Effects of Denervation on the Contents of Cholesterol and Membrane Systems Involved in Muscle Contraction in Rabbit Fast-Twitch Sarcotubular System

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Abstract. Denervated fast-twich rabbit muscles were progressively losing their fresh weight and the yield of sarcotubular protein was increasing. The activity of Ca^{2+}-ATPase was affected but very slightly, the basal Mg^{2+}-ATPase and the Mg^{2+}-ATPase/Ca^{2+}-ATPase ratio however increased together with a simultaneous depression of the membrane-bound acetylcholinesterase activity. We did not observe any differences in density properties of sarcotubular fractions between control and denervated muscle. However, a relative enrichment in SM and H fraction could be seen after denervation with small changes in the content of the Ca^{2+}-pump protein, increased levels of calsequestrin and cholesterol, mostly in the heavy and the SM fraction. After denervation the binding sites for ^{3}H-PN-200-110 did not show any changes in receptor affinity, but the number of putative Ca^{2+}-channels increased twice along with a depression of ^{3}H-ouabain binding sites. We suggest that the denervation of fast-twitch muscle leads to the hypertrophy of the junctional sarcoplasmic reticulum and the T-system. Changes in the cholesterol content, in the number of putative Ca^{2+}-channels and in Na^+, K^+-ATPase can affect the muscle contraction.

Key words: Denervation — Sarcotubular fractions — Cholesterol — Enzyme and binding activities

Introduction

Resection of the motor nerve of skeletal muscle affects not only the total and

Abbreviations used: PMSF-phenylmethylsulphonyl fluoride; B_{max}-maximal binding capacity; K_D-dissociation constant; SDS-PAGE-sodiumdodecylsulphate polyacrylamide gel electrophoresis; DHP-dihydropyridine; EGTA-(2-aminoethyl)glycolether-N,N,N',N'-tetraacetic acid; STS-sarcotubular system; SM-very light fraction; LSR- light sarcoplasmic reticulum; HSR-heavy sarcoplasmic reticulum; j-SR-junctional sarcoplasmic reticulum; T-tubule-transverse tubule; E—C coupling — excitation-contraction coupling.

contractile proteins (Davis and Kiernan 1980; Gillis 1985) but it also changes the composition and transport properties of the surface membrane (Leung et al. 1984). Generally, it raises the concentration of proteins of some outer membrane transport systems, i.e. acetylcholine receptor and dihydropyridine receptor, or sodium channels (Shieh et al. 1988a; Guo et al. 1987; Schmid et al. 1984a).

Previous studies of isolated sarcoplasmic reticulum (SR) after denervation concerned mainly the Ca^{2+}-ATPase and Ca^{2+}-release (Palexas et al. 1981; Tate et al. 1983; Heiner et al. 1984; Zorzato et al. 1989). Conflicting results have been reported concerning changes in the protein and lipid composition of SR after denervation: this was probably due to differences in animal species, the type of fiber composition and in the time interval after denervation.

We addressed this issue by studying the lipid and protein composition and enzyme activities of isolated sarcotubular systems (STS) and separated STS fractions two weeks after denervation. We could show that denervation affects the numbers of putative Ca^{2+}-channels and Na^+, K^+-ATPase. Our results also indicate that the early effects of denervation on STS membranes concern mainly junctional sarcoplasmic reticulum and transverse tubules (T-tubules).

Materials and Methods

+(Methyl)^3-H-PN-200-110, specific activity 3.07 TBq.mmol^{-1}, ^{3}H-ouabain, specific activity 1.03 TBq.mmol^{-1} (Amersham, England), nitrendipine, ouabain (Sigma), Ca^{2+}-ionophore (A-23187), ATP-Na salt, acetylthiocholiniodine, eserine, PMSF, SDS (Serva, FRG); other chemicals were from Lachema (Czechoslovakia).

