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The identification of the phosphorylated 150/160-kDa proteins of sarcoplasmic reticulum, their kinase and their association with the ryanodine receptor

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Abstract

In the present work we studied the relationship between the phosphorylated 150- and 160-kDa proteins and other SR proteins in the 150 000–170 000 range of molecular masses, on SDS-PAGE, the identification of their kinase, as well as the purification and structural interactions between these proteins and the ryanodine receptor (RyR). The phosphorylated 150-kDa protein was identified as sarcalumenin based on: (a) its cross-reactivity with three different monoclonal antibodies specific for sarcalumenin, (b) its mobility in SDS-PAGE which was modified upon digestion with endoglycosidase H, (c) its elution from lentil-lectin column by α-methyl mannoside, (d) its resistance to trypsin, (e) its ability to bind Ca^{2+} and to stain blue with Stains-All. The phosphorylated 160-kDa protein was identified as the histidine-rich Ca^{2+} binding protein (HCP) based on: (a) its Ca^{2+}-binding property and staining blue with Stains-All, (b) phosphorylation with the catalytic subunit of cAMP-dependent kinase, (c) its increased mobility in SDS-PAGE in the presence of Ca^{2+}, (d) its heat stability and (e) its partial amino acid sequence. The endogenous kinase was identified as casein kinase II (CK II) based on the inhibition of the endogenous phosphorylation 160/150-kDa proteins by heparin, 5,6-dichlorobenzimidazole riboside, polyaspartyl peptide and hemin, and its ability to use [γ-^{32}P]GTP as the phosphate donor. The association of CK II with SR membranes, was demonstrated using specific polyclonal anti-CK II antibodies. The luminal location of CK II is suggested because CK II was extracted from the SR by 1 M NaCl only after their treatment with hypotonic medium, and CK II activity was inhibited with the charged inhibitors heparin and polyaspartyl peptide only after their incubation with the SR in the presence of NP-40. The 160- and 150-kDa proteins were purified on spermine-agarose column, and were phosphorylated by CK II. Like the endogenous phosphorylation of the 150/160-kDa proteins in SR, the phosphorylation of the purified proteins by CK II was inhibited by La^{3+} ($CI_{50} = 4$ μM) and hemin. The results suggest the phosphorylation of the luminally located sarcalumenin and HCP with CK II.

Keywords: Sarcoplasmic reticulum; Sarcalumenin; Protein phosphorylation; Ryanodine receptor; Casein kinase II

1. Introduction

One major hypothesis for excitation-contraction (E-C) coupling suggests that a voltage sensor molecule on transverse tubules undergoes conformational changes in response to depolarization. These changes may be transmitted to the ryanodine receptor (RyR)/Ca^{2+}-release channel on the junctional face of the sarcoplasmic reticulum (SR) [1,2]. The voltage sensor is now believed to be the dihydropyridine receptor [2,3] where, conformational changes in it may modulate the opening of the SR Ca^{2+}-release channel. Recently, the involvement of several other proteins in the regulation/modulation of the SR Ca^{2+}-release channel has been suggested [4–11]. It has been shown that a 95-kDa protein, an intrinsic protein of the junctional SR, interacts with both the DHP receptor and the RyR [4]. Electron microscopy [5], as well as biochemical studies [6–9], suggest that calsequestrin is linked with

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)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)dimethylammonio]-1-propanesulfonate; PMSF, phenylmethylsulfonyl fluoride; RyR, ryanodine receptor; ATPase, Ca^{2+}-ATPase; HCP, histidine-rich Ca^{2+}-binding protein; CK, casein kinase.

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the junctional face membrane where the Ca^{2+}-release channel is localized, and that it plays an important role in Ca^{2+} release. Recently, the modification of RyR/Ca^{2+}-release channel behavior by the tightly associated FK-506 binding protein has been demonstrated [10,11].

Several SR proteins in the 150–170kDa range on SDS-PAGE were characterized, and their involvement in the regulation of Ca^{2+} transport was suggested [12–21]: (1) A 160-kDa Ca^{2+} binding glycoprotein was purified, characterized, cloned, sequenced, located in the SR lumen and named sarcalumenin [12–14]. (2) A 170-kDa protein was labeled with [^{14}C]doxorubicin and suggested to be an integral part of the ryanodine receptor [15]. (3) A 170-kDa protein was eluted from reactive-red agarose column by caffeine and suggested to be the caffeine-receptor [16]. (4) A 165-kDa protein that binds Ca^{2+} and low-density lipoprotein, referred to as histidine-rich Ca^{2+} binding protein (HCP), was purified and cloned [17,18]. It has been shown to bind tightly to junctional membrane and to be phosphorylated by cyclic AMP-dependent protein kinase [19]. (5) A 170-kDa Stains-All blue protein was shown to interact specifically on affinity column with a 95-kDa integral protein of junctional SR [5]. This protein, however, was suggested to be identical to HCP [19]. The function of these proteins is unknown. In our previous studies [20,21], we demonstrated the phosphorylation of 150- and 160-kDa proteins by an endogenous phosphorylation system. This work has been extended in the accompanying paper [22], where we show that phosphorylation of the 150- and/or 160-kDa proteins inhibits ryanodine binding due to alterations in the Ca^{2+}- and ryanodine-binding affinities of the RyR.

In the present study, we identified the phosphorylated 150- and 160-kDa proteins as sarcalumenin and HCP as well as their kinase as casein kinase II.

2. Experimental procedures

2.1. Materials

Lentil-lectin Sepharose 4B, α-methyl mannoside, polyaspartate, 5,6-dichlorobenzimidazole riboside, hemin, heparin, trypsin (type III), endoglycosidase H (*Streptomyces griseus*), dithiothreitol, sodium deoxycholate, CHAPS, spermine, spermine-agarose and alkaline phosphatase-conjugated goat anti-mouse IgG were obtained from Sigma. Sephadex G-50 (fine) was obtained from Pharmacia. Casein kinase II was purchased from Boehringer. Other reagents were as in the accompanying paper [22].

2.2. Membrane preparations

SR membranes were prepared from rabbit fast-twitch skeletal muscle as described by Saito et al. [23], Lai et al. [24] or MacLennan [25]. In all these SR preparations, the proteinase inhibitors, PMSF (0.2 mM), benzamidine (0.8 mM), and leupeptin (0.5 μg/ml), were included in all solutions. SR was suspended in a buffer containing 0.2 M sucrose and 10 mM Tricine, pH 8.0, and stored at −70°C. Protein concentration was determined by the method of Lowry et al. [26].

2.3. Protein phosphorylation, gel electrophoresis, autoradiography and [^{3}H]ryanodine binding

These assays were carried out as described in the accompanying paper [22], or in the figure or table legends.

2.4. Immunoblotting

Western blot analysis was carried out as described previously [27]. 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 antibody or to protein A as secondary antibody. The color was then developed (up to 5 min) with 5-bromo-4-chloro-3-indolyl phosphate and Nitro blue tetrazolium.

