Distribution of Ca^{2+}-Modulating Proteins in Sarcoplasmic Reticulum Membranes After Denervation

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Abstract. The early response to the loss of motor innervation to the muscle is connected with an altered Ca^{2+}-homeostasis. Our study, based on Western blotting, indicates that denervation influenced expression of some sarcoplasmic Ca^{2+}-modulating proteins. Evidence has been brought for an increase of the level of calsequestrin and of the putative ryanodine receptor paralleled with a slight decrease of the total amount of Ca^{2+}-pump protein. The expression of unchanged Ca^{2+}-pump isoform and unaltered quantities of other non-junctional Ca^{2+}-binding proteins support the hypothesis that changed cellular Ca^{2+} homeostasis include also an alteration of Ca^{2+}-modulating systems, mainly from the junctional region of sarcoplasmic membranes.

Key words: Ca^{2+}-modulating proteins — Sarcoplasmic reticulum — Denervation

Introduction

The free cytosolic Ca^{2+} concentration is the primary determinant for contractile activation in muscles, and it is also known to be centraly involved in the regulation of a variety of cellular functions in non-muscle cells. The proteins involved in cellular Ca^{2+}-modulation and localized either in the plasma membrane, the cytoplasm or in intracellular stores can be classified as Ca^{2}-pumps, Ca^{2+}-release and Ca^{2+}-binding proteins (Pietrobon et al. 1990).

The Ca^{2+}-uptake by the intracellular stores of striated muscle cells is mediated by at least two different isoforms of the ATP-dependent Ca^{2+}-pump: isoform SERCA 1 which corresponds to the Ca^{2+}-pump in fast-twitch skeletal muscle sarcoplasmic reticulum, and SERCA 2 isoform which is expressed in slow skeletal and

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cardiac muscle cells (Missiaen et al. 1991).

The sequestration of Ca^{2+} in the intracellular Ca^{2+}-stores is thought to be facilitated by luminal Ca^{2+}-binding proteins that lower the concentration of free Ca^{2+}. Storage of Ca^{2+} within the sarcoplasmic reticulum (SR) of striated muscle is enhanced by calsequestrin, a low-affinity high capacity Ca^{2+}-binding protein which is localized in the lumen of the junctional SR (MacLennan and Wong 1971). The cardiac and fast-twitch skeletal muscle isoforms of calsequestrin are products of two different genes (Fliegel et al. 1987).

Skeletal muscle expresses also a high-affinity Ca^{2+}-binding protein, in addition to calsequestrin (Ostwald and MacLennan 1974). This protein is identical to the protein which was independently identified by others in non-muscle tissues and which has been designated calreticulin (Fliegel et al. 1989). It was suggested that calreticulin may function as a non-muscle analogue of calsequestrin (Milner et al. 1992). Furthermore, other acidic Ca^{2+}-binding proteins that may play a role in Ca^{2+} sequestration have been described in the SR of skeletal and cardiac muscle: e.g. the 160 kDa Ca^{2+}-binding glycoprotein or sarcalumenin (Leberer et al. 1989), or the 170 kDa histidine-rich Ca^{2+}-and LDL-binding protein (HCP) (Hofmann et al. 1989).

The aim of this study was to investigate, by SDS-PAGE and Western-blot analysis, the presence and distribution of the Ca^{2+}-modulating proteins in sarcoplasmic reticulum of rabbit fast-twitch skeletal muscle and to see whether their expression is affected by denervation.

Abbreviations: SERCA-sarco/endoplasmic reticulum Ca^{2+}-pump; Ca^{2+}--ATPase-Ca^{2+},Mg^{2+}-activated ATPase; CBB-Coomasie Brilliant Blue-G-250; SDS-PAGE-sodium dodecylsulphate polyacrylamideelectrophoresis; SR-sarcoplasmic reticulum; DHP-receptor-dihydropyridine receptor; MAb-monoclonal antibody; CS-calsequestrin; CR-calreticulin; GP-53-53KDa glycoprotein; GP-160-160 kDa glycoprotein; RyR-ryanodine receptor

Materials and Methods

Materials

All chemicals were of analytical grade and were obtained from Serva, F.R.G. and Sigma Chemicals, U.S.A. Immobilon-P was purchased from Millipore,U.S.A. Phenylmethylsulphonyl fluoride(PMSF), leupeptin, pepstatin, Coomasie Briliant Blue-G-250 were from Serva, F.R.G. The cationic dye Stains All was from Kodak, U.S.A. Molecular mass standard for SDS-PAGE(myosin,205 kD, beta- galactosidase, 116 kD, phosphorylase b, 97,4 kD serum albumin, 68 kD, egg albumin, 45 kD, carbonic anhydrase, 29 kD) were from Sigma Chemicals, U.S.A.

