Subcellular fractionation to junctional sarcoplasmic reticulum and biochemical characterization of 170 kDa Ca\(^{2+}\)- and low-density-lipoprotein-binding protein in rabbit skeletal muscle

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Skeletal-muscle sarcoplasmic reticulum (SR) comprises two distinct domains, corresponding to the free membrane of longitudinal SR (LSR) and the junctional membrane region of the terminal cisternae (TC), respectively. The junctional membrane contains the ryanodine receptor (RyR)/Ca\(^{2+}\)-release channel and additional minor protein components that still require biochemical investigation, in relation to excitation–contraction coupling. Recent findings suggested the involvement in this process of a 170 kDa protein [Kim, Caswell, Talvenheimo & Brandt (1990) Biochemistry 29, 9281–9289], also characterized as a phosphoprotein in junctional TC in independent studies [Chu, Submilla, Inesi, Jay & Campbell (1990) Biochemistry 29, 5899–5905]. We show that this protein is a specific substrate of exogenous cyclic AMP-dependent protein kinase, that it is exposed to the outer surface of intact TC vesicles, and that it co-localizes with the RyR to the junctional membrane. Comparative analysis of LSR and TC subfractions for the 160 kDa glycoprotein sarcalumenin, using Western-blot techniques and specific monoclonal antibodies or concanavalin A as a ligand, revealed that the distribution of this protein within the SR corresponds inversely to both that of the RyR and of the 170 kDa protein. The 170 kDa protein, like sarcalumenin, stains blue with the cationic dye Stains-All and binds \(^{45}\)Ca\(^{2+}\) on blots, but it is uniquely distinguished by its ability to bind \(^{131}\)I-labelled low-density lipoprotein. The similarity of these properties, as well as the pI and solubility properties, to those described for the SR protein, recently purified and cloned and named histidine-rich Ca\(^{2+}\)-binding protein [HCP; Hofmann, Brown, Lee, Pathak, Anderson & Goldstein (1989) J. Biol. Chem. 264, 8260–8270], makes it very likely that our protein and HCP may indeed be identical. The protein described in the present study differs from sarcalumenin because its migration in SDS/PAGE is accelerated in the presence of Ca\(^{2+}\), a previously reported property of other Ca\(^{2+}\)-binding proteins [LeMaire, Lund, Viel, Champeil & Moller (1989) J. Biol. Chem. 265, 1111–1123], arguing for Ca\(^{2+}\)-induced protein-conformational changes. Kinase-dependent phosphorylation of our protein is another distinguishing feature, which, although not previously reported for HCP, is consistent with the presence of potential serine/threonine phosphorylation sites in the middle portion of the cloned HCP molecule. The finding that HCP, contrary to early views, selectively binds to the cytoplasmic side of the junctional membrane, together with its newly characterized properties, seem to provide new clues as to a possible role in electromechanical coupling and/or Ca\(^{2+}\) release.

INTRODUCTION

The sequence of events that transduces depolarization of skeletal muscle fibres into release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR), leading to contraction (Fleischer & Inui, 1989), involves a voltage-dependent membrane charge movement in transverse tubules (TT) as the first event (Schneider & Chandler, 1973). The electrophysiological hypothesis of excitation–contraction (E–C) coupling further postulates that the voltage sensor on TT undergoes a conformational change in response to depolarization. The voltage sensor is now believed to be the dihydropyridine (DHP) receptor, on account both of functional studies (Rios & Brum, 1987) and of its subcellular fractionation to the junctional membrane domain of TT in skeletal muscle (Fosset et al., 1983; Salvatori et al., 1990). The problem of the way such a change may be transmitted to the ryanodine receptor (RyR)/Ca\(^{2+}\)-release channel on the apposed junctional membrane of SR terminal cisternae (TC) at the triad junction of skeletal muscle still revolves on the basic question of whether the DHP receptor and the RyR interact either directly (Block et al., 1988) or, alternatively, through additional bridging proteins.