2.5. Purification of 150-kDa (sarcalumenin), 160-kDa (HCP) and 53-kDa glycoprotein

The 150-kDa, 160-kDa and 53-kDa proteins were partially purified by a new one-step method using spermine-agarose column. HSR [24], R_4 or R_3 [23] membranes (30 to 100 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 slowly 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 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 spermine-agarose column (1.0/2.7 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 20 ml cold (≈ 8°C) buffer A, which elutes the 53-kDa glycoprotein. Sarcalumenin with other proteins was eluted with buffer A containing 5 mM of spermine. By increasing NaCl concentration to 0.3 M, sarcalumenin was eluted. The 160-kDa protein was eluted from the column by 1.0 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 [27]. The purified proteins were stored at −20°C.

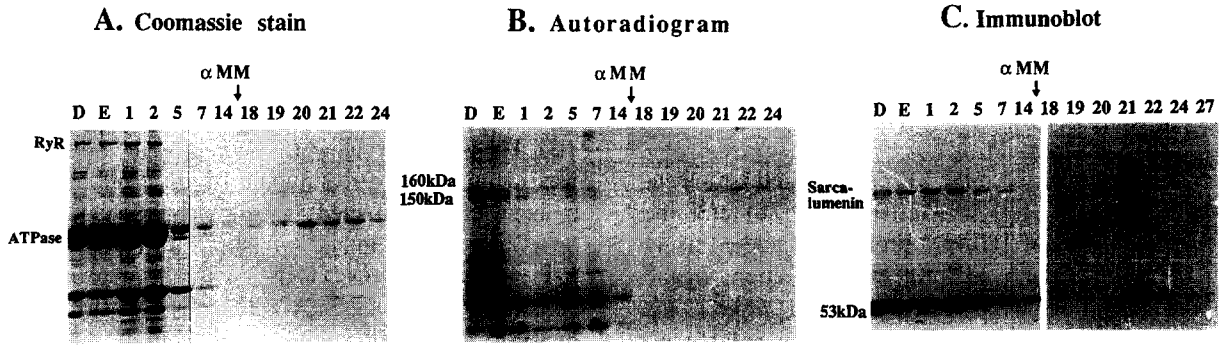

Fig. 1. Elution profiles of phosphorylated 150-kDa protein and sarcalumenin from lentil-lectin column. HSR membranes (6 mg) were phosphorylated with [γ-^{32}P]ATP as described under Section 2, solubilized with deoxycholate (1 mg/mg protein) in the presence of 100 mM NaF, 0.8 mM benzamidine, and 0.2 mM PMSF. After 20 min at ice, the deoxycholate extract was centrifuged at 40000 × g for 20 min. The free [γ-^{32}P]ATP in the supernatant was separated from the proteins using the Sephadex G-50 chromatography-centrifugation method [44]. The eluent was applied (1 ml/10 min) three times to a lentil-lectin-Sepharose column (1.2 × 5 cm) pre-equilibrated with a solution containing: 20 mM Tris, pH 7.5, 100 mM NaF, 0.1% deoxycholate, and 0.2 mM PMSF (buffer-L). The loaded column was washed with buffer-L and the glycoproteins were eluted with buffer-L containing 0.5 M of α-methyl mannoside (αMM). SDS-PAGE profile of the fractions applied and eluted from the lentil-lectin column is shown in (A), the corresponding autoradiogram in (B), and the immunoblot staining using monoclonal antibodies ((XIIC$_4$) against the sarcalumenin (1:1000)), and alkaline phosphatase-conjugated anti-mouse IgG antibodies as secondary antibodies is presented in (C). SDS-PAGE and immunoblot staining were carried out as described in Section 2. D indicates deoxycholate extract and E indicates void fraction.

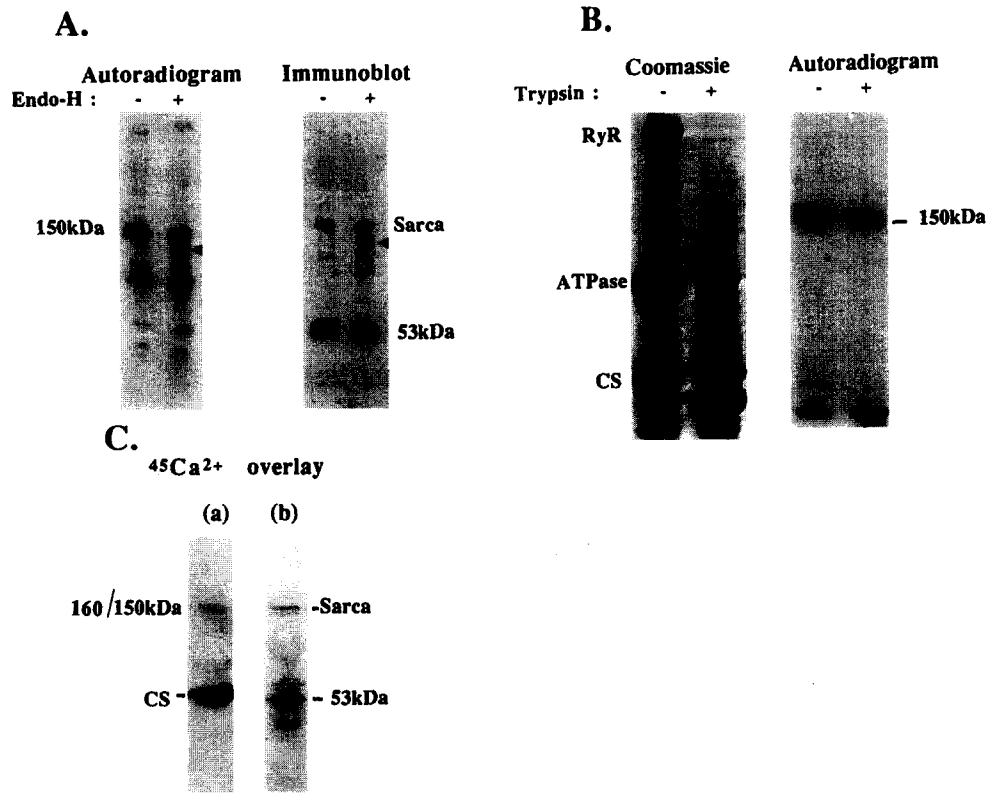

Fig. 2. Effect of Endo-H and trypsin treatment on the phosphorylated 160/150-kDa proteins and their Ca^{2+}-binding capability. HSR membranes were phosphorylated for 1 min with 0.4 mM of [γ-^{32}P]ATP in the presence of 60 mM NaF, and then treated with either Endo-H or trypsin. For Endo-H treatment (A), the phosphorylated membranes (1 mg/ml) were incubated with 0.7% C_{12} E_9 and boiled for 3 min, and then leupeptin (0.5 μg/ml) and Endo-H (0.04 unit/ml) were added. The samples were then incubated for 2 h at 37°C and run on SDS-PAGE (5–9% acrylamide) according to Ref. [45]. The gel was either stained with Coomassie or transferred onto nitrocellulose membranes and immunostained with monoclonal antibodies (XIIC$_4$). The arrow heads point to the new band formed by Endo-H treatment. For trypsin treatment (B), the phosphorylated membranes were treated with trypsin (trypsin/SR mass ratio of 1:50) for 2 min. The treated membranes were subjected to SDS-PAGE (6% acrylamide) and autoradiography, as described in Section 2. $^{45}Ca^{2+}$ ligand overlay of nitrocellulose blot (C) was carried out as described previously [46]. (a) is the autoradiogram of the $^{45}Ca^{2+}$ overlay blot, and (b) is the immunoblot.