Monoclonal antibodies

The MAb specific for the fast-twitch skeletal muscle isoform of the Ca^{2+}-pump (SERCA 1) MAb A52 was kindly provided by dr D MacLennan (Univ Toronto), the MAb specific against isoform SERCA 2 (nondiscriminating between the muscle and non-muscle isoforms) MAb IID8 was kindly provided by dr K Campbell (Univ Iowa) The MAb specific against muscle isoform SERCA 2a MAb AS2 was prepared as described by Eggermont et al (1990), monoclonal antibodies G10D7 directed against both the 160 kDa (sarcalumenin) and 53 kDa glycoprotein were prepared as described by Raeymaekers et al (1992) The MAb specific against calsequestrin was prepared as decribed by Yazaki et al (1990) and Raeymaekers et al (1992) The MAb specific against calreticulin was kindly provided by dr M Michalak (Univ Alberta)

Methods

Male adult rabbits (Velaz, Prague,) were used Under pentobarbital anesthesia, the fast-twitch muscles of the hind leg (m gracilis, m vastus) were denervated by unilateral section of 1 cm of the sciatic nerve as described by Lehotsky et al (1991) The control group of animals were sham-operated

Fourteen days after denervation the sarcoplasmic reticulum vesicles from the above muscles were prepared by a modified method of Caswell et al (1976) as described in Lehotsky et al (1991) using PMSF (0 1 mol l^{-1}), benzamidine (0 5 mmol l^{-1}), leupeptin (0 5 mmol l^{-1}), aprotinin (0 5 μg ml^{-1}) as protease inhibitor

Protein concentration was determined as described by Lowry et al (1951) with bovine serum albumin as standard

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970), using 7 5% slab gels Gels were stained with CBB and then with Stains All (Campbell et al 1983)

Electrophoretic transfer of protein onto Immobilon-P sheets was carried out as reported by Towbin et al (1979) Blots were used for different types of immunoligand overlay Primary antibody reaction was carried out for 1 h at room temperature with specific antibodies, and secondary antibody reaction was performed with peroxidase-conjugated IgG for 1 h at room temperature For antibody staining the system 4-chloro-1 naphtol and H_2O_2 were used

Densitometric records were obtained by scanning the gels with an ERI-65 densitometer (Zeiss Germany)

Results

Fig 1*A* shows the electrophoretic protein profile of sarcoplasmic reticulum membranes of the control rabbit muscle and from animals two weeks after denervation The predominant presence of 105 kDa protein (Ca^{2+} pump), 64 kDa calsequestrin and of two lower molecular weight proteins appeared to be characteristic feature in both the control and the vesicles from denervated muscle The vesicles from the denervated muscle also shared several of the minority protein components detected for control SR, e i the 350 kDa high molecular weight protein (putative ryanodine receptor) and the 160 kDa glycoprotein The 170 kDa Ca^{2+}-binding protein