Male adult rabbits (Velaz, Praha) were used. The fast-twitch muscles (m. gracilis, m. vastus, m. semimembranosus) of the hind leg were denervated by unilateral section of the sciatic nerve under pentobarbital anesthesia. The control group of animals was sham-operated. The animals were killed on postdenervation day 14—16. Sarcotubular system (STS) vesicles from the above muscles were prepared according to the modified method of Caswell et al. (1976) using PMSF (0.1 mmol.l^{-1}) as protease inhibitor. Isolated STS vesicles were contaminated to less than 6 percent by other subcellular membrane components, the protein profil exhibited the three typical fractions, the 102 kD protein (Ca^{2+}-pump) making up 45—50 percent of the total proteins (Lehotský et al. 1986). STS vesicles were fractionated by discontinuous sucrose gradient density centrifugation at 85.000 xg for 16 hours according to Rosemblatt et al. (1981). The heavy and light sarcoplasmic reticulum fractions (HSR and LSR) were collected at the interphase 40—50 % and 35—40 % sucrose layers, the T-tubule fraction (SM) from the limit 0—25 % sucrose. Protein concentrations were determined according to Lowry et al. (1951) with bovine serum albumin as a standard.

Binding assay with the Ca^{2+} antagonist dihydropyridine (+)^{3}H-PN-200-110

Binding was carried out in 50 mmol.l^{-1} Tris-HCl pH-7.4; 1 mmol.l^{-1} $CaCl_2$ in the presence of 1—10 nmol.l^{-1} ^{3}H-PN-200-110. Nitrendipine (0—5μmol.l^{-1}) was added to estimate K_D and B_{max}. Samples of approximately 100 μg protein containing ^{3}H-PN-200-110 in a final volume of 1 ml of

buffer were incubated for 1 hour at 22 °C. Because of the light sensitivity of PN-200-110, all steps were carried out in the dark. After the incubation, 10 μl of albumin (5 mg . ml $^{-1}$) was added and the samples were filtered through Whatman GF C glass filters and washed twice with 5 ml of buffer without dihydropyridine and protein. The radioactivity of the filters was measured in SLT-41 scintillation cocktail (Spolana, Czechoslovakia) on Tri-Carb 300C. The specific binding was determined by subtracting nonspecific binding, obtained in the presence of excess of unlabelled nitrendipine, from total binding. B_{max} and K_D were estimated from Scatchard plots (Scatchard 1949).

Binding assay with ^{3}H-ouabain using saponin

Binding of ^{3}H-ouabain promoted by ATP and sodium ions was carried out according to Hidalgo et al. (1986) in a total volume of 1 ml buffer containing (in mmol . l $^{-1}$): 120 NaCl, 10 $MgCl_2$, 1 EGTA, 10 ATP, 40 Tris-HCl, pH-7.4; 0.3 g . l $^{-1}$ saponin, 60 – 100 μg protein and 1 – 160 nmol . l $^{-1}$ ^{3}H-ouabain. Unlabelled ouabain (50 – 100 μmol . l^{-1}) was added and ATP was omitted to estimate nonspecific binding. The samples were incubated for 60 min. at 37 °C and filtered through Whatman GF C glass filters and washed twice with 4 ml of buffer without ouabain and protein. Scintillation cocktail SLT-41 was added and the radioactivity of each sample was measured.

Determination of enzyme activities

Ca^{2+}-Mg^{2+}-ATPase activity (E.C.3.6.1.36) and basal Mg^{2+}-ATPase was determined according to Lehotský et al. (1987a) with or without Ca^{2+} ionophore (A 23187) at 37 °C by measuring released P_i. Membrane bound sarcotubular acetylcholinesterase (E.C.3.1.1.7) was analyzed spectrophotometrically at pH 8.0 and in the presence of 10 mmol . l $^{-1}$ Na-deoxycholate as described by Vidal et al. (1987) for sarcotubular acetylcholinesterase.

