A V L Q R
4. Skeletal muscle	498 I Y H S S I K P L E Q S Y K 511
Cardiac muscle	I Y (H) S S I K P L E Q S (YK)[a]
5. Skeletal muscle	580 T I E G I V M A A D S A R S F S P L E K 599
Cardiac muscle	T I E G I V M A A D S A R S F S P L E K
6. Skeletal muscle	621 V T F V D T P G I I E N R 633
Cardiac muscle	V T F V D T P G I I E N R
7. Skeletal muscle	723 V Y V S S F W P Q E Y K P D T H R 739
Cardiac muscle	V Y V S S F W P Q E Y K P D T H R
8. Skeletal muscle	805 D I V E D P D K F Y I F K 817
Cardiac muscle	D I V E D P D K F Y I F K

Cardiac sarcalumenin was partially purified, subjected to SDS-PAGE, electroblotted and trypsin digested. HPLC separation and sequencing was performed as described in Materials and Methods. The obtained sequences are presented with sequence for skeletal muscle sarcalumenin, deduced from the nucleotide sequence of the cloned protein [31]. Residues different in both proteins (in peptide No 1) are underlined. (a) Indicates Laser desorption mass spectrometry analysis (MALDI), carried out on the HPLC fraction containing peptide number 4, resulted in a mass of 1696 Da. The Edmann degradation allowed us to read sequence between 498 and 506. The calculated average molecular mass of the peptide 498 to 511 (according to the skeletal muscle sequence) fits the MALDI result of 1696 Da. Therefore, we completed the sequence obtained upon Edman degradation with amino acids EQSYK.

able ATP analogue AMP-PNP does not replace ATP, and the presence of NaF as phosphatase inhibitor is required, it suggests that protein phosphorylation could be involved in the inhibition of ryanodine binding. As shown in Figs. 1*A* and 4*A*, only the phosphorylation of the 150 and 130 kDa is dependent on the presence of NaF. As in the skeletal muscle SR [37], the phosphoprotein phosphatase inhibitor, okadaic acid, when present during the preincubation of the SR with ATP, can not replace NaF either in the inhibitory effect on ryanodine

Table 2. Preincubation with ATP and NaF but not AMP-PNP and NaF inhibits ryanodine binding to cardiac and skeletal SR

Preincubation conditions	Ryanodine bound, pmol/mg	
	Cardiac SR	Skeletal SR
Control	8.3	10.7
AMP-PNP (0.4 mM)	8.8	12.3
AMP-PNP + NaF (20 mM)	8.9	11
AMP-PNP + NaF (50 mM)	8.9 (100%)	11.3 (100%)
ATP (0.4 mM)	9.2	11.3
ATP + NaF (20 mM)	8.0	10.3
ATP + NaF (50 mM)	1.6 (18%)	3.8 (33%)

Cardiac or skeletal muscle SR membranes (1 mg/ml) were preincubated with buffer alone (control) or with buffer and the indicated compounds at 30°C in a solution containing 20 mM Tricine, pH 7.2 and 100 mM NaCl. After 2 min, samples were diluted (1:1) with two-times concentrated reaction mixture for ryanodine binding to final concentrations 20 nM ryanodine 1M NaCl and 50 μM $CaCl_2$, and after 20 min at 37°C assayed for [^{3}H]ryanodine bound, as described in Materials and Methods. The numbers in parenthesis indicate % of control.

binding or in the phosphorylation of the cardiac 150/130 kDa proteins (*data not shown*).

The effect of phosphorylation of the cardiac 150/130 kDa proteins on the ryanodine- and Ca^{2+}-binding affinities are demonstrated in the following experiments. The binding of [^{3}H]ryanodine to membranes incubated with NaF + AMP-PNP (control) or with NaF + ATP (phosphorylated) as a function of ryanodine concentration is shown in Fig. 10. The results indicate that treatment of SR membranes with ATP + NaF, which leads to phosphorylation of 130/150 kDa proteins, alters the ryanodine binding affinity of the RyR. The apparent binding affinity is decreased K_D increased) in the phosphorylated membranes by about 18-fold. The inhibition of ryanodine binding by this treatment decreases from about 88 to 35% with increasing ryanodine concentration from 5 to 60 nM. The results suggest that phosphorylation of 150/130 kDa proteins causes a change in ryanodine binding affinity.

The effect of phosphorylation of 150/130 kDa proteins on the Ca^{2+} activation of ryanodine binding is shown in Fig. 11. Under the conditions used (1.0 M NaCl and pH 7.4), the Ca^{2+} dependence of the phosphorylated (incubated with ATP + NaF) and unphosphorylated (incubated with AMP-PNP + NaF) membranes is different. Half-maximal stimulation (C_{50}) of ryanodine binding was obtained at about 0.24 μM of Ca^{2+} with the control, and at about 1.6 μM of Ca^{2+} with the phosphorylated membranes.