3. Results

3.1. Is the phosphorylated 160- or 150-kDa protein sarcalumenin?

The relationship between the SR proteins in the molecular weight range of 150000–170000 [12–20] and the phosporylated 150- and 160-kDa proteins is studied using several approaches such as: protein purification, ligand binding and specific antibody interaction. Fig. 1 shows the purification of the 150-kDa glycoprotein (sarcalumenin) from the deoxycholate extract of [γ-^{32}P]ATP phosphorylated membranes, using lentil-lectin affinity chromatography [13]. The SDS-PAGE protein profile, the corresponding autoradiogram and immunoblot of the various fractions from the column are illustrated. The unbound or weakly-bound proteins that include part of the phosphorylated 150/160-kDa proteins are washed off the column and then, as reported previously [13], α-methyl mannoside (αMM) elutes three major proteins with molecular masses of 150, 95 and 53 kDa. Among these three proteins, the 150-kDa protein is the only phosphorylated protein (Fig. 1B). The phosphorylated 150-kDa protein, eluted by αMM was identified as sarcalumenin by using monoclonal antibodies ($XIIC_4$) directed against a common domain of the 53-kDa glycoprotein and sarcalumenin (kindly provided by K.P. Campbell, Iowa University). Similar results were obtained with monoclonal antibodies G_7 and G_{10} (kindly provided by Prof. D.H. MacLennan, University of Toronto). It should be noted that although the deoxycholate extract of the HSR was passed through the column three times, only parts of the glycoproteins were bound to the column. This led us to develop a new method for its purification (see Fig. 5).

To ensure that the phosphorylated 150-kDa is the 160-kDa glycoprotein known as sarcalumenin, we carried out

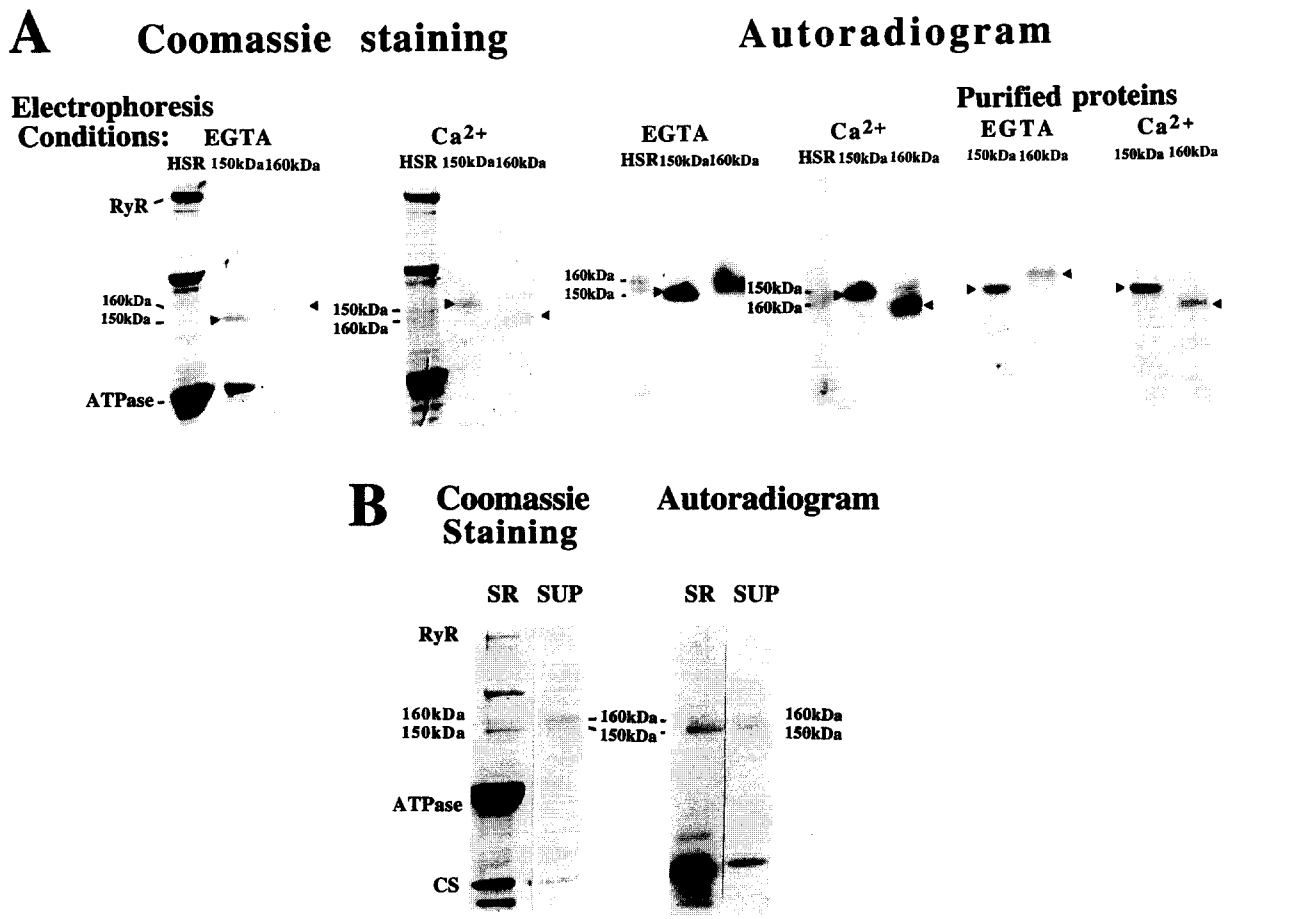

Fig. 3. Identification of the phosphorylated 160-kDa protein as HCP. In A, SR membranes were phosphorylated with 0.3 mM [γ-^{32}P]ATP in the presence of 60 mM NaF, subjected to SDS-PAGE, staining and destaining as described in Section 2. The phosphorylated 160- and 150-kDa protein bands were cut off the gel and run in a second gel (5% acrylamide separating gel and 3% stacking gel) containing 0.1 mM EGTA or 1 mM $CaCl_2$. In each gel the original phosphorylated membranes (applied to the first gel) are also shown. 150-kDa and 160-kDa proteins were purified and phosphorylated with CK II as described in Figs. 5 and 6, respectively and then subjected to electrophoresis in gels containing either 1 mM $CaCl_2$ or 0.1 mM EGTA. In B, the heat stability of the phosphorylated 160-kDa protein is demonstrated. SR membranes, isolated according to MacLennan [25], were phosphorylated as in A. Then, Triton X-100 was added to aliquots to a final concentration of 1% and the samples were incubated for 15 min at boiled water followed by centrifugation for 10 min at 16800 × g. The obtained supernatant was subjected to SDS-PAGE, staining, destaining and to autoradiography as described in Section 2.

several other experiments. The effect of the endoglycosidase H, which digests the carbohydrate chains in the glycoprotein (sarcalumenin) and increases its mobility in SDS-PAGE, on the phosphorylated 150-kDa protein is shown in Fig. 2A. Parallel to a decrease in the 150-kDa phosphorylated band, a new phosphorylated band with a higher mobility appeared. This new protein band cross-reacts with monoclonal antibodies against sarcalumenin ($XIIC_4$), as does the phosphorylated 150-kDa. As shown for sarcalumenin [12], the phosphorylated 150-kDa protein in intact SR membranes is resistant to trypsin (Fig. 2B). It seems, however, that at least part of the 160-kDa is digested by trypsin. The phosphorylated protein that cross-reacted with antibody against sarcalumenin also binds $^{45}Ca^{2+}$ (Fig. 2C) and stained blue with Stains-All (data not shown).

