Alteration of sarcoplasmic reticulum after denervation of chicken pectoralis muscle

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(Received 13 July 1982/Accepted 27 October 1982)

To determine the neural influence on the function of the sarcoplasmic reticulum (SR) of fast-twitch skeletal muscle, the superior pectoralis muscle of adult chicken was denervated, and the SR was isolated at 20 days post-denervation. The isolated SR was probably derived from the longitudinal SR and was relatively free of contaminants. The protein profile of the SR was quantitatively changed after denervation with an increase in the M_{55} and 30000-mol.wt. proteins relative to the Ca^{2+} -ATPase. Ca^{2+} -dependent ATPase activity and phosphoenzyme formation were lower in the denervated-muscle SR; however, the enzyme catalytic-centre activity was similar to the control value. The decrease in Ca^{2+} -ATPase activity in denervated-muscle SR was accompanied by a lower Ca^{2+} accumulation so that the relationship between Ca^{2+} accumulation and Ca^{2+} -dependent ATPase activity was well maintained in the SR from denervated muscle. The data imply that denervation may result in a diminution of functional Ca^{2+} pump sites. Evidence is presented, though, which suggests that denervation affects a single class of Ca^{2+} -binding sites of the Ca^{2+} -ATPase, resulting in a lower affinity for Ca^{2+} .

The importance of the motor nerve on the structural and functional properties of skeletal muscle has been demonstrated in various ways. The contractile properties of fast-twitch muscle are transformed to those resembling slow-twitch muscle when the fast-twitch muscle either is cross-innervated with a nerve to a slow-twitch muscle or is electrically stimulated with a frequency pattern of a slow-twitch motor neuron (Sreter *et al.*, 1975; Pette & Heilmann, 1977). Since the SR is the fundamental regulator of intracellular Ca^{2+} fluxes involved in the excitation-relaxation cycle of striated muscle, it is noteworthy that the Ca^{2+} -transport process, including the Ca^{2+} -dependent hydrolysis of ATP, of the SR *in vitro* is changed in a manner similar to the contractile properties of the muscle from which it is isolated after cross-innervation or electrical stimulation (Margreth *et al.*, 1973; Sreter *et al.*, 1975; Heilmann & Pette, 1979). Furthermore, a comparative analysis of the SR from different muscle fibre types shows that the functional properties of the SR *in vitro* are well correlated with the contractile properties of the

muscle fibre type from which it is isolated (Van Winkle *et al.*, 1978; Heilmann *et al.*, 1977). These studies suggest that the motor nerve is essential to the genetic expression of the SR and of the Ca^{2+} -ATPase, the primary structural and functional protein of the SR.

Sectioning of the motor nerve (denervation) leads to a variety of physiological changes in skeletal muscle (Thesleff, 1974), including a prolongation of contraction and relaxation times (Gutmann *et al.*, 1972; Gutmann & Melichna, 1979). Previous studies, however, present conflicting evidence on the function of the SR from denervated skeletal muscle. Using rat fast-twitch hindlimb muscle, Sreter (1970), Margreth *et al.* (1972) and Hughes & Yasin (1973) found that the Ca^{2+} -ATPase activity of isolated SR was not altered after denervation, whereas Palexas *et al.* (1981) reported a decrease in Ca^{2+} -dependent ATPase activity of SR isolated from a mixture of denervated rat hindlimb muscles. The rate of ATP-dependent Ca^{2+} accumulation by rat denervated-muscle SR is either unchanged (Margreth *et al.*, 1972; Hughes & Yasin, 1973) or decreased with respect to control (Sreter, 1970; Palexas *et al.*, 1981). Thus, the relationship *in vitro* between

Abbreviations used: SR, sarcoplasmic reticulum; Ca^{2+} -ATPase, Ca^{2+} -dependent ATPase.