Discussion

The results presented here demonstrate that cardiac SR membranes contains an endogenous phosphorylation system that phosphorylates two proteins with apparent molecular masses of 150 and 130 kDa in a NaF-dependent manner. This phosphorylation system is similar to that of the skeletal muscle sarcalumenin and the histidine-rich Ca^{2+} binding protein (HCP) [36, 37, 45] with respect to: (i) requirement for Ca^{2+} and NaF (Fig. 1); (ii) phosphorylation with similar ATP concentration dependency, time course and (Figs. 2 and 3); (iii) similar La^{3+} inhibition pattern (Fig. 3); (iv) phosphorylation by GTP as a substrate (Fig. 4); and (v) inhibition of ryanodine binding following phosphorylation (Table 2 and Figs. 10 and 11). These observations suggest that the cardiac and skeletal muscle proteins are phosphorylated by the same protein kinase type, and their phosphorylation modifies the Ca^{2+} and ryanodine binding affinities of the RyR.

The kinase responsible for the phosphorylation of 130 kDa (sarcalumenin) and 150 kDa proteins is of a CK II type. This is strongly supported by the following observations: (i) purified CK II phosphorylates the purified sarcalumenin; (ii) as in the endogenous SR phosphorylation system (Fig. 3), this phosphorylation is inhibited by La^{3+} (Fig. 6). Inhibition by La^{3+} is not specific for the phosphorylation of the 130 kDa protein, but it is observed at the phosphorylation of the 105 kDa protein (Fig. 6). La^{3+} also inhibits the skeletal muscle sarcalumenin and HCP phosphorylation [36, 45]. Moreover the phosphorylation of several luminal proteins in the Golgi complex by CK II was also inhibited by La^{3+} [56]. Thus, La^{3+} inhibition seems to be due to its interaction with the kinase. (iii) Hemin and heparin, inhibitors of casein kinase II [24], also inhibit the endogenous phosphorylation of the skeletal [36] and cardiac proteins (*data not shown*). (iv) [γ-^{32}P]GTP phosphorylates the 150/130 kDa proteins by the SR endogenous kinase (Fig. 4). GTP is known to be a phosphate donor to CKII, but not to other kinases [24]. (v) Using antibodies against CK II, we demonstrate the presence of CK II in both cardiac (Fig. 4) and skeletal muscle SR preparations [45]. Furthermore, CK II has been found to be tightly associated with the SR membranes, and was not extracted by EGTA at alkaline pH, but was extracted from these leaky vesicles (not from intact SR) by high NaCl concentration (Fig. 4). The existence of an endogenous CK II activity in cardiac SR has been demonstrated [4]. Calsequestrin has been isolated from heart SR in phosphorylated form, and the phosphorylation site is a CK II one [4]. The phosphorylation of calsequestrin, in vivo and in vitro, by CK II is a further indication for the luminal location of CKII [5]. It was suggested that the cellular site of calsequestrin phosphorylation is in the Golgi complex [5, 6]. The phosphorylation of sarcalumenin, a luminal SR protein, however, suggests CK II location in the SR lumen. It has been shown that CK II that phosphorylates several intralumenal proteins of the Golgi complex is intraluminally located [8].

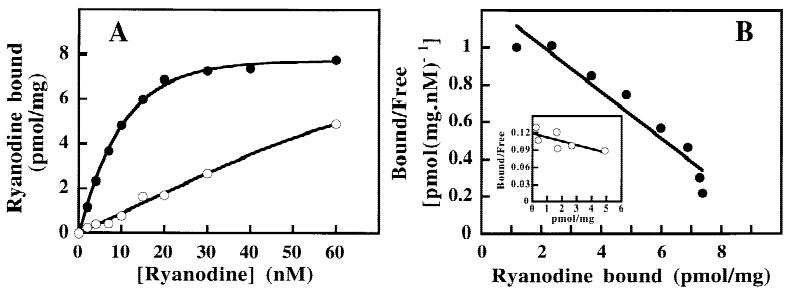

Fig. 10. Effect of phosphorylation of 130 and 150 proteins on the ryanodine binding affinity of the RyR. Cardiac SR membranes were incubated, at pH 7.2, with 0.4 mM ATP or AMP-PNP and 60 mM NaF. [^{3}H]ryanodine binding to unphosphorylated- (incubated with AMP-PNP and NaF) (●) and phosphorylated membranes (○) (incubated with ATP and NaF) was assayed, as a function of ryanodine concentration, for 20 min, as described in Materials and Methods. The binding is about 90% of the maximal binding obtained after 2 h of incubation. The isotherms are shown in *A* and the Scatchard plots in *B.*