3.2. Identification of the 160-kDa protein as HCP

Since the phosphorylated 160-kDa protein stained blue with Stains-All (not shown) and binds Ca^{2+} (Fig. 2B), it is possible that it is the HCP. To test this possibility we carried out the following experiments. Fig. 3A shows that, as it has been shown previously for HCP [19], the phosphorylated 160-kDa protein exhibited a Ca^{2+}-induced shift in its electrophoretic mobility. The Ca^{2+}-induced shift in the mobility of 160-kDa protein was demonstrated on the SR phosphorylated 150- and 160-kDa proteins and on purified 160- and 150-kDa proteins (see Fig. 5) phosphorylated by added casein kinase II (see Fig. 6). The phosphorylated bands were cut off the gel and subjected to a second electrophoresis in the presence of EGTA or Ca^{2+}. In either case, Ca^{2+} induced a significant shift in the

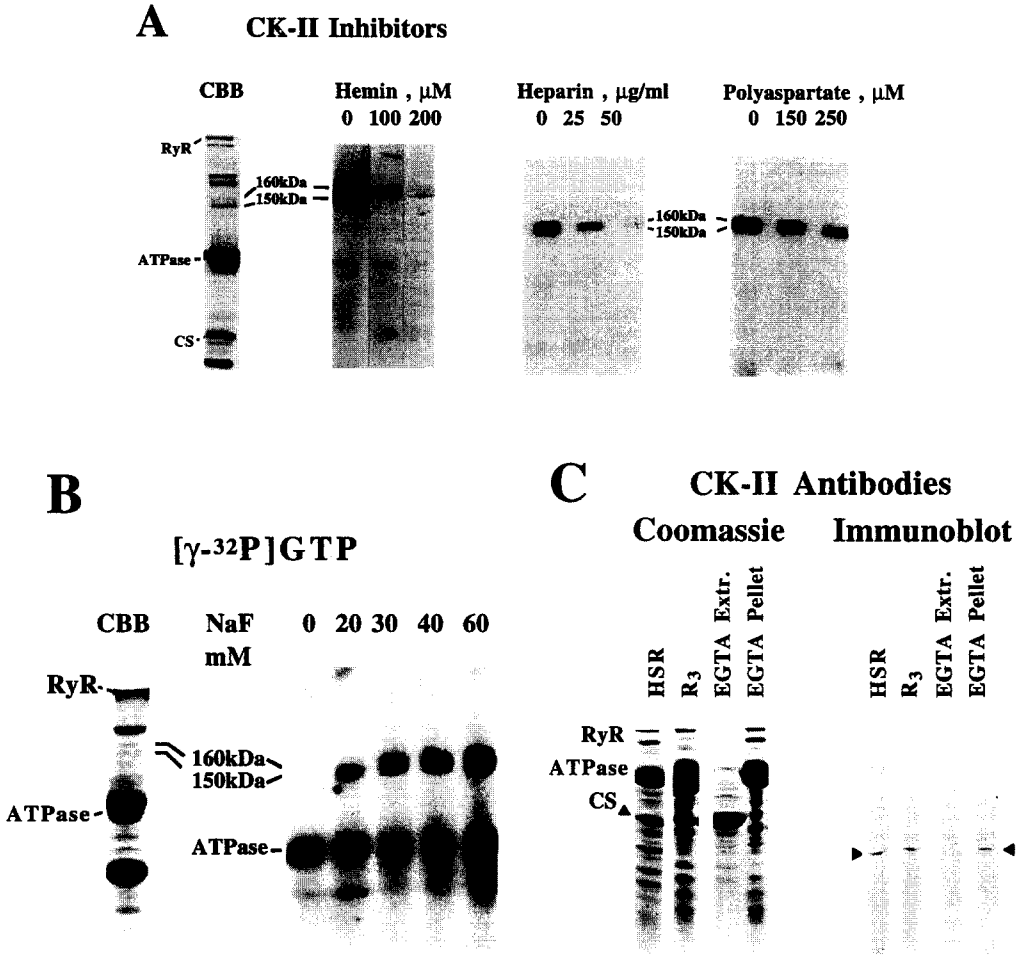

Fig. 4. Endogenous phosphorylation of 160/150-kDa proteins is carried out by casein kinase II. In A, SR membranes were phosphorylated with 0.2 mM [γ-^{32}P]ATP in the presence of 60 mM NaF and the indicated hemin, heparin or polyaspartate concentrations. Heparin and polyaspartate were incubated for 10 min at 30°C with the SR in the presence of 0.03% NP-40 prior to the phosphorylation reaction. In B, SR membranes were phosphorylated with 0.3 mM [γ-^{32}P]GTP in the presence of the indicated NaF concentrations, as described in Fig. 1. In C, different SR preparations R_3 [23] and HSR [24] and SR sub-fractions were analyzed by SDS-PAGE, and either stained with Coomassie or electroblotted and immunostained with polyclonal antibodies against CK II (15% acrylamide) (provided by Dr. Issinger) as described in Fig. 1C, except that alkaline phosphatase-conjugated protein A was used as the secondary antibody. The arrows indicate the cross-reactive band (45-kDa).

mobility of 160-kDa but not in 150-kDa, supporting the identification of 160-kDa as HCP. It has been shown previously that HCP is stable to boiling [19]. Fig. 3B shows that boiling of phosphorylated SR membranes resulted in soluble phosphorylated 160-kDa protein.

We also carried out amino acid sequence of the phosphorylated 160-kDa protein as described previously [28,29]. The phosphorylated membranes were subjected to SDS-PAGE and then electroblotted onto PVDF membranes that were subjected to in situ trypsin digestion. The resulting peptide fragments were separated by reverse phase HPLC. The phosphopeptide-containing peak was applied to a gause phase sequenator and the following sequence was obtained: Ala-Leu-Ala-Asp-Met-Leu-Glu-Thr-Pro. This sequence is identical to the C-terminal of the HCP sequence as deduced from the cDNA (positions 842–850). Interestingly, the same tryptic fragment was obtained and sequenced from the purified HCP [20].

