Effects of Disuse on the Function of Fragmented Sarcoplasmic Reticulum of Rabbit *M. Gastrocnemius**

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Abstract. A preparation method has been described to obtain a relatively pure and functionaly intact fragmented sarcoplasmic reticulum (SR) vesicles fraction from normal and atrophied muscles. Purified SR preparations from rabbit gastrocnemius muscle atrophied by disuse showed similar protein composition (gel electrophoresis; Laemmli 1970) and similar vanadate induced crystallization (Dux and Martonosi 1983) properties of Ca^{2+}-ATPase as those of control preparations. In the early period of atrophy (1—2 weeks) both the Ca^{2+}-ATPase activity and Ca^{2+} uptake showed a 2—3-fold increase (from 3.42 ± 0.24 to 7.34 ± 0.25 μmol Pi . mg^{-1} prot . min^{-1} and from 1.26 ± 0.10 to $3.36 \pm \pm 0.22$ μmol/l Ca^{2+} . min^{-1} . mg^{-1} prot. respectively).

Key words: Disuse of muscle — Ca^{2+}-ATPase — Ca^{2+} uptake — Sarcoplasmic reticulum — Rabbit skeletal muscle

Introduction

It is a well-established experimental observation that muscles fixed at a length shorter than normal undergo atrophy due to the disuse (the e.g., Thomsen and Luco 1944; Hollosi et al. 1977; Booth 1977). The atrophy is accompanied by considerable changes in the metabolic, morphological and physiological properties of the muscle (Booth and Kelso 1973a, b; Brody 1966; Cooper 1972; Maier et al. 1976; Max 1973; Mészáros et al. 1979; Rifenberick et al. 1973; Sohár et al. 1977; Sréter 1970). Disuse also affects the contractile properties of the muscle. The subunit structure of the contractile proteins changes, and hence a shift can also be observed in the muscle type (Guba 1980; Takács et al. 1981; Witzmann et al. 1982).

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The changes in the biomechanical parameters, and particularly in the relaxation half time ($T_{1/2r}$), are closely correlated with changes in the free Ca^{2+} concentration within the muscle fibres (Briggs et al. 1977; Fitts et al. 1980). The free Ca^{2+} concentration in the skeletal muscle fibre is well known to be regulated by the sarcoplasmic reticulum (SR). The observations that disuse induced considerable changes in the duration of the isometric twitch (Fischbach and Robbins 1969; Török et al. 1980; Witzmann et al. 1982) indicated that disuse also influenced the function of the SR. Additional information concerning variations in SR secondary to disuse was further provided by ultrastructural observations (Mészáros et al. 1979). Changes in the SR function have been shown in denervated muscles and in dystrophic muscles (Verjovski and Inesi 1979). Only few data are available on the effects of immobilization (Heiner et al. 1984; Jakab et al. 1980; Kim et al. 1982). The present study was designed to describe the effects of disuse on the function of SR. The two main functional characteristics of SR the Ca^{2+} uptake and Ca^{2+}- ATPase activity of samples, were measured. Both the Ca^{2+} uptake rate and the Ca^{2+}-ATPase activity increased significantly in the initial stage of disuse.

Materials and Methods

Adult New Zealand white male rabbits were used. The right hind limbs were immobilized in an extended position (there was no strain applied on the muscles studied) with plaster casts for 1; 2; 4 or 6 weeks as described earlier (Hollosi et al. 1977). After the immobilization period, the animals were killed by stunning and decapitation, and the gastrocnemius muscle was excised and the medial head was used in experiments. The muscle was freed of the connective tissue and fat, and minced with scissors (individual pieces measured less than 5 mm^3). All subsequent steps were carried out at 0—5 °C.