Evidence of protein–protein interactions, both of the DHP receptor (Brandt et al., 1990) and of the RyR (Kim et al., 1990), with a 95 kDa integral protein of junctional SR has been reported recently. It was further shown (Kim et al., 1990) that the 95 kDa protein specifically interacted on affinity columns with a 170 kDa Stains-All blue-staining protein. The SR membrane origin of this protein is in agreement with early findings by Campbell et al. (1983a,b). The relationship of this protein to the phosphoprotein described in an independent study by Chu et al. (1990) in junctional TC, as well as with the 170 kDa doxorubicin-binding protein of Zorzato et al. (1986), is still controversial, however. The proposal that E–C coupling in skeletal muscle could involve a quaternary protein complex (Kim et al., 1990), is also still largely speculative, as is likewise the suggestion that the 170 kDa protein may in turn interact with calsequestrin (CS) on the luminal side of the junctional face membrane (JFM) of TC (Kim et al., 1990).

Several results have been gathered since the original findings by Campbell et al. (1983a,b) supporting the existence in rabbit skeletal-muscle SR of two distinct proteins in the 160–170 kDa range of molecular masses on SDS/PAGE, both of which stain blue with Stains-All and bind Ca\(^{2+}\). These two proteins are the 160 kDa glycoprotein sarcalumenin (Leberer et al., 1989a, 1990)

Abbreviations used: CS, calsequestrin; DHP, dihydropyridine; E–C, excitation–contraction; FITC, fluorescein isothiocyanate; HCP, 170 kDa Ca\(^{2+}\)- and LDL-binding protein (histidine-rich Ca\(^{2+}\)-binding protein); JFM, junctional face membrane; LDL, low-density lipoprotein; LSR, longitudinal SR; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TT, transverse tubule; TC, terminal cisternae.

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and the 165–170 kDa low-density lipoprotein (LDL)- and Ca\(^{2+}\)-binding protein (referred to also as histidine-rich Ca\(^{2+}\)-binding protein: HCP) of Hofmann et al. (1989a,b). Co-localization to longitudinal SR of the Ca\(^{2+}\)-ATPase and the 160 kDa glycoprotein is supported by subcellular-fractionation studies (Leberer et al., 1990), whereas an immunolocalization study of HCP suggested that this protein might be also an intraluminal protein, but diffusely distributed in the SR (Hofmann et al., 1989a).

The present study was undertaken to investigate the pattern of distribution within the SR membrane system of HCP more closely, relative to that of sarcalumenin. To this purpose, we used SR membrane subfractions, consisting of longitudinal tubules, junctional TC (Saito et al., 1984), or the purified JFM (Costello et al., 1986), and specific probes to detect HCP (Hofmann et al., 1989a) and sarcalumenin (Leberer et al., 1989a, 1990) respectively. Our results demonstrate that HCP, as identified by its electrophoretic, LDL- and Ca\(^{2+}\)-dependent protein kinase, TC-bound HCP, beside being shown to contain phosphorylation sites, which seems to be consistent with the deduced primary structure of this protein (Hofmann et al., 1989a), exhibited a Ca\(^{2+}\)-induced shift in electrophoretic mobility, akin to that described for other Ca\(^{2+}\)-binding proteins and which was correlated with Ca\(^{2+}\)-induced changes in protein structure (leMaire et al., 1989). Our results, although incompatible with the model proposed by Kim et al. (1990), seem to provide new clues as to a possible role of HCP (Hofmann et al., 1989a,b) in E–C coupling and/or in control of Ca\(^{2+}\) release in skeletal muscle.