Other determinations

One dimensional SDS-PAGE was carried out according to Laemmli (1970), using 12 percent slab gels stained with Coomassie brilliant blue. Densitometric scanning of slab gels was carried out in a ERI-65 densitometer (Zeiss, GDR). Total lipid phosphorus in different fraction was determined in lipidic extracts (Folch et al. 1957) according to Lowry and Lopez (1946), and cholesterol plus cholesterol esters contents were determined enzymatically by the method of Sale et al. (1984).

Results

Two weeks after denervation of rabbit fast-twitch muscles a progressive loss of fresh weight could be observed similarly as reported earlier for amphibian and mammalian muscles (Barany et al. 1965; Sreter 1970), and the yield of STS protein increased (data not shown) similarly as described in other studies, (Muscatello et al. 1965; Palexas et al. 1981; Salvatori et al. 1988). SDS-PAGE analysis of microsomal proteins of the denervated and control vesicles showed three main typical bands (102, 64, 55 kD) without marked differences in the protein range examined. Only a slight decrease (from 57 to 53 %) of the 102 kD protein (Ca^{2+}-pump) could be observed together with an insignificant growth of

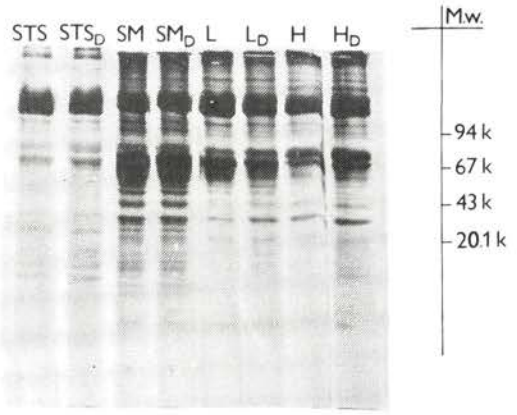

Fig. 1. SDS-PAGE of total STS membranes and separated fractions from control and denervated muscle according to Laemmli (1970). STS, STS$_D$-sarcotubular membranes from control and denervated muscle; SM, SM$_D$-very light fractions from control and denervated muscle; L, L$_D$- light fractions from control and denervated muscle; H, H$_D$- heavy fractions from control and denervated muscle. Standards used: phospohorylase b (94 kD); bovine serum albumin (67 kD); ovalbumin (43 kD); carbonic anhydrase (30 kD); soyabean trypsin inhibitor (20.1 kD); lactalbumin (14.4 kD); (Pharmacia Fine Chemicals, Sweden).

the content of two main minor 64 and 55 kD proteins (calsequestrin and Ca^{2+}-binding protein) (Fig. 1, 2).

The inherent sarcotubular "extra" Ca^{2+}-ATPase activity in denervated and control vesicles in the presence of Ca^{2+}-ionophore A-23187 (which uncouples Ca^{2+}-transport from ATP hydrolysis) showed no remarkable changes in the extent of enzyme stimulation. This suggests that STS vesicles from both sources are equally sealed. However, in denervated vesicles, we observed a slight decrease in the activity of microsomal Ca^{2+}-ATP-ase, in agreement with the protein profile as determined by SDS-PAGE (Table 1).Simultaneously, changes of the STS basal-Mg^{2+}-ATPase activity were pronouced after nerve sectioning. We could observe an increase of basal ATPase (by 30 %) and an increased Mg^{2+}-ATPase- Ca^{2+}-ATPase ratio (from 0.208 to 0.281, $P < 0.05$.)

As was previously shown, STS membranes prepared by the modified method of Caswell consist of vesicles derived from free and junctional sarcoplasmic reticulum as well as of a small proportion of T-tubules (Lehotský et al. 1987b). Density gradient centrifugation analysis of STS vesicles according to Rosemblatt et al. (1981) revealed in denervated muscles three typical fractions with buoyant density properties similar to those of the controls. However, as can be seen from the protein yields of the individual fractions (mg prot/g wet weight),