Muscle fragments were washed in an amount of homogenizing solution equal to the wet weight of the muscle. Minced muscle was homogenized in 5 volumes of 0.25 mol/l sucrose, 0.2 mmol/l Na_2EDTA + 0.1 mol/l Tris, at pH 7.6 for 2 × 20 s in an MSE homogenizer at half maximal speed (8000 rpm). The homogenate was poured through one layer of cheesecloth and centrifuged for 10 min at 900 × *g*. The supernatant was poured through four layers of cheesecloth and centrifuged again for 10 min at 10,000 × *g*. The supernatant was further centrifuged for 50 min at 40,000 × *g* in an L5-50 Beckman centrifuge. The pellet was resuspended and gently homogenized in a small volume of solution containing 0.25 mol/l sucrose + 2.5 mmol/l HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanosulphonic acid), pH 7, in an Ultra Turax homogenizer.

The resulting suspension was the crude SR vesicle fraction, which was further purified on a continuous sucrose density gradient, essentially as described by Sréter (1969).

Usually, 5 ml crude SR fraction (about 15—20 mg protein/ml) were layered on top of a 50 ml sucrose density gradient system, prepared from equal volumes of 0.25 and 2 mol/l neutralized solutions of sucrose, containing 1 mmol EDTA + 3 mmol HEPES. After centrifugation for 90 min at 2500 × *g* in a Heraeus-Christ 20—30 cryofuge, two layers were obtained. The upper one did not penetrate into the gradient, while the lower one was usually located 6—8 cm below the meniscus.

The upper layer was enriched in FSR as demonstrated by vanadate crystallisation procedure (Dux and Martonosi 1983). The myofibrils-contaminated lower fraction was discarded.

The upper fraction was collected and centrifuged at 90,000 × *g* for 1 h. The pellet thus obtained was resuspended in 8 volumes of 0.6 mol/l KCl, 0.3 mol/l sucrose + 2.5 mmol/l HEPES buffer (pH 7.4) and stirred gently for 30 min. The FSR was then repelleted by centrifugation at 90,000 × *g* for 1 h, and washed once with and finally suspended in 0.3 mol/l sucrose + 2.5 mmol/l HEPES buffer (pH 7.4).

Freshly prepared FSR vesicles were used in the experiments.

Contamination with membrane fragments from other organelles was monitored via 5'-nucleotidase (Bodansky and Schwartz 1963) and Na^+, K^+-ATPase (Sulakhe 1971) activities for sarcolemma, and cytochrome C oxidase (Schnaitman et al. 1967) activity for mitochondrial membrane.

Mitochondrial contamination was also checked according to Katz et al. (1970) by measuring Ca^{2+}-Mg^{2+}-dependent ATPase activity in the presence of sodium azide, while the acid phosphatase activity was used to detect lysosomal contamination (Wattiaux and De Duve 1955).

Contamination by structural proteins was checked using myofibrillar ATPase activity (Mommaerts and Parrisch 1951).

The ATPase activities of the FSR preparations were determined essentially as described by Sarzala et al. (1975). The (Mg^{2+}, Ca^{2+})-ATPase activity was usually measured in a medium of 100 mmol/l KCl, 20 mmol/l Tris-maleate (pH 7), 5 mmol/l $MgCl_2$, 5 mmol/l potassium oxalate, 5 mmol/l ATP, 0.5 mmol ethyleneglycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetate (EGTA) + 0.45 mmol/l $CaCl_2$, in a temperature range from 20 to 37 °C. The protein concentration was adjusted to between 0.02 and 0.1 mg protein/ml. The enzyme was pre-incubated in the medium for 2 min during constant stirring, and the reaction was started by the addition of ATP (in a total volume of 2 ml) and stopped by the addition of 2 ml 10 % trichloroacetic acid after different incubation times. The liberated inorganic phosphate was determined by the turbidimetric method (Eibl and Lands 1969). For the determination of the Mg^{2+}-ATPase activity, the medium contained 0.5 mmol/l EGTA and $CaCl_2$ was omitted. Ca^{2+}-ATPase activity was calculated as the difference between (Mg^{2+}, Ca^{2+})-ATPase and Mg^{2+}-ATPase activities.