3.3. Identification of the endogenous kinase as casein kinase II

Screening of the sarcalumenin and HCP amino acid sequence for consensus phosphorylation sites [30], using the prosite subroutine (GCE program), revealed 30 and 26 sites for casein kinase II (CK II), 9 and 15 for protein kinase C, none for either calmodulin-dependent or cAMP-dependent protein kinases for sarcalumenin and HCP, respectively. The involvement of CK II in the endogenous phosphorylation of 160/150-kDa proteins was demon-

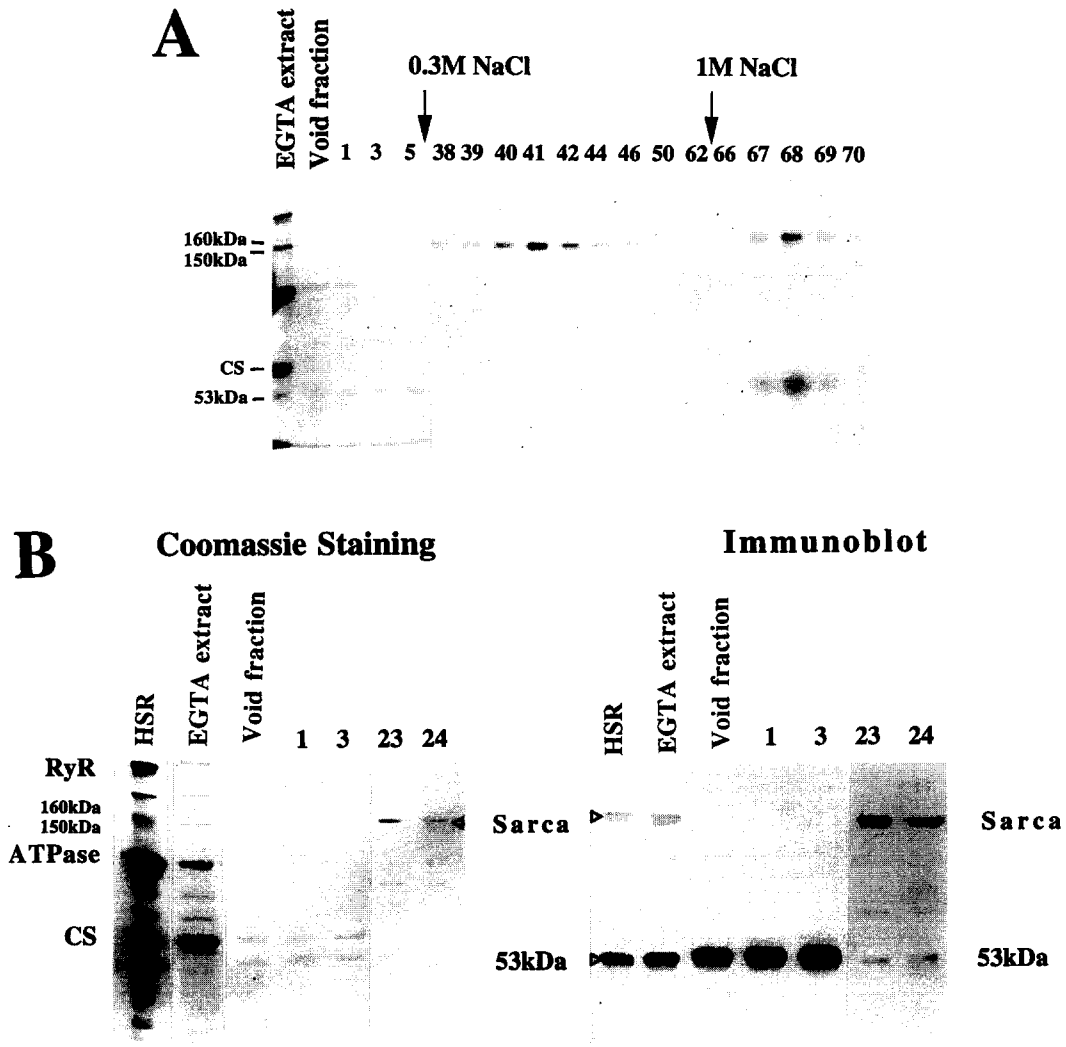

Fig. 5. A simple, one-step method for purification of sarcalumenin, HCP and the 53-kDa glycoprotein, using a spermine-agarose column. In A, EGTA extract of HSR was obtained, and HCP, sarcalumenin and 53-kDa glycoproteins were obtained as described in Section 2. The EGTA extract (lane 1), the void fraction of the spermine-agarose column (lane 2), the washed fractions (fractions 1,3,5, 53-kDa), and the proteins eluted with NaCl; 0.3 M (fractions 38–62, 150-kDa) or 1 M (fractions 66–70,160-kDa) were subjected to SDS-PAGE (7% acrylamide). In B, samples from *another* purification were subjected to SDS-PAGE (7% acrylamide) and the gels were either stained with Coomassie blue or electroblotted to nitrocellulose membrane and immunostained with anti-53-kDa glycoprotein monoclonal antibodies G_{10}. The indicated samples are as in A, except fractions 23 and 24 that are fractions eluted from the column with 0.12 M NaCl + 8 mM spermine. Sarca indicates sarcalumenin.

strated by the effects of hemin, polyaspartate and heparin, inhibitors of CK II [31,32], on their phosphorylation. Hemin inhibited the endogenous phosphorylation of 160/150-kDa proteins (Fig. 4A). Heparin and polyaspartate were found to inhibit the HCP and sarcalumenin phosphorylation only when incubated with the SR in the presence of relatively low concentrations of detergent such as NP-40 (Fig. 4A). In the *absence* of NP-40 no significant inhibition of the phosphorylation of 160-/150-kDa proteins was obtained with heparin (up to 100 μM) and about 30% inhibition was obtained with 250 μM polyaspartate. The non-charged compound 5,6-dichlorobenzimidate riboside inhibited the phosphorylation in the absence of NP-40 (not shown). These results may indicate on the luminal location of the CK II.

Another unique character of CK II is that it utilizes GTP as the phosphate donor almost as effectively as ATP [31]. Fig. 4B shows that the endogenous kinase phosphorylates these proteins using [γ-^{32}P]GTP. Therefore, these results strongly suggest that the endogenous kinase responsible for the phosphorylation of 160/150-kDa protein is of a CK II type. [γ-^{32}P]GTP labeled the Ca^{2+}-ATPase, a labeling which was not observed under the same conditions with [γ-^{32}P]ATP. This labeling may represent the phosphorylated enzyme (E-P) that remains stable under the electrophoresis conditions.

The presence of CK II in different SR preparations is demonstrated in Fig. 4C by using specific antibodies against CK II (kindly provided by Dr. Issinger, Hamburg). Moreover, CK II remains associated with the SR membranes after their EGTA-extraction which releases luminal and peripheral proteins. CK II was extracted from the EGTA-treated SR membranes by their extraction with 0.5 to 1 M NaCl (data not shown).

3.4. Purification of sarcalumenin and HCP

To demonstrate direct phosphorylation of sarcalumenin and the HCP by CK II, we purified both proteins from the EGTA extract of SR membranes. Sarcalumenin was previously purified using sequential column chromatography on DEAE-Sephadex and lentil-lectin Sepharose 4B column [13]. HCP was also purified from SR by heat treatment and chromatography on Mono-Q column [17]. Here we developed a simple and rapid method for purification of these proteins and of the 53-kDa glycoprotein in one-step and with higher yield, using chromatography on spermine-agarose column. Sarcalumenin, HCP and the 53-kDa glycoprotein were isolated from the EGTA extract using the method described in Section 2, and the results are presented in Fig. 5. On the spermine-agarose column, under the ionic strength and pH used (0.12 M NaCl, pH 8.5), the 53-kDa glycoprotein did not bind, and it was effectively washed off with 0.12 M NaCl. Addition of 5 mM spermine to the washing buffer elutes several proteins with apparent molecular masses of about 150 000 (sarcalumenin), 190 000, 85 000, and 53 000 (glycoprotein). Sarcalumenin was eluted with 0.3 M NaCl or with 0.12 M NaCl + 8 mM spermine and HCP with 1.0 M NaCl. Both purified sarcalumenin and HCP were stained blue with Stains-All (not shown). The immunoblot of fractions obtained from another purification (Fig. 5B), shows the separation between the two glycoproteins, 53-kDa and sarcalumenin.