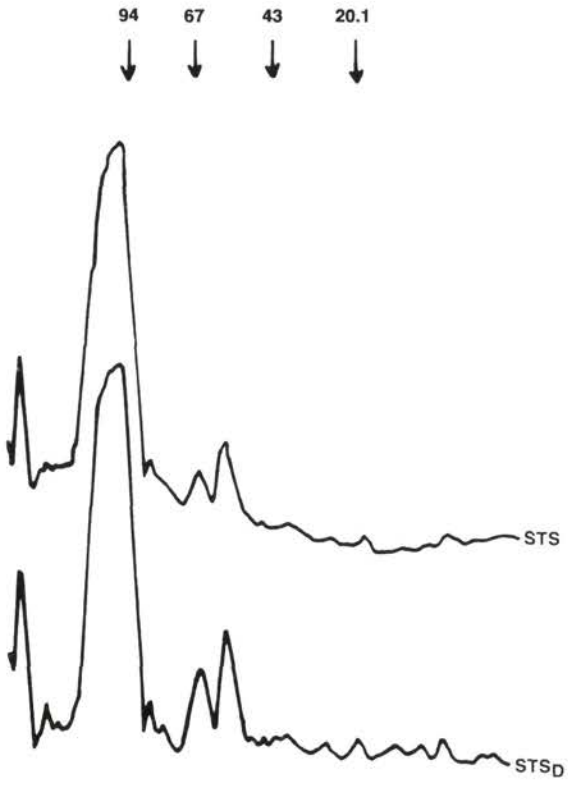

Fig. 2. Densitometric patterns of total STS membrane proteins from control (STS) and denervated muscle (STS_D) as analyzed by SDS-PAGE according to Laemmli (1970). Standards used, see Fig. 1.

Table 1. Specific activities of Ca^{2+}, Mg^{2+}-ATPase, basal Mg^{2+}-ATPase and membrane bound acetylcholinesterase in STS membranes from control and denervated animals. For experimental conditions, see Materials and Methods. Results are means ± double SD from 6—8 experiments

	Control	Denervated
Ca^{2+}, Mg^{2+}-ATPase (μmol P_i/mg/min)	2.218 ± 0.125	2.104 ± 0.145
Mg^{2+}-ATPase (μmol P_i/mg/min)	0.461 ± 0.09	0.592 ± 0.08
Mg^{2+}-ATPase/Ca^{2+}, Mg^{2+}-ATPase	0.208	0.281
Acetylcholinesterase(μmol/mg/h)	1.76 ± 0.03	1.04 ± 0.07

Table 2. Relative protein yields and content of cholesterol in total STS membranes and in separated fractions in controls and after 16 days of denervation. For experimental conditions, see Materials and Methods. Results are means ± double SD from 6–8 experiments

	Fraction			
	STS	H	L	SM
protein yield in % (control/denerv.)	100 ± 9.8/109.9 ± 7.3	100 ± 7.2/107 ± 6.9	100 ± 3.6/104 ± 5.1	100 ± 6.4/115 ± 7.1
cholesterol in % (control/denerv.)	100 ± 10.9/128 ± 11.3	100 ± 9.1/120 ± 7.4	100 ± 7.3/111 ± 9.1	100 ± 16.1/147 ± 18.1

microsomes from denervated muscle appear relatively enriched in SM vesicles as well as in heavy vesicles though to a lower extent (Table 1).

Although the profile of total microsomal proteins after denervation showed only slight changes in the Ca^{2+}-pump content (Fig. 1), the vesicle separation on density gradient revealed further distinctions. Very light fractions (SM) prepared from control and denervated muscles showed in comparison to other fractions increased levels of the 55 kD protein.The content of the 102 kD protein (Ca^{2}-pump) in all fractions showed only small differences, but the light and especially the heavy vesicles were enriched in the 64 kD protein (calsequestrin).

When examined 16 days after denervation the STS vesicles did not show any significant changes in total lipid phosphorus (data not shown). Contrary to the unchanged level of phospholipids, surprising were the findings of increased membrane cholesterol levels in the total denervated STS vesicles; the elevation concerned all fractions with the most obvious change being observed in the SM band (Table 2).