**MATERIALS AND METHODS**

**Materials**

All chemicals were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany) and Carlo Erba (Milano, Italy). Sucrose (ultra-pure grade) was purchased from Beckman Instruments (Palo Alto, CA, U.S.A.). Nitrocellulose was purchased from Hoefer Scientific Instruments (San Francisco, CA, U.S.A.). Phenylmethanesulphonyl fluoride, leupeptin, the cationic dye Stains-All {1-ethyl-3-[3-(1-ethylnaphthyl)l,2-dithiazol-2-ylidene]-2-methylpropenyl]-naphthyl[1,2-d]thiazolium bromide}, Nitro Blue Tetrazolium and 5-chlorobromo-3-indolyl phosphate, cyclic AMP-dependent protein kinase (bovine heart; 1–2 picomolar units/µg) and the catalytic subunit of the cyclic AMP-dependent protein kinase (bovine heart; 30–65 picomolar units/µg) were obtained from Sigma. Molecular-mass standards for SDS/PAGE (phosphorylase b, 97 kDa; BSA, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21 kDa; lysozyme, 14 kDa) were from Bio-Rad (Richmond, CA, U.S.A.). Human \(^{131}I\)-labelled LDL (0.122 µCi/µg) was purchased from Biomedical Technologies Inc. (Stoughton, MA, U.S.A.), \([\gamma-^{32}P]ATP (6000\text{ mCi/mmol}) from New England Nuclear (Du Pont De Nemours, Bad Homburg, Germany), and \(^{45}CaCl_2 (1 \text{ mCi/0.066 mg Ca}^{2+}) was purchased from Amersham International (Amersham, Bucks., U.K.).

**Isolation of muscle membranes**

Predominantly fast-twist muscles (back and hind-leg muscles) from New Zealand male adult rabbits (body weight approx. 2.5 kg) were used. SR membranes were isolated from 20 % (w/v) muscle homogenates, prepared in 0.3 M-sucrose containing phenylmethanesulphonyl fluoride (2 mg/l) and leupeptin (0.5 µg/ml), by the method of Saito et al. (1984), as modified by Inui et al. (1987). Fractionation of SR membranes by isopycnic sucrose-density centrifugation was carried out as described by Saito et al. (1984). The JFM was prepared from TC essentially as described by Costello et al. (1986), by using either the detergent C₆H₄E₆ (Nikko Chemical Co., Tokyo, Japan) or CHAPS instead of Triton X-100. TC membranes were extracted for 30 min at 37 °C with 10 mM-Tris (pH 8.0)/1 mM-EDTA, essentially as described by Duggan & Martinoni (1970).

Protein concentration was determined as described by Lowry et al. (1951), with BSA (Boehringer, Mannheim, Germany) as standard.

**Phosphorylation of SR membranes**

SR membranes (50–60 µg) were incubated for 5 min at 30 °C in 0.1 ml of 20 mM-histidine (pH 7.0)/2.5 mM-MgCl₂/1 mM-dithiothreitol/2.5 mM-EGTA/20 mM-NaF, without or with 2.5 µM cyclic AMP, and in the absence or in the presence of type II protein kinase (10 µg) or of its catalytic subunit (2 µg). The concentration of \([\gamma-^{32}P]ATP was 50 µM. The reaction was stopped by solubilizing in SDS buffer.

**Trypsin treatment of SR membranes**

Isolated TC were incubated in 0.3 M-sucrose/5 mM-imidazole, pH 7.0, at 22 °C for the times and at the trypsin/protein ratios indicated in the legends to Figs. 5 and 6. The reaction was stopped by adding soybean trypsin inhibitor (2:1 ratio to trypsin) and SDS/solubilizing buffer (Laemmli, 1970) to the samples.

**Gel electrophoresis, Western blotting, ligand-overlay experiments and autoradiography**

SDS/PAGE was carried out as described by Laemmli (1970), unless specified otherwise. In some experiments, either 1 mM-CaCl₂ or 0.1 mM-EGTA was included in both the stacking and the separating gels, as described by leMaire et al. (1989). Gels were stained with Coomassie Brilliant Blue, and then with Stains-All (Damiani et al., 1990). Densitometric records of Stains-All-stained gels were obtained with a dual-wavelength Chromato Scanner CS-930 (Shimadzu, Kyoto, Japan). Electrophoretic transfer of protein on to nitrocellulose sheets was carried out as reported previously (Damiani et al., 1990). Blots were used for different types of ligand overlay.

(a) **Fluorescein isothiocyanate (FITC)–concanavalin A binding to SR proteins.** This was done on blots as described previously (Damiani et al., 1986).