Ca^{2+} uptake by the SR was measured by the filtration method (Martonosi and Feretos 1964). The incubation medium was similar to that used for ATPase assays, except that labelled $^{45}CaCl_2$ was present (20,000—30,000 c.p.m./ml). The reaction was started by the addition of FSR, and at various time intervals 0.3 ml of the reaction medium were removed and filtered through a membrane filter (0.4 μm pore size, Sympor, Chemapol, Prague). The protein content was determined according to Lowry et al. (1951).

The protein composition of the FSR samples were followed with 6—18 % gradient polyacrylamide gel electrophoresis (Laemmli 1970). From 20 to 150 μg protein were loaded in each lane after solubilization in 1 % sodium dodecylsulphate and 20 mmol/l dithiothreitol containing sample buffer (protein concentration 2 mg/ml). The gels were run at 20 mA per slab for 14 h.

Proteins were stained with Coomassie brillant blue and destained in a 10 % acetic acid + 34 % methanol mixture.

The SR vesicles were identified, and their surface structure was studied by the method described by Dux and Martonosi (1983). For crystallization of the Ca^{2+}-ATPase, the microsomes were diluted in 0.1 mol/l KCl, 10 mmol/l imidazole (pH 7.4), 0.5 mmol/l EGTA, 5 mmol/l $MgCl_2$ + 5 mmol/l Na_3VO_4 at 2 °C to a final concentration of 1 mg protein/ml. The samples were examined 16 h later in a JEOL JEM 100B electron microscope, using negative staining with 1 % uranyl acetate.

Results

Figure 1 shows vanadate-induced two-dimensional Ca^{2+}-ATPase crystals of outher surface of SR vesicles prepared from normal *m.gastrocnemius* and from *m.gastrocnemius* disused for 1; 2; 4 or 6 weeks.

It can be seen that the SR vesicles were not ruptured in either the samples of control or immobilized animals.

The preparations did not contain connective tissue elements or myofibrillar proteins.

