Characteristics of sarcoplasmic reticulum from normal and denervated rat skeletal muscle

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Denervation of rat skeletal muscle produces after 14 days a decrease in Ca^{2+} uptake of a heterogeneous population of sarcoplasmic-reticulum vesicles, when measured in the presence of oxalate. The Mg^{2+} -dependent ATPase (Ca^{2+} -independent) activity increased after the same period and the Ca^{2+} + Mg^{2+} -dependent ATPase activity decreased. Concomitant with these changes, there was an increase in vesicle size and calcium content. The observations are discussed in terms of changes in altered membrane structure, manifested in the shift of the equilibrium of the ATPase from an enzyme involved in calcium transport to a phosphoenzyme giving rise to an increase in the Mg^{2+} -dependent ATPase activity.

The pathogenesis of muscle disease has intrigued scientists for a long time, but the causes of the various types of muscular disorders are still unknown (Peter & Furukawa, 1977). The surface membrane of the muscle fibre has been most frequently considered to be the site of the primary lesions in muscular dystrophy because one of the earliest signs of the disease is elevated serum concentrations of muscle-specific enzymes. This view is supported by the finding of Mokri & Engel (1975) that the muscle fibres of patients with Duchenne dystrophy have a structural lesion in the plasma membrane. However, membrane abnormalities in muscular dystrophy and other muscle disorders are not confined to the plasma membrane, and many reports have appeared during the last decade showing defects in the sarcoplasmic reticulum and mitochondria of diseased skeletal and cardiac muscle.

It is well established that the sarcoplasmic reticulum is responsible for release and uptake of Ca^{2+} in the excitation relaxation cycle of skeletal muscle (Fuchs, 1974) and that sarcoplasmic reticulum plays the major role in physiological Ca^{2+} movements in skeletal muscle (Kitazawa, 1976). Alterations in sarcoplasmic-reticulum and mitochondrial Ca^{2+} transport properties are believed to be implicated in disorders of skeletal muscle (Wrogemann & Pena, 1976). In a wide range of

muscle diseases studied, the principal finding has been a decrease in the initial rate of total uptake of Ca^{2+} by the sarcoplasmic reticulum (Heffron, 1979).

Marked differences in ATPase activities and Ca^{2+} -uptake characteristics exist between sarcoplasmic reticulum isolated from fast-twitch and slow-twitch mammalian muscle. Both initial Ca^{2+} uptake and total Ca^{2+} uptake are higher in the sarcoplasmic reticulum of fast-twitch muscle. However, Ca^{2+} uptake by sarcoplasmic reticulum is linked in both slow- and fast-twitch muscle to a Ca^{2+} -transport system with similar or identical molecular and kinetic properties (Pette & Heilmann, 1979).

In an attempt to establish the sequence of events occurring in diseased muscle when the pathology is known to be secondary to nerve disease, it was decided to use an animal and induce denervation atrophy. This model, produced by sciatic-nerve severance at the hip region of the rat, was used to observe the biochemical functions of the sarcoplasmic reticulum with time with respect to Ca^{2+} uptake, Mg^{2+} -dependent and Ca^{2+} + Mg^{2+} -dependent ATPase activities, protein profiles, Ca^{2+} content and morphological characteristics of a heterogeneous population of sarcoplasmic reticulum. In the present paper we show that within 14 days of denervation of rat skeletal muscle there is a significant decrease in the Ca^{2+} + Mg^{2+} -dependent ATPase activity and an associated impaired uptake of Ca^{2+} by the sarco-

Abbreviation used: SDS, sodium dodecyl sulphate.

plasmic reticulum. The Mg^{2+} -dependent ATPase activity increases significantly at this stage.

Experimental

The calf and rear-thigh muscles (gastrocnemius, soleus, biceps femoris, extensor digitorum longus, anterior tibialis) of female Wistar rats weighing 200–250 g were denervated by removal of 1 cm segments of the sciatic nerve in the upper half hind-leg hip region. The muscles of the unoperated contralateral hind-leg were taken as controls from each animal. The muscles were removed from the respective legs, cleared of fat, nerve and connective tissue, and used for the preparation of sarcoplasmic reticulum as described by Green *et al.* (1976).