(b) **Immunoblotting.** Immunoblotting with mouse monoclonal antibodies to the rabbit 53 kDa glycoprotein (kindly given by Dr. Francisco Michelangeli, University of Southampton, U.K.), was carried out as described by Damiani et al. (1990).

(c) **\(^{125}I\)-LDL overlay.** Overlay with \(^{125}I\)-LDL was carried out under conditions identical with those described by Hofmann et al. (1989a), on blots of SDS gels after electrophoresis in the absence of β-mercaptoethanol.

(d) **\(^{45}Ca overlay.** This was carried out by the technique of Murayama et al. (1984), as described by Damiani et al. (1988). Autoradiography of SDS gels, both after protein phosphorylation with \([\gamma-^{32}P]ATP (Salvatori et al., 1990) and after overlay with \(^{45}Ca (Damiani et al., 1988) or with \(^{125}I\)-LDL (Hofmann et al., 1989a), was done by using Beta-max films (Amersham).

**RESULTS**

**Substrate proteins of cyclic AMP-dependent protein kinase in purified JFM versus intact TC vesicles**

Chu et al. (1990) reported that cyclic AMP-activated kinase-dependent phosphorylation of isolated TC from rabbit skeletal
muscle, in the absence of Ca\(^{2+}\), involves a 170 kDa component, as well as other minor SR proteins, in addition to the RyR. The endogenous phosphorylating activity found in TC was attributed to the presence of TT contaminants, i.e. junctional TT, on account of our independent studies (Salvatori et al., 1990).

We first investigated the full potential of protein phosphorylation sites present in the junctional membrane domain of the SR, using the purified JFM obtained by detergent treatment of TC (Costello et al., 1986), i.e. leaky vesicles, in order to make all internal sites accessible to the endogenous kinase activity, and of calsequestrin, as detected by metachromatic staining with Stains-All

SDS/PAGE was carried out as described by Laemmli (1970), in a 5–10%–polyacrylamide linear gradient gel. Slab gels were stained with Stains-All (Damiani et al., 1990). About 50 μg of protein was loaded per lane. Protein bands indicated by arrows were stained blue. (a) SR subfractions were obtained by the method of Saito et al. (1984), as described by Inui et al. (1987), and are numbered from bottom to top of the gradient. Lanes: 1, heavy fraction, corresponding to junctional TC; 2, intermediate fraction; 3, fraction enriched in LSR; 4, light fraction. (b) JFM was obtained from junctional TC as in Fig. 1(a), except that 1% CHAPS was used. Lanes: 1, molecular-mass standards (see the Materials and methods section); 2, solubilized protein after detergent treatment; 3, purified JFM; 4, TC before treatment. Abbreviation: GP160, 160 kDa Ca\(^{2+}\)-binding glycoprotein (sarcalumenin).
was found to be fully retained after this treatment. When phosphorylated with \([\gamma-32P]\)ATP in the presence of EGTA, and then analysed by SDS/PAGE and autoradiography, the purified JFM showed a pattern of phosphorylated proteins (Fig. 1a), similar to that described by Chu et al. (1990) in isolated TC, under assay conditions also similar to ours, i.e. the RyR (apparent molecular size about 350 kDa), a 170 kDa protein and components in the 98–85 kDa range of mobilities. Furthermore, in the case of JFM, despite these proteins, the protein CS (64 kDa) appeared to be markedly phosphorylated (Fig. 1a).

Protein phosphorylation was enhanced by incubating the purified JFM in the presence of cyclic AMP (Fig. 1a, lane 4), as expected (Salvatori et al., 1990; Chu et al., 1990). It was further enhanced in the presence of exogenous cyclic AMP-dependent protein kinase, the pattern of protein phosphorylation being, however, virtually the same under all conditions (Fig. 1a, lane 2).

The specific cyclic AMP-dependent phosphorylation of the RyR (Seiler et al., 1984) in the purified JFM (Fig. 1a, lane 2) was verified by parallel immunoblot analysis (results not shown) of SDS gels with a polyclonal anti-RyR antibody that had been previously characterized by Zorzato et al. (1989). Western-blot analysis (results not shown) also allowed identification of a relatively more heavily labelled band of about 330 kDa, corresponding to a major proteolytic fragment of the RyR, in agreement with previous evidence (Caswell & Brandt, 1989).