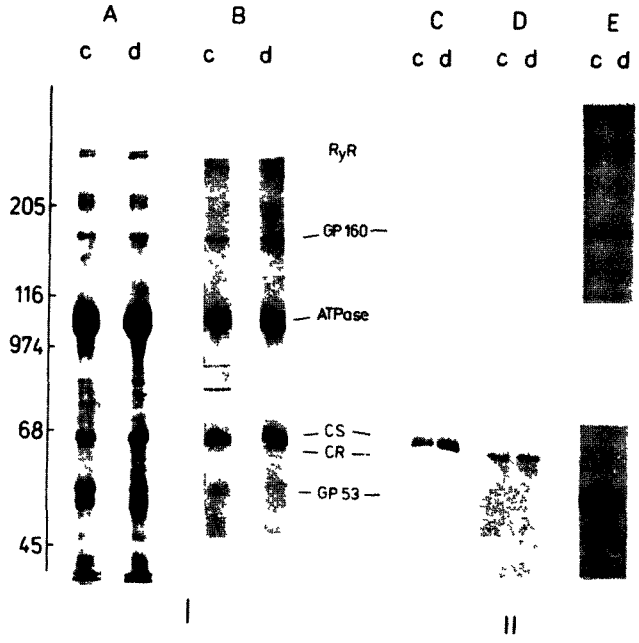

Figure 1. I. SDS-PAGE protein pattern of sarcoplasmic reticulum membranes of control and denervated muscles as detected by CBB (*A*) or by metachromatic staining with Stains All (*B*). II. Identification of luminal Ca^{2+}-binding proteins in SR membranes by immunoblot technique (*C*, *D*, *E*). Thirty μg of SR protein was loaded per lane. Protein bands corresponding to the glycoproteins GP-160 and CS in (*B*) stained ultramarine blue,other proteins were not quantitatively stained red or pink (Campbell et al. 1983). SR protein after electrophoresis was transferred onto Immobilon-P sheets. Blots were incubated with monoclonal antibodies to calsequestrin (*C*), with monoclonal antibodies to calreticulin (*D*) and monoclonal antibodies to 53 kDa and 160 kDa glycoproteins (MAb 10D7) (*E*). In all experiments, SR from control muscles is denoted (c), SR from denervated muscles (d)

(HCP) and calreticulin were barely detectable on gels stained by CBB. In addition, Fig. 1*B* shows the unambiguous presence of the 160 kD Ca^{2+}-binding protein sarcalumenin and calsequestrin (metachromatic staining with Stains All). Fig. 1*C* and 1*D* show the results of probing both the low-affinity Ca^{2+}-binding protein calsequestrin and the high affinity calreticulin by immunoblotting. Although calreticulin was barely detectable by CBB staining, immunoanalysis clearly showed the presence of this protein. However, the calreticulin contents of controls and of vesicles from denervated muscle were very similar.

When using anti-SERCA 1 (MAb A52) and anti-SERCA 2a (MAb AS2) antibodies on Western blots of sarcoplasmic reticulum membranes (Fig. 2) only fast

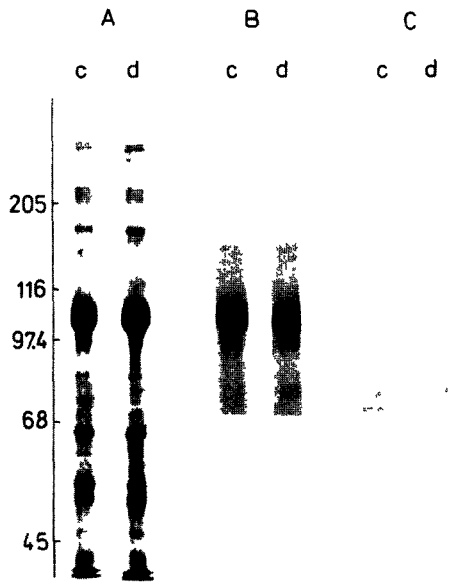

Figure 2. Expression of SERCA protein isoforms in control and denervated sarcoplasmic reticulum membranes. SR proteins were run as described in legend to Fig. 1 and transferred onto Immobilion-P sheets. Blots were incubated either with monoclonal antibodies to SERCA 1 (fast) (MAb A52) (*B*) or with monoclonal antibodies to SERCA 2a (slow-cardiac) (MAb AS2) (*C*). SR from control muscles is denoted (c), SR from denervated muscle (d).

isoform (SERCA 1) could be detected in control as well as in denervated muscle. Western blots were also negative for SERCA 2 when using the SERCA 2 specific (MAb IID8) antibody, which does not discriminate between "a" (muscle) and "b" (non-muscle) isoforms (results not shown).

Fig. 3 illustrates the results of a quantitative analysis of densitometric scanning of different stainings and blotting of sarcoplasmic proteins. Like in previous experiments (Salvatori et al. 1988; Lehotský et al. 1991), SR vesicles from denervated muscle displayed only a slight decrease of the Ca^{2+}-pump protein (93% of control level). Further, an increase of calsequestrin content after denervation and an increase from the 0.121 to 0.179 of the ratio of calsequestrin to the Ca^{2+}-pump protein could be shown. Also, the content of the high molecular weight ryanodine receptor was increased about twice in denervated muscle.