Ca^{2+} uptake by sarcoplasmic reticulum

This was measured at time intervals after incubation of sarcoplasmic reticulum with $^{45}CaCl_2$ followed by Millipore filtration (Martonosi & Feretos, 1964). The assay medium contained, in a total volume of 1 ml, 100 mM-KCl, 40 mM-histidine, pH 6.8, 5 mM- $MgCl_2$, 5 mM-oxalate, 0.4 mM- $^{45}CaCl_2$ (0.26 $\mu Ci/ml$) and 0.15 mM-EGTA. The reaction was conducted in a shaking water bath at 25°C with 48 μg of sarcoplasmic-reticulum protein [1:1 (w/w) ratio of protein to calcium] added to the incubation medium. The reaction was initiated by the addition of ATP at a final concentration of 5 mM. At the appropriate time, transport was terminated by filtering 1 ml of the assay medium through the Millipore filters and washing with 2 ml of 100 mM-KCl/40 mM-histidine buffer, pH 6.8. After drying, the Millipore filters were placed in scintillation vials containing 8 ml of Aquagel (Chemlab) and counted for radioactivity in a Packard liquid-scintillation spectrometer.

ATPases

The initial rates of Mg^{2+} -dependent ATPase and $Ca^{2+} + Mg^{2+}$ -dependent ATPase activities were determined by the method of McIntosh *et al.* (1977), with the modification that the P_i released was measured by the method of Taussky & Schorr (1953). Both assays were performed as time-course experiments, with appropriate enzyme blanks. The preparation of the sarcoplasmic reticulum and assaying of the samples were completed within 6 h of killing the animals.

Protein determination

The protein concentration of the sarcoplasmic reticulum was determined by using a combination of A_{280} measurements and the Bio-Rad protein assay method (Bradford, 1976).

Electrophoresis

The sarcoplasmic-reticulum fractions were examined by SDS/polyacrylamide-gel electrophoresis in a slab-gel apparatus by using a discontinuous buffer system (Laemmli, 1970) and 10% (w/v) acrylamide concentration. The gel slabs were stained with Coomassie Blue.

Calcium determination

The calcium contents of the sarcoplasmic reticulum were assayed by atomic absorption spectroscopy by the method of Pleasure *et al.* (1979).

Chemicals

ATP was obtained from Boehringer. Sucrose was Aristar grade from BDH. Millipore filters used were HA type, 0.45 μm pore size. $^{45}CaCl_2$ (10 mCi/ml) was obtained from The Radiochemical Centre, Amersham. The Bio-Rad reagent was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Results

The results reported in the present study were obtained from sarcoplasmic-reticulum preparations which contained a heterogeneous population of vesicles, i.e. a mixture of light and heavy vesicles. In all cases the sarcoplasmic-reticulum fraction was prepared from a mixture of both fast and slow muscles removed from the hind legs of the rat.

In the present study, with both fast and slow muscles from the hind-leg of the rat, there was an increased yield of sarcoplasmic reticulum (expressed as mg of protein/g wet wt. of muscle) and a corresponding decrease in the weight of the denervated muscle with time as compared with the normal contralateral leg (see Fig. 1).

Effect of denervation on Ca^{2+} uptake by sarcoplasmic reticulum

Denervated-muscle sarcoplasmic reticulum showed a significant decrease in both the initial rate of Ca^{2+} uptake and total amount of Ca^{2+} taken up, as reflected in Fig. 2. There is an average decrease of 33% in the total amount of calcium accumulated by denervated sarcoplasmic reticulum. There is also a 78% decrease in initial velocity of uptake of calcium into the sarcoplasmic reticulum of 14-day-denervated muscle.

Effect of denervation on ATPase activities

The results obtained from the ATPase assays on normal- and denervated-muscle sarcoplasmic reticulum show that already after 14 days significant differences in the respective activities are

manifested. The Mg^{2+} -dependent ATPase activity in the denervated sarcoplasmic reticulum shows a significant increase (40%; Table 1), in comparison to the normal sarcoplasmic reticulum, the respective values being 0.82 and 0.59 μmol of P_i /min per mg of sarcoplasmic-reticulum protein.