Phosphorylation of the 170 kDa SR protein component (Chu et al., 1990), like that of the RyR and its proteolytic fragment, was found to be considerably enhanced in the presence of exogenous cyclic AMP-dependent protein kinase (Fig. 1a, lane 2).

As opposed to the prominent phosphorylation of CS in the purified JFM, as well as in heavy SR preparations, probably consisting of leaky vesicles, as reported previously (Campbell & Shamoo, 1980), we were unable to find any detectable phosphorylation of this protein in junctional TC, even in the presence both of cyclic AMP and of the catalytic subunit of cyclic AMP-dependent protein kinase, in order to obtain maximal levels of protein phosphorylation (Fig. 1b, lane 2). The apparent inaccessibility of intraluminal CS to the kinase in purified TC vesicles, reflecting their membrane integrity, should consequently mean that kinase-dependent protein phosphorylation in TC might occur exclusively or preferentially at surface-exposed regions, such as the cytoplasmic domain of the RyR (Takeshima et al., 1989; Zorzato et al., 1990).

Fig. 4. Identification of the 170 kDa LDL-binding and Ca\(^{2+}\)-binding protein in SR membrane subfractions

(a) SR proteins after electrophoresis in 7.5 % polyacrylamide Laemmli slab gels under non-reducing conditions (Hofmann et al., 1989a) were transferred on to nitrocellulose. Sheets were stained with Ponceau Red (lanes 1–4), and then incubated with \([\gamma-32P]\)ATP for 60 min at 37 °C (see the Materials and methods section), in the presence of 5 mM-EDTA. \([\gamma-32P]\)-labelled proteins were detected by autoradiography after a 90 min exposure at −80 °C. Lanes: 1 and 5, LSR, 100 μg; 2 and 6, TC, 100 μg; 3 and 7, TC after Tris/EDTA treatment, 40 μg (see Fig. 4c); 4 and 8, supernatant obtained from TC, after Tris/EDTA treatment, 40 μg (see Fig. 4e). (b) \(^{45}Ca^{2+}\) overlay was carried out as described previously (Damiani et al., 1988). First 150 μg of TC (lanes 1 and 3) and LSR (2 and 4) membrane proteins were resolved in a 5–10 % polyacrylamide linear gradient gel, and transferred on to nitrocellulose. Blots were stained with Ponceau Red (lanes 1 and 2), and then incubated with \(^{45}CaCl_2\) (see the Materials and methods section). \(^{45}Ca\)-labelled proteins (lanes 3 and 4) were detected by autoradiography after a 7-day exposure. (c) TC membranes and subfractions, after treatment with Tris/EDTA, were phosphorylated with \([\gamma-32P]\)ATP in the presence of cyclic AMP and of the catalytic subunit of cyclic AMP-dependent protein kinase, as described in the Materials and methods section. Electrophoresis and autoradiography were carried out as in Fig. 1. About 50 μg of protein was loaded per lane. Lanes: 1, supernatant obtained from TC, after Tris/EDTA extraction; 2, TC after extraction; 3, TC before treatment.
The results, although showing that the 170 kDa LDL-binding protein is also a Ca\(^{2+}\)-binding protein, provided further evidence that the same protein, at variance with the 160 kDa glycoprotein, co-localizes with the RyR. It was verified that the 170 kDa Stains-All blue-staining LSR- and Ca\(^{2+}\)-binding protein, like the protein of similar electrophoretic mobility described by Hofmann et al. (1989a,b), was highly acidic. As determined by the method of O'Farrell (1975), our protein electrofocused at pH 5.2–5.4 (results not shown).