3.5. Phosphorylation of the purified sarcalumenin and HCP by CK II

Our results suggest that the 150-kDa and 160-kDa proteins, identified as sarcalumenin and HCP, respectively, are phosphorylated by the same kinase type, most probably CK II (Fig. 4). Thus, we expect the purified proteins to be phosphorylated by CK II. CK II but not the catalytic subunit of the cAMP-dependent kinase, stimulated the phosphorylation of the purified sarcalumenin (Fig. 6A).

The maximal phosphate incorporated into the purified sarcalumenin (6 to 8 mol/mol) is about 20% higher than in the SR protein. Also, phosphopeptide maps of the purified and the SR proteins indicate the presence of several identical and a few non-identical phosphopeptides.

Protein kinase C slightly stimulated the phosphorylation of sarcalumenin (data not shown). HCP is phosphorylated by both CK II and by the catalytic subunit of cAMP-dependent kinase (Fig. 6). Interestingly, although no consensus phosphorylation sites for PK-A were found in HCP, the protein was phosphorylated by PK-A, as also reported previously [19].

La^{3+} inhibited the phosphorylation of the purified sarcalumenin and HCP by CK II, with half-maximal inhibition obtained at about 4 μM of La^{3+} (Fig. 6B), similar to the SR-associated proteins (Ref. [20] and Fig. 6B). Hemin (Fig. 6B) as well as heparin and polyaspartate (data not shown) inhibited the phosphorylation of the purified sarcalumenin. No protein phosphorylation was obtained in the absence of CK II (data not shown).

3.6. The distribution of sarcalumenin and HCP in SR subfractions

The distribution of RyR, HCP and sarcalumenin in different SR preparations and SR subfractions is presented in Fig. 7 and Table 1. As expected, RyR is enriched in the heavy fractions of the SR (HSR) (Fig. 7A, Table 1, compare HSR, R_4 to R_1, R_2 and light SR (LSR)). The distribution of sarcalumenin, as followed by Stains-All, phosphorylation and immunoblot staining, indicates that this protein is present in light, intermediate (R_2 and R_3) and heavy SR (R_4), although it is enriched in the light SR, R_2 and in SR isolated according to MacLennan [25] (Fig. 7B and Table 1). The 53-kDa glycoprotein distribution is similar to that of sarcalumenin. The heavy fractions of SR are enriched with both RyR and HCP (Table 1), in agreement with previous findings [19].

The sarcalumenin and HCP phosphorylation system (which represents the phosphatase(s) and kinase(s) activities) is present in all SR preparations isolated according to Saito et al. [23], Lai et al. [24], and MacLennan [25] (Table 1).

3.7. The structural interaction between ryanodine receptor and sarcalumenin

In the accompanying paper [22], we show that the level of ^{32}P incorporation into 150/160-kDa proteins affects the binding of ryanodine to RyR, suggesting an interaction between these proteins. The inhibitory effect of sarcalumenin and HCP phosphorylation on ryanodine binding is reversed in the presence of relatively low concentrations of CHAPS (Table 2). This effect of CHAPS is due to alteration of the ryanodine binding affinity (K_D) (Fig. 8). The phosphorylation decreases the total binding sites (B_{max}) by about 90% (from 12.1 ± 2.8 pmol/mg, $n = 3$ to 1.0 ± 0.4 pmol/mg, $n = 3$) and the K_D by 1.5-fold (from 11.3 ± 2 nM, $n = 3$ to 16.4 ± 2 nM, $n = 3$). Addition of 0.1% CHAPS to control membranes increased K_D for ryanodine binding by 3.1-fold (from 3.5 ± 0.3 nM, $n = 3$ to 11.2 ± 0.7 nM, $n = 3$) with no significant effect on the B_{max} value (from 11.8 ± 0.7 pmol/mg, $n = 3$ to 12.9 ± 2.4 pmol/mg, $n = 3$). Thus, addition of CHAPS to the *phosphorylated* membranes resulted in 3.2-fold lower affinity compared to that of control membranes, suggesting modification of the phosphorylation effect on ryanodine binding by CHAPS.

To test the interaction between the ryanodine receptor and sarcalumenin, we purified the RyR (from the CHAPS extract) both by chromatography on spermine-agarose column [33], and density gradient centrifugation [24]. Using the spermine-agarose column, a phosphorylated 150-kDa protein which cross-reacted with monoclonal antibodies ($XIIC_4$) against sarcalumenin is co-purified with the RyR (not shown). However, using sucrose-gradient centrifugation [24], the phosphorylated sarcalumenin (identified with $XIIC_4$ antibodies) is distributed at the middle of the gradient while RyR at the bottom of the gradient (not shown). These results suggest that, under the SR solubilization and separation conditions used, the phosphorylated sarcalumenin is not tightly associated with the RyR.

4. Discussion

In our accompanying paper [22], we have shown that phosphorylation of 160/150-kDa proteins resulted in alteration of the properties of the RyR as reflected in changes in Ca^{2+}- and ryanodine-binding affinities. In this paper we have identified the 160- and 150-kDa phosphorylated proteins, their kinase, and provided some data on the interaction of these proteins with the RyR.

4.1. The identity of the 160 / 150-kDa proteins as sarcalumenin and HCP

The phosphorylated 150-kDa protein is identified as sarcalumenin based on the following: The 150-kDa phosphorylated protein was eluted from the lentil-lectin column by α-methyl mannoside (Fig. 1), and its mobility was modified by treatment with Endo-H (Fig. 2A), suggesting that it is a glycoprotein. This protein cross-reacted with monoclonal antibodies, G_{10}, G_7 and $XIIC_4$, specific for the sarcalumenin and the 53-kDa glycoproteins. It stained blue with Stains-All, bound Ca^{2+} and was resistant to trypsin (Fig. 2). These results are in agreement with the reported sarcalumenin properties.

The identification of the phosphorylated 150-kDa protein as sarcalumenin and its phosphorylation by CK II (Fig. 6) allow us to reach a conclusion about the possible location of phosphorylation site(s). The 53-kDa glycoprotein sequence is included in the sarcalumenin sequence [14], but is not phosphorylated either in the SR by the endogenous kinase or as purified protein by CK II. This suggests that the phosphorylation site(s) is *not* located in the common sequence (amino acid residues 437 to the carboxyl terminus). This leaves the location of the phos-

Table 1
Distribution of 150- and 160-kDa proteins and RyR in different SR preparations

SR preparations	Bound [^{3}H]ryanodine (pmol/mg)	Phosphorylated proteins (relative units)		Proteins amount(relative units)		
		150-kDa	160-kDa	150-kDa	160-kDa	RyR
HSR (Saito et al., [23])	2.7	0.37	0.36	0.42	0.3	0.31
Fractions from HSR:						
R_1	1.2	0.49	0.32	0.57	nd	0.06
R_2	4.9	0.65	0.49	0.69	0.37	0.46
R_3	9.1	0.44	0.63	0.60	0.76	0.74
R_4	13.5	0.39	0.61	0.54	0.75	1.00
HSR (Lai et al., [24])	8.3	1.00	0.92	1.00	1.00	0.73
SR (MacLennan, [25])	5.6	0.98	0.99	0.73	0.46	0.62

The different SR preparations were prepared and phosphorylated with the endogenously present kinase in the presence of 0.3 mM [γ-^{32}P]ATP, and 60 mM NaF, as described in Section 2. Ryanodine binding was assayed for 20 min. Quantitative analysis of the protein bands was performed by densitometric scanning of the Coomassie stained gel (for RyR) or Stains-All stained gel (for 160- and 150-kDa proteins), as described in Section 2. The results are the average of 2–4 experiments which differ by 5 to 15%. n.d. = not determined.

phorylation site(s) in one or more of the 12 possible sites for CK II (1–436 amido acids). The exact location of the phosphorylation site(s) in sarcalumenin will be determined in the future by sequencing the isolated phosphorylated peptide(s).