ATP-dependent Ca^{2+} accumulation and Ca^{2+} -ATPase activity of SR from denervated muscle either may be maintained or may be altered. One of the potential problems in using SR isolated from rat hindlimb muscle is that the rat, depending on its environmental space, may passively stretch the denervated muscle by dragging the incapacitated limb. The degree of passive stretch may therefore evoke the various results noted, as discussed by Goldspink (1978) for muscle protein synthesis-degradation rates.

To obviate the potential problems noted above, we elected to examine the effect of denervation on the SR with the chicken fast-twitch pectoralis muscle as our model in which no environmental stretch problems exist. We demonstrate that the Ca^{2+} -ATPase activity of SR isolated from denervated chicken pectoralis muscle is significantly lower than control. The maximal phosphoenzyme formation is also decreased, so that the enzyme turnover is unchanged. These alterations in Ca^{2+} -dependent ATPase activity are accompanied by a lower Ca^{2+} accumulation, so that the relationship between ATP-dependent Ca^{2+} accumulation and Ca^{2+} -ATPase activity is well preserved in the SR isolated from denervated muscle. Evidence is presented, though, which suggests that the affinity of a single class of Ca^{2+} -binding sites on the Ca^{2+} -ATPase is lower after denervation.

Experimental

Choice of chicken fast-twitch muscle

Adult Leghorn roosters (3–6 kg) were used in the study. The chicken pectoralis muscle was chosen for a variety of reasons. (1) The muscle is primarily a homogeneous fast-twitch glycolytic muscle (Thakar, 1977). (2) The muscle is rarely, if ever, used by the caged bird, so that the contralateral muscle is an excellent control, obviating the possible variable experimental difficulties with denervation of mammalian hindlimb skeletal muscle. (3) The denervation technique is relatively simple and poses no apparent stress to the birds.

Denervation technique

Under diethyl ether anaesthesia, a small incision was made under the wing, and the median nerve of the pectoral nerves of the brachial plexus was severed, thus denervating the superior pectoralis muscle. The sham-operated contralateral pectoralis muscle served as the control. (In preliminary studies we determined that the functional characteristics of SR isolated from the contralateral muscle does not differ from the SR isolated from a sham-operated control muscle.) No observable wing droop was noted at any time after the procedure. Furthermore, the chickens had functional use of both wings. The birds were killed 20 days post-denervation.

SR isolation

The birds were first anaesthetized with halothane followed by decapitation. The SR was isolated from 100 g of tissue (trimmed of connective tissue and fat) from both the denervated and contralateral muscles by the technique of Scales *et al.* (1977), except that the total homogenization time in 3 vol. of 10 mM-Mops (4-morpholinepropanesulphonic acid, pH 6.8) and 10% sucrose was 90 s rather than 180 s. The resultant SR pellet was suspended in 10 mM-Mops (pH 6.8) and 28% sucrose. Protein was estimated by a biuret procedure. Contamination by sarcolemmal and mitochondrial fragments was estimated by ouabain-inhibitable ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity both in the presence and in the absence of deoxycholate (Besch *et al.*, 1976) and succinate dehydrogenase activity (Singer, 1974) respectively.

Ca^{2+} accumulation

ATP-dependent Ca^{2+} accumulation was monitored by the dual-wavelength spectrophotometric method previously described (Tate *et al.*, 1980), which employs the chelometric dye, murexide, at the wavelength pair, 507–542 nm. The reaction medium included 10 mM-Mops (pH 6.8), 100 mM-KCl, 10 mM- MgCl_2 , 100 μM -murexide, and an ATP-regenerating system (2 mM-phosphoenolpyruvate and 20 units of pyruvate kinase/ml) in a final volume of 3 ml. For Ca^{2+} accumulation in the absence of oxalate (Ca^{2+} binding), the SR protein concentration was 0.33 mg/ml. For oxalate-supported Ca^{2+} accumulation (Ca^{2+} uptake), the protein concentration was 0.1 mg/ml. After a 5 min equilibration period at 30°C, 40 μM - CaCl_2 was added. After a stable baseline was recorded, the reaction was initiated by the addition of 1 mM-disodium ATP.