In additional experiments, the specific cyclic AMP-dependent phosphorylation of the 170 kDa junctional SR protein was demonstrated, after solubilization of the same protein from junctional TC by treatment with Tris/EDTA at alkaline pH. The effective solubilization of the 170 kDa protein was monitored by staining with Stains-All (Fig. 5a) and by overlay with 1\(^{31}\)P-labelled human LDL, under assay conditions identical with those described by Hofmann et al. (1989a). The results in Fig. 4(a) demonstrate that, in the presence of 5 mM-EDTA, LDL binding was restricted to a single band, corresponding to Ponceau-Red-stained material at about 170 kDa. However, in other experiments, we also noticed a weak labelling of CS, most probably due to the highly acidic properties of this protein (MacLennan & Wong, 1971), and since it is known that polyamionic compounds bind to LDL (see Hofmann et al., 1989a).

Both the 170 kDa protein and the 160 kDa glycoprotein could be identified as Ca\(^{2+}\)-binding proteins on blots by \(^{45}\)Ca-overlay techniques. As shown in Fig. 4(b), the \(^{45}\)Ca-ligand assay conditions used (see Zorzato & Volpe, 1988) were sensitive and specific enough to detect also the Ca\(^{2+}\)-ATPase protein and the RyR, in addition to CS. These results, although showing that the 170 kDa LDL-binding protein is also a Ca\(^{2+}\)-binding protein, provided further evidence that the same protein, at variance with the 160 kDa glycoprotein, co-localizes with the RyR. It was verified that the 170 kDa Stains-All blue-staining LSR- and Ca\(^{2+}\)-binding protein, like the protein of similar electrophoretic mobility described by Hofmann et al. (1989a,b), was highly acidic.
As opposed under iso-osmotic conditions and evidence cleavage i.e. Ca$^{2+}$-induced changes of electrophoretic mobility of SR proteins

Effect of Ca$^{2+}$ on susceptibility to trypsin and the electrophoretic properties of the 170 kDa LDL- and Ca$^{2+}$-binding protein

The results in Fig. 5(b) show that mild trypsin digestion (1 min at 22°C, trypsin/protein ratio 1:5000) of TC vesicles incubated under iso-osmotic conditions and in the absence of salts led to extensive cleavage of the RyR, in agreement with previous evidence (Chu et al., 1988; Shoshan-Barmatz & Zarka, 1988; Meissner et al., 1989), and to the concomitant total disappearance of the 170 kDa protein, as detected by staining with Stains-All. As opposed to these proteins, neither of the two intraluminal SR proteins, i.e. CS (Campbell, 1986) and sarcalumenin (Leberer et al., 1990), was found to be appreciably affected by the same treatment. The most obvious explanation for this finding was that the brief exposure to trypsin did not make the membrane leaky. Therefore, it was verified that CS contained in TC vesicles was not phosphorylated by ATP (see above), by incubating trypsin-treated TC in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase (results not shown).

A specific effect of Ca$^{2+}$ on the susceptibility to trypsin of the 170 kDa protein, as confirmed by the protective effect of EGTA, could be observed at lower concentrations of trypsin, as shown by the densitometric records in Fig. 6, with the 160 kDa glycoprotein as an internal control. These experiments were carried out on a SR subtraction of buoyant density intermediate between that of the LSR and of junctional TC (fraction R 3 from the gradient), and a correspondingly intermediate content of this glycoprotein.

Ca$^{2+}$-dependent changes in susceptibility to proteolytic enzymes, depending on Ca$^{2+}$-induced changes in protein structure, had been previously demonstrated, both for the low-affinity high-capacity Ca$^{2+}$-binding protein CS (Mitchell et al., 1988; Damiani et al., 1989) and for SR Ca$^{2+}$-ATPase (leMaire et al., 1989). It was likewise reported that both these proteins and calmodulin exhibited a Ca$^{2+}$-dependent shift in mobility on SDS gels (leMaire et al., 1989).

When SDS/PAGE of SR subfractions was performed in the presence of 0.1 mM-EGTA, the rate of mobility of main protein components was the same as under standard conditions. In the presence of 1 mM-CaCl$_2$, both the Ca$^{2+}$-ATPase and CS exhibited a faster mobility, as a distinctive feature (Fig. 7a), in agreement with the findings by Campbell & Shamo (1980) and leMaire et al. (1989). Furthermore, the 170 kDa and 160 kDa Ca$^{2+}$-binding proteins, which are resolved in the presence of EGTA, were found to co-migrate in the presence of Ca$^{2+}$ (Fig. 7a). The band that was accelerated by Ca$^{2+}$ was found to be the 170 kDa protein band. In the experiment illustrated in Fig. 7(b), SDS/PAGE was run in the presence of EGTA in the first dimension, and in the presence of Ca$^{2+}$ in the second dimension. The 170 kDa protein was the only protein other than CS exhibiting a strikingly aberrant electrophoretic behaviour, i.e. it also fell distinctly below the diagonal line.