The identity of the phosphorylated 160-kDa protein as the histidine-rich Ca^{2+}-binding protein (HCP) [19] is based on the partial amino acid sequence of the phosphorylated 160-kDa protein, on its staining with Stains-All, its ability

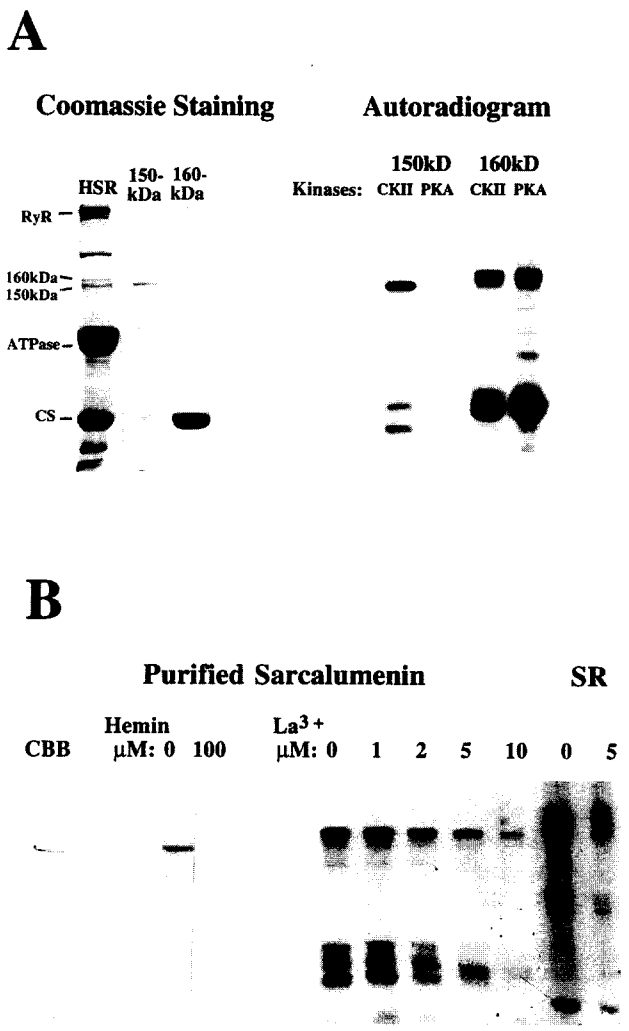

Fig. 6. Phosphorylation of sarcalumenin and HCP by casein kinase II, and cAMP-dependent protein kinase. In (A), the purified sarcalumenin or HCP were phosphorylated in the absence or in the presence of the catalytic subunit of cAMP-dependent kinase (2 μg) or casein kinase II (CK II) (0.05 unit). For CK II, the standard reaction mixture contained 5 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (0.5 μCi/nmol), 20 mM Tris-HCl, pH 7.1, 130 mM NaCl, 2 mM DTT, and also the indicated compounds. For cAMP-dependent phosphorylation the reaction mixture contained 25 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, and 1 mM DTT. The reaction was initiated 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 were subjected to SDS-PAGE (7% acrylamide) and autoradiography, as described in Section 2. (B) shows the inhibition of the phosphorylation of the purified and SR associated sarcalumenin by La^{3+} and of purified sarcalumenin by hemin. HSR membranes were phosphorylated in the absence and the presence of 5 μM La^{3+} as described in Fig. 2.

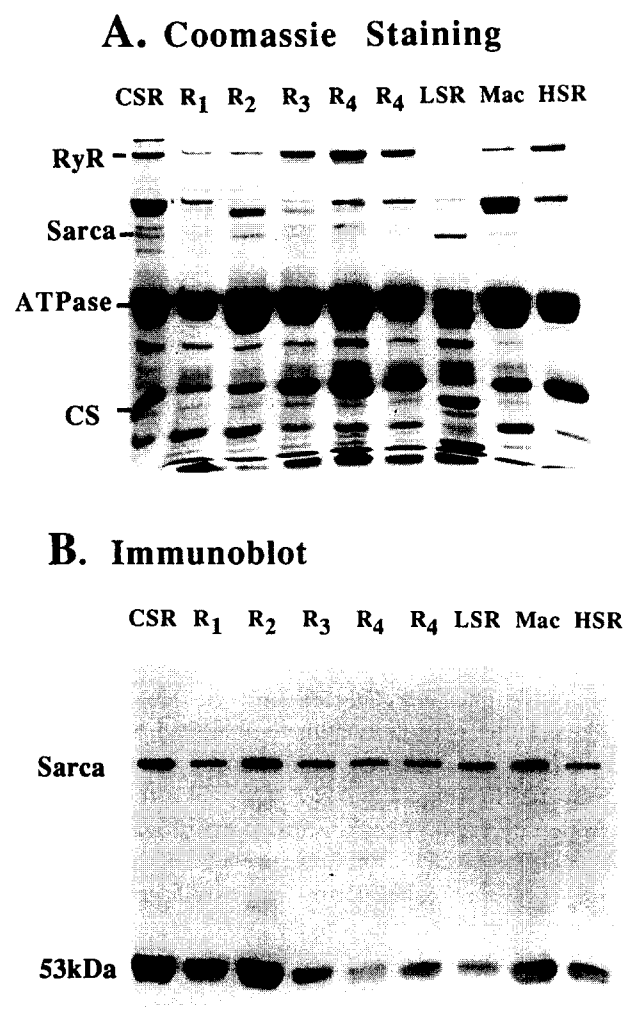

Fig. 7. The distribution of sarcalumenin and the 53-kDa glycoprotein in different SR preparations and SR sub-fractions. LSR (light SR), CSR (crude SR), R_1, R_2, R_3 and R_4 were obtained as in Ref. [23]. HSR and Mac (MacLennan) SR were obtained as described (Refs. [24,25], see also Table 1). The different SR preparations and SR sub-fractions (50 μg) were subjected to SDS-PAGE (6% acrylamide), and to immunoblot staining as described in Fig. 1C. A shows the Coomassie blue staining and B the immunoblot staining, using monoclonal antibodies G_{10}.

to bind Ca^{2+}, and to shift its mobility in SDS-PAGE after Ca^{2+} binding, its heat stability, and its phosphorylation by CK II (identified as the endogenous kinase). Also, as expected for HCP [19], this protein is phosphorylated by the catalytic subunit of cAMP-dependent kinase (Fig. 6). The phosphorylation of HCP by 60-kDa calmodulin-dependent protein kinase and its possible involvement in the modulation of the functional state of the RyR has been presented recently [34].