To investigate the mechanisms by which the denervation- induced changes in protein and lipid composition of STS affect the membrane systems responsible for spreading of action potential and E—C cycle, we determined the activities of some of these systems, including acetylcholinesterase and the numbers of binding sites of putative Ca^{2+}-channel and Na^{+}, K^{+}-ATPase.

The sarcotubular acetylcholinesterase activity, the enzyme being an interal part of the STS system (Vidal et al. 1987) showed a substantial decrease in vesicles from denervated muscle (about 40 %) in comparison to the control group (Table 1).

The Ca^{2+}-antagonist dihydropyridine PN-200-110 was used as a biochemical marker of the voltage sensitive Ca^{2+} channel concentrated in skeletal muscle T-tubules (Hidalgo et al. 1986; Sabbadini and Dahms 1989). Increased protein yield of the SM band after denervation along with elevated levels of basal ATPase activity and cholesterol content in microsomal vesicles suggested a membrane enrichment of T-tubular origin. As was shown by DHP-binding analysis (Fig. 3), this suggestion appeared to be correct. After saturation binding, denervated vesicles exhibited a single family of ^{3}H-PN-200-110 receptors with maximum binding capacity B_{max} = 4.5 pmol/mg (5.49 pmol/g wet weight) for denervated and B_{max} = 1.98 pmol/mg (2.21 pmol/g wet weight) for control vesicles. The dissociation constants of the DHP-receptor complexes were very similar for both vesicle type (K_D = 11.81 nmol/l and 12.18 nmol/l for denervated and control vesicles).

Specific binding of ^{3}H-ouabain in muscle was estimated to study denervation-induced changes in Na^{+}, K^{+}-ATPase which is important for the maintenance of the muscle electric activity. In denervated microsomal vesicles maximum specific ^{3}H-ouabain binding was significantly decreased and reached only

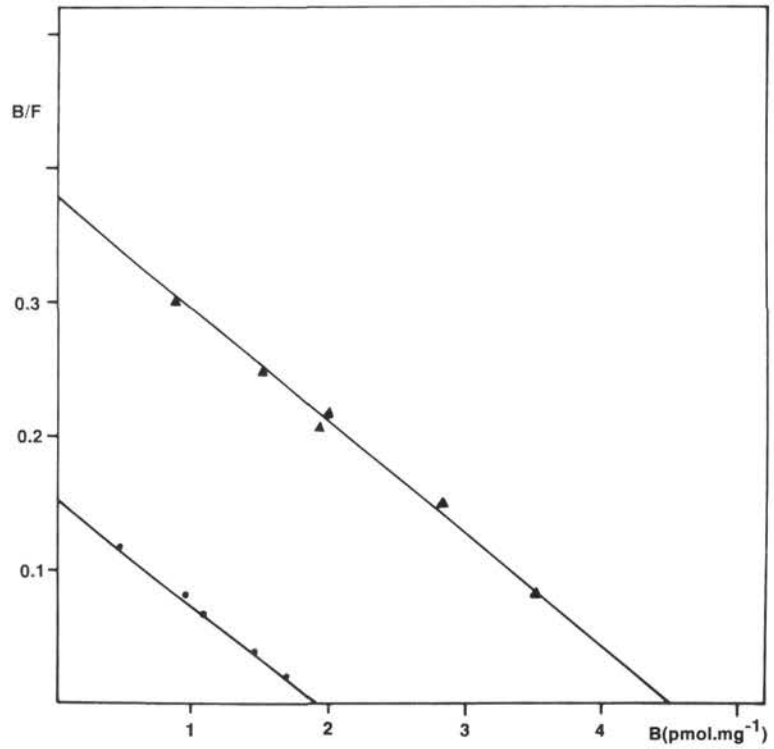

Fig. 3. Scatchard analysis of specific dihydropyridine ^{3}H-PN-200-110 binding to STS membranes from control (-·-·) and denervated (-▲-▲) muscles. For experimental conditions, see Materials and Methods. Results are means from 6 independent experiments run in duplicate and analysed using linear regression.