DISCUSSION

The 170 kDa protein that we have identified in purified SR membrane preparations as a Ca$^{2+}$-binding and LDL-binding protein by SDS/PAGE and ligand-blot techniques appears to have many significant properties in common (see Results section) with the SR protein originally characterized and cloned by Hofmann et al. (1989a,b) and named HCP. Our findings further demonstrate that our protein and HCP are not only...
closely related to each other, but also to the 170 kDa Stains-All blue-staining phosphoprotein of junctional SR, investigated by Chu et al. (1990) and by Kim et al. (1990), thus suggesting that they may indeed be the same protein. The 170 kDa protein that we had earlier identified in junctional TC by photoaffinity labelling with [3H]doxorubicin, in addition to the RyR (Zorzato et al., 1986), also shares some common properties with the 170 kDa Ca\(^{2+}\)- and LDL-binding phosphoprotein reported here. The selective association of the 170 kDa protein with the junctional membrane domain of the SR, as indicated by our previous results, fits well with the present evidence of kinase-dependent phosphorylation of this protein in intact junctional TC, in conjunction with that of the RyR occurring at its cytoplasmic domain (Takeshima et al., 1989; Zorzato et al., 1990). As our results also indicate that under the same conditions intraluminal CS was not accessible to either endogenous or exogenous cyclic AMP-dependent protein kinase (see the Results section), that would further argue for a localization of the 170 kDa protein on the external side of JFM, as supported also by its high susceptibility to trypsin, i.e. similar to that of the RyR itself (Chu et al., 1988).

Our findings supporting the contention that HCP, rather than sarcalumenin (Chu et al., 1990), is the actual substrate of cyclic AMP-dependent protein kinase in isolated TC also define a previously undescribed property for the same protein. Studies of HCP by cDNA cloning (Hofmann et al., 1989b) depict a molecule with a calculated molecular mass of about 93 kDa, i.e. much smaller than its apparent molecular mass of 165–170 kDa as determined by SDS/PAGE, having a single hydrophobic segment at the N-terminus, probably acting as a signal sequence for membrane targeting, and a cysteine-rich region in its C-terminal portion, probably involved in binding to LDL. Another striking structural feature of HCP is the presence of acidic repeats in the middle of the protein. Each repeat begins with a histidine-rich sequence, followed by a highly acidic central core, probably corresponding to a Ca\(^{2+}\)-binding site, and then by a serine/threonine-containing segment (Hofmann et al., 1989b). Such hydroxyamino acid residues are potential phosphorylation sites that could account for kinase-dependent phosphorylation of the TC-bound protein. We have been able to confirm that HCP is a specific substrate of cyclic AMP-dependent protein kinase, following purification of the solubilized protein from TC (results not shown) (see below).

Hofmann et al. (1989a), from an immunolocalization study by electron microscopy, suggested that HCP is a luminal protein, diffusely distributed within the SR membrane system. Considering that indirect immuno-gold labelling techniques involving the sequential addition of many antibodies are subject to serious errors of interpretation, such as concerning the precise localization of peripheral membrane proteins, the discrepancy between these findings and our subcellular-fractionation data on HCP could therefore be more apparent than real, given the different methodological approaches. Within this context, the strength of interaction of HCP with SR membrane is also an important variable that needs to be further investigated. The affinity-chromatography data of Kim et al. (1990) provide an indication that HCP is associated with the SR membrane through binding to a 95 kDa integral membrane protein of junctional SR. The high content of anionic amino acids which can bind Ca\(^{2+}\) in HCP (Hofmann et al., 1989b), together with our present evidence that this protein can be detached from TC by EDTA treatment at alkaline pH (see the Results section, Fig. 5), would in turn suggest the possible involvement of Ca\(^{2+}\) bridges in its binding to the membrane. We have further found (E. Picello, E. Damiani & A. Margreth, unpublished work) that HCP remained tightly bound to membrane in CS-depleted JFM preparations obtained by treatment with 0.5 M-NaCl, as described by Ikemoto et al. (1989), and that the subsequent treatment with low-ionic-strength solutions containing 1 mM-EDTA or -EGTA, and at alkaline pH, solubilized it completely.