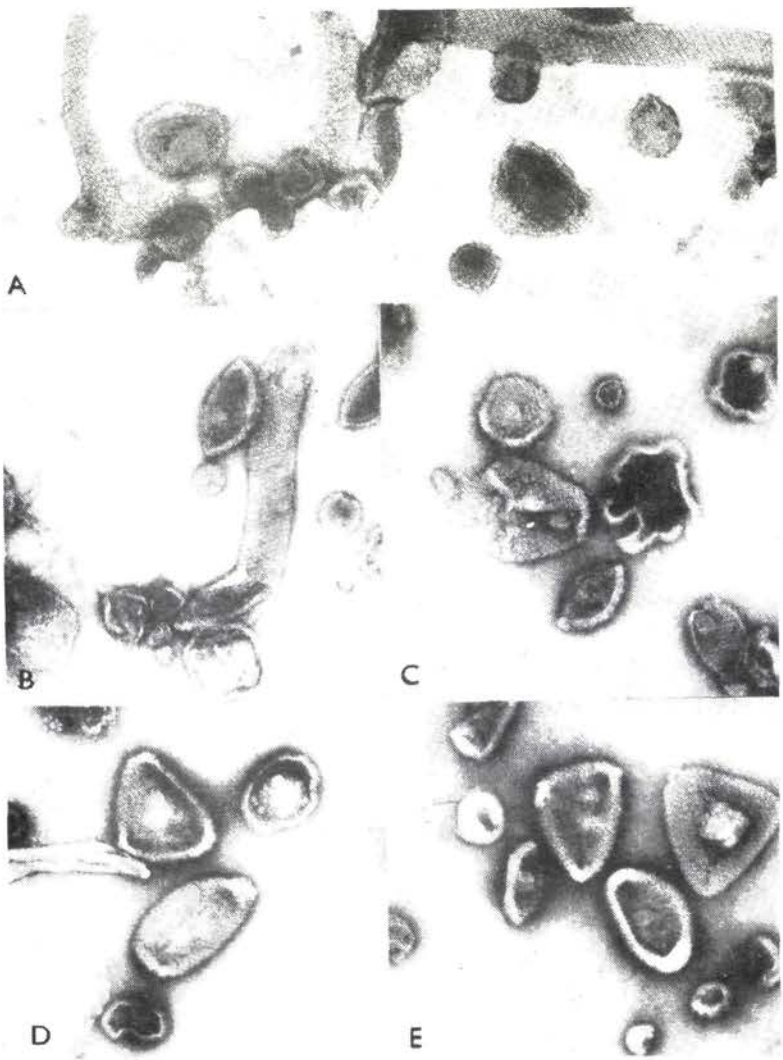

Fig. 1. Electron micrographs of vanadate-induced crystals of Ca^{2+}-ATPase from FSR of rabbit *m. gastrocnemius* microsomes; negatively stained with 1 % uranyl acetate at pH 4.3 (Dux and Martonosi 1983). (*A*) control microsomes, (*B*) after one week, (*C*) after two weeks, (*D*) after four weeks, (*E*) after six weeks of disuse by hind limb immobilization with a plaster cast. Magnification: *A*: 78,000 ×; *B*: 65,000 ×; *C*: 71,000 ×; *D*: 65,000 ×; *E*: 72,000 ×

The micrographs show that structurally identical, two-dimensional crystals of Ca^{2+}-ATPase were induced by vanadate in all samples.

Markers for contaminating cell fractions revealed that the SR of *m. gastrocnemius* was any negligible contaminated by sarcolemma and lysosomes. 5′-nucleotidase and $Na^+ + K^+$-ATPase activities were not detectable. The acid phosphatase activity was 0.003—0.007 μmol $P_i min^{-1} . mg^{-1}$ protein, indicating a negligible contamination of the SR by lysosomes. Myofibrillar ATPase activity was not detected. Also, the biochemical analysis of marker enzymes excluded any significant contamination by mitochondria, as judged by the lack of inhibition of ATPase by sodium azide, and a very low activity of cytochrome C oxidase (0.01—0.04 μatom oxygen $. mg^{-1}$ protein $. min^{-1}$).

The purities of the samples and the changes in the polypeptide composition were checked by 6—18 % gradient SDS-PAGE according to Laemmli (1970).

To establish the minor protein components of the different samples, experiments were performed with very heavily protein loaded gels (140 μg protein/lane) (Fig. 2).

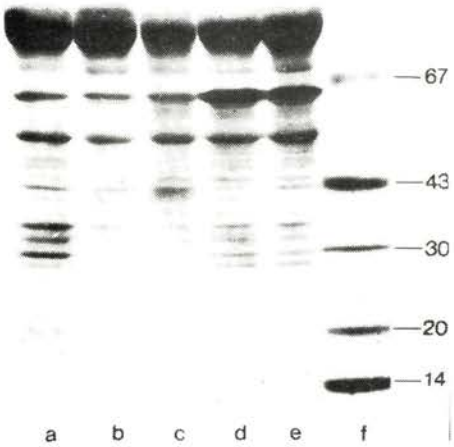

Fig. 2. SOS gel electrophoretic pattern of SR proteins of *m. gastrocnemius:* (*a*) control, (*b*) after one week, (*c*) after two weeks, (*d*) after four weeks, (*e*) after six weeks of immobilization. Gradient gel electrophoresis was carried out according to Laemmli (1970). The gels were loaded with 140 μg FSR protein and stained with Coomassie blue. The molecular weight standards are indicated (*f*).

As shown in Fig. 2, immobilization of the muscles did not produce any substantial changes in the protein composition as compared to the control.

Table 1 lists the ATPase activities of FRS samples of different origin.

The Table shows that, in the early period of muscle inactivity, there was a marked increase in the total ATPase activity. The change in the Mg^{2+}-ATPase activity was negligible during the experiment.