4.2. The identity of the endogenous kinase that phosphorylates HCP and sarcalumenin

The identification of the SR endogenous kinase that phosphorylates the sarcalumenin and HCP as a CK II type is strongly supported by the following:

Table 2
Effect of CHAPS on the inhibition of ryanodine binding by preincubation with ATP + NaF

Preincubation with	Incubation with CHAPS (%)	Ryanodine bound pmol/mg
AdoPPNH]P		
AdoPP[NH]P + NaF	0	4.7
AdoPP[NH]P + NaF	0.1	7.1
AdoPP[NH]P + NaF	0.6	7.0
ATP + NaF	0	1.1 (77)
ATP + NaF	0.1	7.1 (0)
ATP + NaF	0.6	5.9 (16)

HSR membranes (1 mg/ml) were incubated with 60 mM NaF and 0.4 mM of AdoPP[NH]P (control) or of ATP. After 2 min at 30°C, aliquots were incubated for 5 min at 20°C with the indicated concentration of CHAPS in the presence of 0.5 M NaCl, and then assayed for ryanodine binding as described previously for soluble RyR [33]. The numbers in parentheses indicate % of inhibition. This is a representative experiment from four similar experiments.

(a) CK II phosphorylates the purified sarcalumenin and HCP, and this phosphorylation is inhibited by La^{3+} and hemin (Fig. 6), similar to their endogenous phosphorylation in the SR (Fig. 4 and Ref. [20]). We found that the inhibition by La^{3+} is not specific to sarcalumenin or HCP phosphorylation, but also observed with CK II phosphorylation of purified calsequestrin and casein and of cardiac muscle sarcalumenin (data not shown). In Golgi vesicles inhibition of luminal casein phosphorylation by La^{3+} has been previously reported [34]. Thus, we suggest an interaction of La^{3+} with the kinase and inhibition of its activity.

(b) Inhibitors of CK II inhibited the endogenous phosphorylation of 160/150-kDa proteins (Fig. 4A).

(c) [γ-^{32}P]GTP phosphorylated the 160/150-kDa proteins by the SR endogenous kinase (Fig. 4B), which is known to be a phosphate donor unique to CK II [31].

(d) CK II is present in different SR preparations and subfractions as demonstrated by using specific antibodies against CK II (Fig. 4C). This is in accord with the finding that 160/150-kDa proteins are phosphorylated by the endogenous kinase in these fractions (Table 1). Our results show that CK II is associated with the SR membranes, and it was *not* extracted by EGTA at alkaline pH, but was extracted from these membranes by high NaCl concentration, suggesting its luminal location with its substrate sarcalumenin. Furthermore, inhibition of its activity, in the SR, by the charged inhibitors, hemin and polyaspartate was obtained only in the presence of low concentrations of NP-40. These findings are in accord with the observations that Golgi complex is permeable to ATP and contains an intraluminal CK II which phosphorylates several intraluminal proteins [35,36].

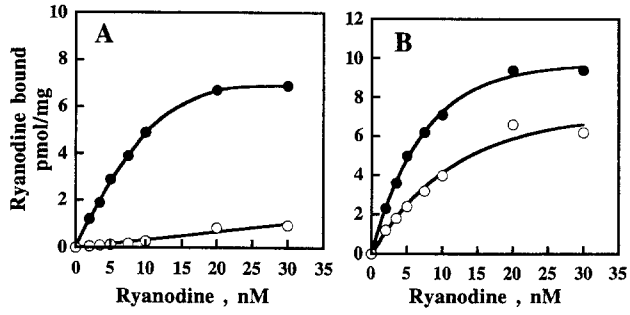

Fig. 8. Effect of CHAPS and phosphorylation of the 150/160-kDa proteins on the total ryanodine binding sites and their affinity. HSR membranes were incubated for 2 min at 30°C, with 0.4 mM ATP or AdoPP[NH]P and 60 mM NaF and then to aliquots CHAPS was added to a final concentration of 0.1%, and the membranes were incubated for 5 min. [^{3}H]Ryanodine binding to unphosphorylated (incubated with AdoPP[NH]P and NaF) (●) and phosphorylated membranes (○) (incubated with ATP and NaF) and then both without (A) or with CHAPS (B), as a function of ryanodine concentration was assayed for 60 min in the presence of 10 μM $CaCl_2$ as described in Table 2.

4.3. The distribution and localization of the HCP and sarcalumenin in SR

HCP and sarcalumenin are present in all structural parts of the SR (Table 1 and Fig. 7). This is in agreement with previous observations indicating uniform distribution of sarcalumenin and HCP through the SR [14,17,19,37,38]. The use of trypsin proteolysis and Endo-H digestion as topological probes (Fig. 2), as well as the extraction with EGTA at alkaline pH (Fig. 5), support the luminal location of the 150-kDa phosphorylated protein/sarcalumenin [12,14]. Our results are in agreement with HCP enrichment in the junctional SR [19]. However, the lumenal location of HCP [17] has been questioned, and its localization on the external side of the junctional SR has been suggested [19].

The phosphorylation of the luminally located sarcalumenin suggests that ATP must cross the SR membrane. Our recent results show that ATP is transported to the SR lumen by a specific 4,4′-diisothiocyanostilbenedisulfonic acid (DIDS)-sensitive transport system [39].

4.4. The structural interactions between RyR and sarcalumenin and / or HCP

The alteration of ryanodine and Ca^{2+}-binding properties of the RyR by sarcalumenin and/or HCP phosphorylation (Fig. 8, Table 2 and Ref. [22]) suggests an interaction between RyR and these proteins. The reversal of the inhibitory effect of sarcalumenin and HCP phosphorylation on ryanodine binding by relatively low concentrations of CHAPS (Fig. 8 and Table 2) indicates that this interaction is modified by the presence of detergent. The separation between the phosphorylated or non-phosphorylated proteins and RyR by sucrose density centrifugation suggests a weak interaction, if at all, between the RyR and these proteins. It is possible that the phosphorylation of these proteins modulates the RyR properties via protein–protein

interaction involving other proteins such as calsequestrin, the 53-kDa glycoprotein, annexin VI and/or triadin. The regulation of Ca^{2+}-release activity by the luminally located calsequestrin, and its specific attachment to the RyR by an anchoring protein, has been suggested [6,9,40,41]. Annexin VI, a Ca^{2+}/phospholipid-binding protein, that is associated with the luminal surface of the SR, has been shown to modify the gating of the Ca^{2+}-release channel [42]. It is possible that sarcalumenin and/or HCP modulates the RyR activity by being part of the Ca^{2+}-mobilizing machinery (movement of Ca^{2+} from the non-junctional SR, where it is pumped by the Ca^{2+}-ATPase to the Ca^{2+}-release channel at the junctional membranes [43]), where its/their effect is controlled by their phosphorylation state. Recently, the phosphorylation of HCP and triadin but not of the RyR/Ca^{2+} channel by the membrane-bound 60-kDa protein kinase has been demonstrated, and a possible involvement of these phosphorylated proteins in the modulation of the functional state of the channel has been proposed [34].

The identification of the HCP and sarcalumenin associated protein(s) is important for the elucidation of the molecular components of the RyR complex, and for understanding the mechanism by which the RyR is regulated by the phosphorylation/dephosphorylation of these proteins.

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