The presence of the 160 kDa glycoprotein (sarcalumenin) and the 53 kDa sarcoplasmic glycoprotein was demonstrated unambigously by staining with Stains All as well as by immunoprecipitation with monoclonal antibody (MAb 10D7) (Fig. 1*E*). Although the amount of 160 kD glycoprotein (stained by CBB) seemed to rise after denervation (Fig. 1*A*), this finding was not confirmed by the data from immunoblots (Fig. 1*E*). No significant differences could be found between the control and denervated muscle (Fig. 3).

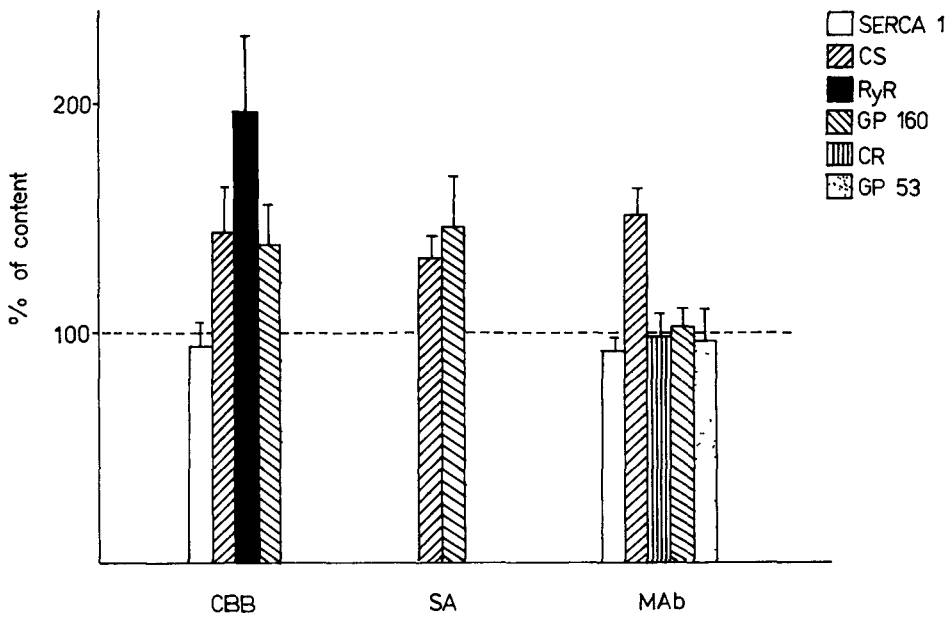

Figure 3. Relative contents of SERCA 1 protein, calsequestrin, 350 kDa protein, 160 kDa or 53 kD glycoproteins and calreticulin of sarcoplasmic reticulum membranes from denervated muscles, as quantified by densitometric scanning of Coomassie brilliant blue (CBB), Stains All (SA), and antibodies (MAb) stained gels and blots and normalized to 100% of the corresponding proteins from control muscles. Values are means S.E. ± from 6 determinations from 3 different preparations. The S.E. of controls ranges between 5 to 10% of the means. The contents of calsequestrin and the 350 kDa protein were significantly different from that of the controls ($P < 0.005$).

Discussion

The early response to loss of motor innervation to the muscle is associated besides other changes, with an altered Ca^{2+} homeostasis and an increase of Ca^{2+} in the muscle cell (Picken and Kirby 1976; Palexas et al.1981; Zorzato et al.1989). These findings can in general be explained by a modification of the major cellular Ca^{2+} modulating systems. Thus, the response to the denervation of the surface membrane is associated primarily with an increase of the density of ionic channels such as the extrajunctional acetylcholine receptors, DHP-receptors (Ca^{2+}-channel) as well as Na^{+}-channels isoform transitions (Shieh et al.1988; Yang et al.1991; Lehotský et al. 1991). In contrast, the cytosolic Ca^{2+}-binding protein parvalbumin was shown to decrease in the myoplasm of denervated muscle (Leberer and Pette 1986).

At the level of the sarcoplasmic reticulum membrane the possible alterations

could include the Ca^{2+}-uptake and Ca^{2+}-release mechanisms as well as the Ca^{2+}-binding luminal proteins. The changes might result either from an alteration in gene expression, e.g. in protein synthesis rate or in expression of other protein isoforms. Another possibility could be the functional modification of major Ca^{2+} modulating systems.