73 % of the value obtained for control vesicles. Similar results were obtained with whole muscle homogenates prepared from denervated muscles, although the decrease of maximum ouabain binding did not show any such marked differences (Fig. 4). When relating the binding parameters to muscle wet weight (data not shown) maximum binding showed only slight difference (approximately 80 %).

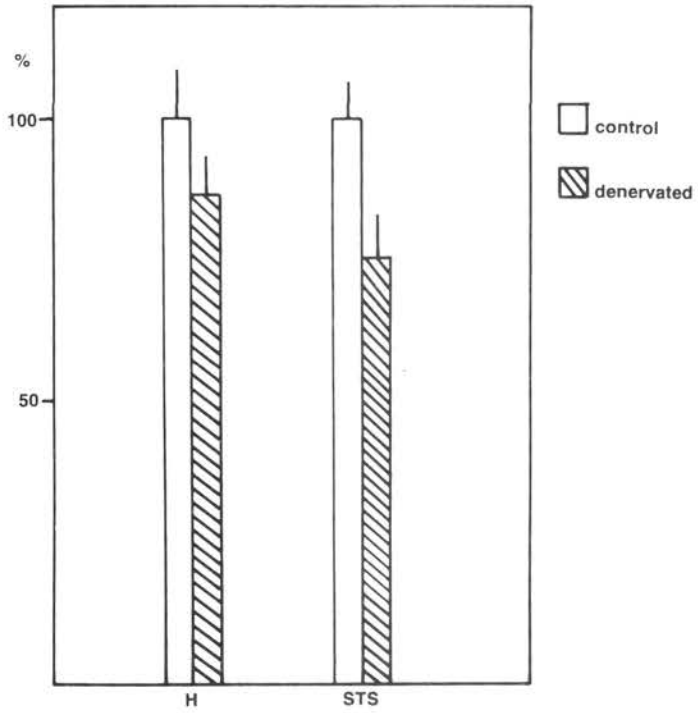

Fig. 4. Specific ATP-supported binding of ^{3}H-ouabain to homogenate (H) and STS membranes from control and denervated muscles according to Hidalgo et al. (1986). For experimental conditions, see Materials and Methods. Results are means ± double SD from 6 independent experiments run in duplicate.

Discussion

Denervation of skeletal muscle results in many structural, metabolic and functional changes (Jolesz and Sreter 1981). Electron microscopis studies have shown that an early morphological response along with tissue atrophy is the proliferation of membrane structure (Davis and Kiernan 1980; Schiafino and Settembrini 1970), which was biochemically supported also by an increase of microsomal protein yield (Muscatello et al. 1965; Salvatori et al. 1988). The protein profile of the protein classes examined of denervated microsomes showed a decrease of the 102 kD protein (Ca^{2+}-pump) along with a reduction of Ca^{2+}-ATPase activity and a non-significant increase of minor proteins; how-

ever, these changes were not as obvious as these reported by Margreth et al. (1972) and Wan and Boegman (1981) after surgical and chemical muscle denervation.

The pronounced increase of basal ATPase preferentially localised in T-tubules (Sabbadini and Dahms 1989) and the basal ATPase/Ca^{2+}-ATPase ratio are in agreement with the results obtained by other investigators (Sreter 1970; Palexas et al. 1981; Salvatori et al. 1988) and suggested that microsomal membrane proliferation mainly concerned the T-tubule structure.

Density gradient analysis of vesicles from denervated muscle did not reveal any marked differences in the density properties of the STS fraction in comparison to the control. Enrichment of microsomes in the SM and the heavy fraction (derived from T-tubule, triads and junctional SR) following denervation, and also the increase of the 64 kD protein (calsequestrin), classified as a relative specific marker of j-SR (Collins et al. 1990), in the light and especially in heavy fractions strongly support the suggestion concerning denervation-induced proliferation of T-tubules and j-SR structure.