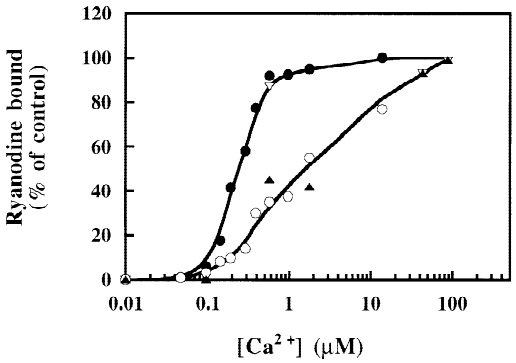

Fig. 11. Ca^{2+} dependency of ryanodine binding in control and phosphorylated SR membranes. Cardiac SR membranes were phosphorylated at pH 7.2 with 0.4 mM ATP and 60 mM NaF (○, ▲) and then diluted 1:1 with two times concentrated reaction mixture for ryanodine binding containing 0.4 mM EGTA and the indicated free Ca^{2+} concentrations. Free Ca^{2+} concentrations were calculated using a computer program, as described [18]. Control membranes (●, ▽) were incubated under the same conditions but with AMP-PNP instead of ATP. Ryanodine binding (10 nM) was assayed for 20 min, as described in Materials and Methods. Using higher Ca^{2+} concentrations the same maximal binding was obtained for phosphorylated and nonphosphorylated membranes (*not shown*). Control activity (100%) were 4 and 1.5 pmol ryanodine bound/mg protein for the two SR preparations (○, ● and ▲, ▽), respectively.

Casein kinase II has no requirement for Ca^{2+} [24]. However, while the phosphorylation of the purified 130/150 kDa proteins by exogenously added CK II is independent of Ca^{2+} (*data not shown*), the phosphorylation of the membrane-associated proteins is absolutely dependent on the presence of Ca^{2+} (Fig. 1). The mechanisms underlying the requirement for Ca^{2+} for the phosphorylation of the SR proteins are not known. Ca^{2+} could be involved in the transport of ATP into the SR lumen [44], or it is involved in the protein-membrane association-dissociation processes. It should be noted that the skeletal muscle SR HCP and sarcalumenin bind Ca^{2+} and as the cardiac proteins were extracted from the SR by EGTA [14, 31].

While the results clearly indicate that the cardiac and skeletal muscle proteins are phosphorylated by the same kinase type, structural studies indicate that the cardiac sarcalumenin is distinct from the skeletal muscle protein. Both the cardiac skeletal muscle phosphorylated proteins belong to the family of so-called calsequestrin-like proteins [41] because of their staining blue with Stains-All, and their ability to bind Ca^{2+} using the Ca^{2+} overlay technique. The cardiac and the skeletal muscle sarcalumenin are different in the following: (i) The electrophoretic mobility of the cardiac protein appears to be different from that of the skeletal muscle proteins as visualized by their phosphorylation (Fig. 1) and their staining with Stains-All (Fig. 7). It should be noted that the skeletal muscle sarcalumenin MW is 98,000 (based on its amino acid sequence) [31], but the mobility in SDS-PAGE of the expressed protein is that of 150–160 kDa [41]. (ii) Immunological analysis of the cardiac protein after SDS-PAGE with three different monoclonal antibodies against the skeletal muscle sarcalumenin demonstrated that only one of the antibodies is able to react with the cardiac protein, and the other two are able to distinguish between the skeletal and cardiac muscle sarcalumenin (Fig. 8). (iii) The peptide and phosphopeptide pattern after partial digestion with *S. aureus* V_8 protease show differences between the cardiac and skeletal muscle proteins (Fig. 9). (iv) Amino acid sequence of eight internal peptides derived from the cardiac 130 kDa protein indicate a very high similarity to the skeletal muscle sequence, but in one of the eight peptides sequenced, two amino acids of the total of 10 are different. These results suggest either the cardiac sarcalumenin is an isoform different from the skeletal muscle protein, or these differences result from species differences (dog *vs.* rabbit).

The results presented here and in our previous work with skeletal muscle SR show that under the conditions of sarcalumenin and HCP phosphorylation, ryanodine binding is inhibited. The relationship between the phosphorylation of the two proteins and the inhibition of ryanodine binding is reflected in the following findings: (i) Both the phosphorylation of these two proteins and the inhibition of ryanodine binding is absolutely dependent on the presence of NaF (Figs. 1, 2 and 4 and Ref. 36). (ii) In both activities okadaic acid (as a phosphoprotein phosphatase inhibitor) did not replace NaF and AMP-PNP did not replace ATP. (iii) Both activities have the

same time course and ATP concentration dependence. (iv) Low Ca^{2+} concentrations were required (during the pre-incubation with ATP and NaF) for the phosphorylation and the inhibition of ryanodine binding. The mechanism by which this phosphorylation affects ryanodine binding is not clear. Both the Ca^{2+} and the ryanodine-binding affinities are modified when one or both proteins are phosphorylated (Figs. 10 and 11). Both in cardiac and in skeletal muscle SR [15, 45] there are no indications for a direct interaction between the RyR and either or both HCP and sarcalumenin. It is possible that HCP and sarcalumenin exert their effect on the RyR via calsequestrin [43]. This, however, required demonstration of an interaction between calsequestrin and sarcalumenin and/or HCP. Calsequestrin was shown to interact with RyR and to modulate its activity [26, 27]. Recently calsequestrin has been shown to control Ca^{2+} release through RyR from the SR [48]. The involvement of protein-protein interaction in the modulation of RyR activity has been suggested recently for several other proteins, such as annexin VI [16], FK-506 binding protein [49, 50], and sorcin [32]. Phosphorylation of HCP and triadin by the membrane-bound 60 kDa protein kinase has been suggested to modulate the functional state of the RyR [15].

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