Fig. 1. Time study of the effect of denervation on the wet weight of the muscle and yield of protein of the sarcoplasmic reticulum

For details see the Experimental section. Data are expressed as percentages of normal controls. Symbols: $\bigcirc$, muscle weight; $\bullet$, yield of sarcoplasmic reticulum (mg of protein/g wet wt. of muscle).

The $Ca^{2+} + Mg^{2+}$ -dependent ATPase activity in muscle sarcoplasmic reticulum (Fig. 3) shows a significant decrease in comparison with that of the normal muscle, implying that there is a decrease in the efficiency of Ca^{2+} transport into the sarcoplasmic reticulum, as reflected in the calcium-uptake study shown in Fig. 2. There is possibly also a decrease in the formation of phosphoenzyme intermediate after denervation as shown by Sreter

Fig. 2. Rate of Ca^{2+} uptake in sarcoplasmic-reticulum vesicles from control and denervated muscle

Vesicles ($48\mu\text{g}$ of protein) from control ($\bullet$, $n = 11$) and denervated (14 day) ($\blacksquare$, $n = 4$) muscle were incubated at 25°C in $100\text{mM-KCl}/40\text{mM-histidine}$ ($\text{pH } 6.8$)/ $5\text{mM-MgCl}_2/5\text{mM-oxalate}$ ($\text{pH } 6.8$)/ $0.4\text{mM-}^{45}\text{CaCl}_2$ ($0.26\mu\text{Ci/ml}$)/ 0.15mM-EGTA in a total volume of 1ml (protein:calcium ratio 1:1). Error bars denote $\pm$ S.E.M. for n experiments.

Table 1. Initial rates of Ca^{2+} uptake, total Ca^{2+} uptake, ATPase activities and Ca^{2+} content of sarcoplasmic reticulum from normal and 14-day denervated muscles

Conditions for Ca^{2+} uptake and ATPase assays are as described in the legends to Figs. 2 and 3. Numbers of experiments are given in parentheses. Significance of differences by unpaired t test: $*P < 0.05$, $**P < 0.02$, $***P < 0.01$, $****P < 0.001$.

Treatment	Ca^{2+} content (nmol/mg of protein)	Enzyme activities (μmol of P_i /min per mg of protein)		Calcium uptake (nmol of Ca^{2+} /mg of protein)	
		Mg^{2+} -dependent ATPase	$Ca^{2+} + Mg^{2+}$ - dependent ATPase	Initial rate (per min)	Total uptake
Control	13.9 (3)	0.59 ± 0.05 (13)	2.03 ± 0.10 (5)	897.97 ± 73.8 (11)	2086.33 ± 87.72 (8)
14-day denervation	20.9 (2)	0.82 ± 0.08 (5)*	1.09 ± 0.06 (4)****	201.54 ± 100.8 (4)***	1390.32 ± 273.39 (4)**

Fig. 3. $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase activities of sarcoplasmic reticulum from normal (●, $n = 6$) and 14-day-denervated (▲, $n = 4$) muscle

The activity was assayed for 15 min at 25°C in the presence of 50 mM-Tris/HCl, pH 7.4, 100 mM-KCl, 5 mM-MgCl₂, 0.1 mM-EGTA, pH 7.4, 5 mM-oxalate, pH 7.4, 5 mM-NaN₃, 0.1 mM-CaCl₂ and 54 μg of sarcoplasmic-reticulum protein in a total volume of 1 ml. Reaction was commenced by the addition of ATP at a final concentration of 10 mM. Error bars denote $\pm$ S.E.M. for n experiments.

(1970). The respective $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase activities for denervated and normal sarcoplasmic reticulum are 1.09 and 2.03 μmol of P_i /min per mg of sarcoplasmic-reticulum protein, the denervated muscle showing a 46% decrease in this activity (Table 1). The enzyme activities and Ca^{2+} uptakes of the sarcoplasmic reticulum were determined at intervals for a period of up to 67 days after denervation, and there was little change from the results observed at 14 days.