In agreement with the results of other authors (Graaf et al. 1965; Zatti et al. 1969; Bunch et al. 1970; Karaba and Tweedle 1981) neither we could detect any remarkable changes in the microsomal content of total lipid phosphorus after denervation.

However, Zatti et al. (1969) as well as Karaba and Tweedle (1981) could show an increased level of total non-polar lipids in denervated microsomes, but they could not estimate their exact types. In our preliminary report (Lehotský et al. 1988), we could identify this lipid as cholesterol and show an increase of its levels in total microsomes of denervated muscle with prevailing accumulation in the SM fraction.

Although the high content of cholesterol is a distinguishing marker of T-tubule versus SR (Rosemblatt et al. 1981; Hidalgo et al. 1986; Sabbadini and Dahms 1989) and a predominant enrichment of the SM fraction can be observed after denervation, no satifactory explanation for its massive increase in STS membranes as a whole is available. However, changes similar to those induced by denervation can be detected in cholesterol binding and metabolism (Drgová, unpublished results) and also in ontogenic experiments (Boland et al. 1974), and in some muscle disorders (Tovar and Verjovski-Almeida 1985; Dobrota et al. 1988). Thus, it is highly probable that the changes in microsomal cholesterol are an accompanying phenomenon of a general response to the tissue disorder.

Similarly as was shown in the studies on total acetylcholinesterase in denervated muscle, which plays an important role in synaptic transmission (Khaskye 1986; Berman et al. 1987), a decrease of specialised sarcotubular membrane-bound acetylcholinesterase activity could be detected in STS vesicles following denervation (40 % smaller than in the control group).

Essential for electric activity and E—C cycle in the muscle are the T-tubular DHP-sensitive Ca^{2+}-channel (Hidalgo et al. 1986; Sabbadini and Dahms 1989) and membrane Na^+, K^+-ATPase which is important for the maintenance of ionic gradient and for the membrane potential (Stefani and Chiarandini 1982). Contrary to diminution of total acetylcholinesterase (Khaskye 1986; Berman et al. 1987) and membrane-bound acetylcholinesterase after denervation, we could detect also an approximately twofold increase in maximum binding sites for ^{3}H-PN-200-110 in STS vesicles without any marked changes in the affinity. Since denervation induced an increase of protein yield mainly in the SM fraction (derived from vesicles of the T-tubule origin), the increase of the DHP receptor provides biochemical evidence for the proliferation of this vesicle type. An increase of DHP binding sites was earlier observed in muscle homogenates from adult rats and chickens (Schmid et al. 1984a), and denervation was shown to affect DHP receptor also during *in vivo* myogenesis (Schmid et al. 1984b); so far however, no reports have been available concerning of muscle microsomes.

The effects of denervation on ^{3}H-ouabain binding activity were already shown by Clausen et al. (1981) in *in vitro* experiments with whole mouse muscle. Similarly to these results, Schmid et al. (1984a) could observe in another type of experiments a decrease of ^{3}H-ouabain binding (by 40—60 %) also in denervated rat and chicken muscle homogenates. In our experiments with microsomal vesicles lysed by saponin, the changes in Na^+, K^+-ATPase numbers differ substantially from those of the DHP receptor and is in agreement with the results obtained with whole muscle and homogenates. We observed an approximately 30 % decrease of binding sites below the control values and together with an obvious reduction of Na^+, K^+-ATPase phospohrylation (Walkiewicz and McEwen 1985) it may be expected that the denervation-induced changes in Na^+, K^+-ATPase are related to altered electric muscle activity.

Finally, it can be assumed that denervation of rabbit fast-twitch skeletal muscle leads to proliferation of predominantly the j-SR and T-tubules with simultaneous increases of basal ATPase activity and cholesterol content. The denervation-induced changes in the numbers of Ca^{2+}-channel receptors and Na^+, K^+-ATPase together with an elevated Ca^{2+}-content in the SR observed previously (Palexas et al. 1981) could affect the E—C cycle after denervation.

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