There is still little knowledge of the Ca\(^{2+}\)-binding characteristics of HCP, in terms of affinity and maximum binding capacity for Ca\(^{2+}\), as compared with other Ca\(^{2+}\)-binding proteins (Hofmann et al., 1989a,b). However, a previously undescribed property, linking HCP to other muscle Ca\(^{2+}\)-binding proteins, is the observed acceleration in its electrophoretic mobility on SDS gels run in the presence of Ca\(^{2+}\). For CS, the Ca\(^{2+}\)-dependent shift in mobility was attributed to the more compact molecular structure of this protein in the Ca\(^{2+}\)-bound form (LeMaire et al., 1989), as directly shown previously by Cozens & Reithmeier (1984) and by Mitchell et al. (1988). Although it remains to be demonstrated that HCP may undergo somewhat analogous Ca\(^{2+}\)-dependent changes in conformation, the hypothesis is indirectly supported by the present evidence of an increased susceptibility to trypsin of TC-bound HCP in the presence of Ca\(^{2+}\), implying that surface-exposed regions become expressed when Ca\(^{2+}\) binds to the protein, and consequently might be more accessible to trypsin. A previously noted effect of Ca\(^{2+}\) on the 170 kDa protein is the inhibition by Ca\(^{2+}\) of kinase-dependent phosphorylation of this protein in isolated TC (Chu et al., 1990). We were able to confirm this effect in the presence of cyclic AMP and of exogenous protein kinase (results not shown).

On account of the present experimental findings, several arguments can be made as to a possible role of the 170 kDa junctional SR protein in control of Ca\(^{2+}\) release (Zorzato et al., 1986) and/or in electromechanical coupling in skeletal muscle (Kim et al., 1990). One simple view that encompasses other, still fragmentary, pieces of information on specific components of junctional SR, in addition to the RyR (Zorzato et al., 1986; Kim et al., 1990; Chu et al., 1990; Damiani & Margreth, 1990), which also seems to be able to put them together with available structural knowledge of HCP (Hofmann et al., 1989b), can be expressed as follows. It appears that the solubility properties of HCP satisfy the characteristics of a peripheral membrane protein. Its selective binding to the junctional membrane of the SR might involve a specific interaction with the 95 kDa integral protein, but unlike that proposed by the model of Kim et al. (1990), at the cytoplasmic domain of this protein, rather than at the luminal domain. There is in addition no evidence, as investigated by protein-overlay techniques, that the 170 kDa junctional SR protein may be able to interact with CS, either in the presence of Ca\(^{2+}\) or in its absence (Damiani & Margreth, 1990), and that it might therefore correspond (Kim et al., 1990) to the anchoring protein filaments described morphologically by Franzini-Armstrong et al. (1987). Co-localization to junctional TT of the DHP receptor and of cyclic AMP-dependent protein kinase, taken together with the present evidence that TC-bound HCP is freely accessible to the kinase, whether TT-bound endogenously or exogenously added, suggest that HCP is exposed to the cytoplasmic side of the junctional membrane of TC, facing TT in the intact triad structure. What is much less clear, on account of the present experimental evidence, is whether Ca\(^{2+}\)-dependent changes in protein structure, as well as in protein phosphorylation (Chu et al., 1990), might influence the interaction of HCP with other junctional SR proteins, such as the 95 kDa protein or a third protein (Kim et al., 1990). Although the present evidence thus seems to provide new interesting clues as to a possible functional role of HCP in SR function (Hofmann et al., 1989a,b), much further work is required to answer these various questions.

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