The protein profiles of the sarcoplasmic reticulum of normal and denervated muscle showed qualitatively little differences, with both fractions showing the 105 000–110 000-mol.wt. ATPase component as the major band.

Discussion

The decrease in muscle weight after denervation observed in this study confirms previous findings (Hnik, 1962). This decrease is associated mainly

with myofibrillar disruption and decreased protein content (Pellegrino & Franzini, 1963; Stonnington & Engel, 1973; Cullen & Pluskal, 1977), with either an increase in protein degradation or a decrease in protein synthesis after denervation (Goldspink, 1976). Furthermore, from the results obtained in the present study it can be stated that significant changes take place in the sarcoplasmic reticulum after 14 days of denervation. The most important finding in terms of the functional properties of the sarcoplasmic reticulum is the decrease in activity of the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase with the associated impairment of Ca^{2+} uptake. The reason for this decrease is not apparent at this stage. A study of the protein profiles of the normal- and denervated-muscle sarcoplasmic reticulum showed no apparent decrease in the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase content. It is possible that alterations in either lipid composition or other proteins in the sarcoplasmic reticulum could produce conformational or other changes which lead to a decrease in the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase activity and an impaired capacity to take up Ca^{2+} . It is notable that Zatti *et al.* (1969) reported a marked increase in cholesterol content of isolated microsomal fractions after denervation. Changes in phospholipid composition and increased turnover of membrane lipids were observed by Bunch *et al.* (1970).

The role of Mg^{2+} -dependent or basal ATPase is controversial. Fernandez *et al.* (1980) claim that the basal ATPase is not found in sarcoplasmic-reticulum vesicles which are highly purified, and is thus not part of the sarcoplasmic reticulum. These findings are difficult to reconcile with the view that the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase can occur in two forms, E_1 and E_2 , one of which is active in the presence of Mg^{2+} (E_1) and the other (E_2) which is activated by both Ca^{2+} and Mg^{2+} and is responsible for the transport of Ca^{2+} (Inesi *et al.*, 1976). It is tempting to speculate that the increased activity of the Mg^{2+} -dependent ATPase of the sarcoplasmic reticulum observed in the present study after denervation is a consequence of an altered configuration in the membrane of the sarcoplasmic reticulum with a transition to the E_1 form of the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase enzyme. This idea is not novel. Verjovski-Almeida & Inesi (1979) observed an increased Ca^{2+} -independent ATPase activity in sarcoplasmic reticulum vesicles isolated from the pectoralis muscle of dystrophic chickens, and they suggested that a fraction of the ATPase of dystrophic-muscle sarcoplasmic reticulum is not activated by Ca^{2+} , owing to an altered protein assembly within the membrane bilayer.

Our findings of an increased Mg^{2+} -dependent ATPase activity after denervation confirms the finding of Sreter (1970), who used white and red muscle of male rats. He also found that denervation

produces a transient increase (maximum at 15 days) in Ca^{2+} uptake by the sarcoplasmic reticulum, but the uptake was back to normal or decreased at 6 weeks. The initial rate of Ca^{2+} uptake by the sarcoplasmic reticulum after denervation did not increase. These results appear contradictory, for in our study the initial rate of Ca^{2+} uptake, the total Ca^{2+} accumulated by the sarcoplasmic reticulum and the Ca^{2+} + Mg^{2+} -dependent ATPase activity were all significantly decreased after denervation at day 14.

An alternative explanation for the decrease in Ca^{2+} + Mg^{2+} -dependent ATPase activity and impaired uptake of Ca^{2+} by the sarcoplasmic reticulum after denervation could be the possibility of an overloading of the Ca^{2+} -binding capacity. In a limited number of samples we have observed an increased Ca^{2+} content in the denervated-muscle sarcoplasmic reticulum fractions, a finding also reported by Sreter (1970). We have also found a significantly increased Ca^{2+} content in homogenates of denervated muscle (M. Joffe, N. Savage & H. Isaacs, unpublished work). These conditions may lead to an increased leakiness of the membrane, as evidenced by decreased Ca^{2+} uptake by the sarcoplasmic reticulum shown in this study.

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