A significant increases in the Ca^{2+}-stimulated ATPase activity of SR isolated

Table 1. Total basic and (calculated) specific activities of FSR from (disused) rabbit *m. gastrocnemius* after various periods of hind limb immobilization

Immobilization period	(Mg^{2+}, Ca^{2+})-	Mg^{2+}-	Ca^{2+}-ATPase
	μmol P_i mg^{-1} prot. min^{-1}		
control* (8)	3.67 ± 0.29	0.25 ± 0.05	3.42 ± 0.24
1 week (7)	6.52 ± 0.20	0.35 ± 0.03	6.17 ± 0.28
2 weeks (10)	7.74 ± 0.30	0.40 ± 0.02	7.34 ± 0.25*
4 weeks (5)	5.97 ± 0.33	0.37 ± 0.04	5.60 ± 0.30
6 weeks (6)	3.95 ± 0.25	0.14 ± 0.03	3.82 ± 20

Values are means ± SE; numbers of experiments (2—4 pooled muscles in each experiment) are given in parentheses; in each experiment 3 parallel assays were performed; * Control vs disused, $P < 0.01$. P_i means inorganic phosphate. The enzymic assays were performed at 37 °C.
* Untreated, normal animals.

Table 2. Effect of disuse (plaster cast immobilization of the hind limb) on the yield and the Ca uptake of FSR prepared from rabbit *m. gastrocnemius*

	Control (16)	Immobilization period			
		1 week (5)	2 weeks (7)	4 weeks (5)	6 weeks (4)
FSR protein yield mg/g muscle (wet weight)	1.63 ± 0.08	1.81 ± 0.08	1.71 ± 0.09	1.54 ± 0.12	1.42 ± 0.10
Initial rate of Ca^{2+} uptake μmol Ca^{2+} min^{-1}. mg^{-1} prot.	1.26 ± 0.10	1.93 ± 0.09	3.36 ± 0.22*	1.03 ± 0.15	0.91 ± 0.07
Ca^{2+} uptake capacity, μmol Ca^{2+} 12 min mg^{-1} prot.	4.25 ± 0.19	4.85 ± 0.20	4.92 ± 0.25	4.72 ± 0.18	4.55 ± 0.22

Values are means ± SE; figures in the parentheses have the same meaning as described in Table 1.
* Control vs. disused, $P < 0.01$. In determining the initial rate of Ca^{2+} uptake 30—60 g FSR prot/ml were used and samples were taken at 10 s intervals. The results were obtained in experiments performed at 23 °C.

from atrophied muscles has to be assumed. The increase in the enzymatic activity after 1—2 weeks of disuse was about twofold.

During a lasting manipulation the values declined and tended to approach the control level.

The Ca^{2+} uptake (Table 2) also showed a marked change in the early stage of disuse. An approximated 3-fold increase in both the initial rate and the extent of Ca^{2+} uptake could be observed after 1—2 weeks of disuse.

Table 2 also shows that a prolonged treatment of the limbs resulted in values similar to those found in the controls.

Discussion

In previous investigations we concluded (in agreement with other authors) that muscles undergo changes very dynamically and that the static muscle type designations are not valid (Guba 1980). Limb immobilization has proved a good model in studies of these adaptive muscle changes (Booth 1982; Guba et al. 1980; Kim et al. 1982).

Our present results indicate that in the initial stage of disuse, during which also other considerable changes occur, (see e.g. Sohár et al. 1977), the SR likewise undergoes a functional change; its features subsequently become similar to the functional properties of the control SR. It was rather surprising that both the Ca^{2+}-ATPase and the Ca^{2+} uptake increased greatly over the first two weeks of disuse. As concerns the Ca^{2+} uptake, our results agree with the data of Kim et al. (1982) and Heiner et al. (1984) obtained on rat and rabbit muscle, respectively: there was an increase in the Ca^{2+} uptake in the initial stage of limb immobilization. However, our finding of an increase in the ATPase activity seems to be in contrast with the observations reported by Kim et al. (1982) on rat muscles; this can be explained by a high scatter of the data presented by the latter authors.

The interpretation of these findings is a more difficult problem. One possibility explaining the increased values might be a change in the proteins. The great similarity in the protein composition of the samples, and the unchanged level of contamination of the SR preparations by other membrane elements does not seem to support this explanation.

Other possibilities are a change in the lipid composition and the role of lipid — protein interactions. Observations of both are known (Scott and Coe 1983). Further studies are necessary to clarify this question.

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