In fact, denervation induces the proliferation of junctional SR (Salvatori et al. 1988; Lehotský et al. 1991), a significant modification of functional properties of the Ca^{2+}-release channel (Zorzato et al. 1989) with a parallel decrease of Ca^{2+}-uptake and Ca^{2+}-ATPase activities (Palexas et al. 1981). Although in our experiments we could demonstrate no appreciable differences in total qualitative protein pattern between denervated and control sarcoplasmic vesicles, the quantitative results are in good agreement with these findings. Differences in the protein profile were manifested by a decrease of Ca^{2+}-pump protein parallelled mainly by an increase of the ratio of calsequestrin to Ca^{2+}-ATPase as well as an increase in the relative amount of the 350 kDa protein (putative ryanodine receptor).

Another possible explanation for the reduced Ca^{2+}-uptake and ATPase activities might be the Ca^{2+}-pump protein isoform transition as was described earlier on avian skeletal muscle sections after long-term denervation (Kaprelian et al. 1991). This fenomenon, however can be excluded in our experiments because after denervation we were not able to detect other SERCA isoforms besides SERCA 1. It should be mentioned in this respect that, similar to the study of Kaprelian et al. (1991), the two-week period of denervation may be too short to allow expression of other SERCA isoforms.

Alternatively, the decrease of SR transport activity after denervation could also be due to a modification of the enzyme itself, e.g. at its nucleotide binding site or due to an increase of the turnover (degradation) of the ATPase protein, similarly as demonstrated in chronic muscle stimulation experiments or chemically denervated muscle cells in culture (Dux et al. 1990; Charuk et al. 1992).

The major representatives of luminal proteins of SR/ER membranes are calsequestrin and calreticulin. Both proteins bind and store Ca^{2+} accumulated by the Ca^{2+}-pump protein (Fliegel and Michalak 1991). However, in contrast to calsequestrin which is a muscle specific protein of the SR membrane, calreticulin is an endoplasmic reticulum house-keeping protein of muscle and non-muscle cells (Milner et al. 1992). The increase of the relative amount of calsequestrin indicates that denervation influences expression of at least some luminal Ca^{2+}-binding proteins. Based on the localisation of calsequestrin this finding also supports a previous conclusion on denervation-induced proliferation of junctional SR (Zorzato et al. 1989). Unfortunately, due to the source of the antibody it was not able to detect the possible presence of the cardiac isoform (a component of slow-twitch muscle) (Fliegel and Michalak 1991) in addition to fast calsequestrin. Thus, the question remains open whether calsequestrin isoform transition also occur after denervation, as has

been observed for SERCA or Na^+-channel isoforms (Kaprelian et al. 1991; Yang et al. 1991).

Sarcalumenin (160 kDa glycoprotein) and 53 kDa glycoprotein are other luminal sarcoplasmic proteins residing at the inner side of the membrane and are thought to be involved in sequestration of Ca^{2+} but in the non-junctional region of SR (Leberer et al. 1990). The proposed role of the 53 kDa glycoprotein in the regulation of Ca^{2+} transport (Kutchai et al. 1991) was however not confirmed in reconstitution experiments (Grimes et al. 1991). Our present findings demonstrating no changes in the levels of both glycoproteins are not in contrast to the view that denervation affects mainly junctional SR membrane.

In summary, the present study indicates that denervation of fast-twitch skeletal muscle influenced expression of several Ca^{2+} modulating proteins of the sarcoplasmic reticulum. Evidence was brought for an increase of the levels of calsequestrin and of the putative ryanodine receptor paralleled by a decrease of the total amount of the Ca^{2+}-pump protein. Unchanged expression of SERCA protein isoforms and unaltered quantities of other non-junctional Ca^{2+}-binding proteins support the hypothesis that after denervation the changed cellular Ca^{2+}-homeostasis includes an alteration of Ca^{2+}-modulating systems, mainly from the junctional region of sarcoplasmic membranes. It might be worthwhile to check out whether denervation also leads to modification of the 170 kDa Ca^{2+} and the LDL-binding HCP protein (also a protein of the junctional region) (Damiani and Margreth 1991). An argument in favor of this can be an elevated level of sarcoplasmic membrane cholesterol after denervation (Lehotský et al. 1991) where a possible role of the HCP protein in the accumulating process can not be excluded.

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