ATPase activity

ATPase activity was monitored through the oxidation of NADH by using the linked enzyme method of Albers *et al.*, (1968) and a Gilford continuous recording spectrophotometer. The reaction mixture included 10 mM-Mops (pH 6.8), 100 mM-KCl, 2 mM-phosphoenolpyruvate, 20 units of pyruvate kinase/lactate dehydrogenase/ml (Sigma), 0.4 mM-NADH and 20 μg of protein/ml in a final volume of 2.0 ml. To determine the K_m and $V_{\max}$ for free Ca^{2+} , Ca^{2+} -EGTA buffers were utilized. The computer programs and the stability constants of Fabiato & Fabiato (1979) were used, as modified (Tate *et al.*, 1981), to derive the total concentrations of Ca^{2+} , Mg^{2+} and EGTA necessary to maintain the specified free Ca^{2+} at pH 6.8. Under our reaction conditions, the total EGTA concentration was 2 mM, the free Mg^{2+} concentration was 5.0 mM and the MgATP concentration was 1.0 mM. Free $[\text{Ca}^{2+}]$ was varied between 0.2 and 30 μM . After a 5 min equilibration period at 30°C, the reaction was

started by the addition of 1 mM-disodium ATP. Ca^{2+} -ATPase activity was determined by subtracting the rate in the presence of Mg^{2+} and EGTA (Mg^{2+} -dependent ATPase activity) from the rate in the presence of Mg^{2+} , EGTA and Ca^{2+} (total ATPase activity).

Phosphoenzyme formation

Steady-state phosphoenzyme formation at 0°C was determined by the method of Shigekawa *et al.* (1976) under identical reaction conditions listed above for Ca^{2+} -ATPase activity except that the protein concentration was increased to 0.3 mg/ml, and the free $[\text{Ca}^{2+}]$ was maintained at $10\ \mu\text{M}$. The reaction was initiated by the addition of 1.0 mM-disodium $[\text{P}^{32}]\text{ATP}$ (sp. radioactivity, 2000–3000 c.p.m./nmol).

Electrophoresis

Electrophoresis of isolated SR in 7.5% sodium dodecyl sulphate/polyacrylamide gels was performed as described by Laemmli (1970). The samples (25 μg) were solubilized in 1% sodium dodecyl sulphate, 1% β -mercaptoethanol, 10% glycerol and 0.008% Bromophenol Blue (tracking dye) in 20 mM Tris/HCl buffer, pH 6.8, at room temperature. The gels were stained with Coomassie Blue, destained and scanned with a Zeineh soft laser scanning densitometer (LKB Products). Molecular weights of the individual bands were determined by comparison with known standards. The percentage area of the individual protein peaks under the curve was determined by planimetry and verified by computer analysis (Apple II Plus Computer). Staining of 10% gels was also accomplished with Stains-All by the procedures of King & Morrison (1976).

Data analyses

The K_m and $V_{\max}$ of Ca^{2+} -ATPase activity as a function of free $[\text{Ca}^{2+}]$ were determined as described by Barnes & Waring (1980). Data are means $\pm$ S.E.M. for three observations with five birds used per observation. The data were statistically analysed by using the Mann-Whitney U test for paired observations (Runyon & Haber, 1971) with the 0.05 probability level established for significance. The muscle-weight data were statistically analysed with the paired Student's *t* test.

Results

Muscle atrophy, SR yield and protein profile

As previously shown for mammalian fast-twitch skeletal muscle (Sreter, 1970; Hughes & Yasin, 1973), a significant 43.2% ($P < 0.001$) loss of muscle weight in chicken fast-twitch skeletal muscle occurred at 20 days post-denervation. The tissue weights were $72.5 \pm 2\text{ g}$ wet wt. ($n = 15$) for the contra-

lateral muscle and $41.1 \pm 2\text{ g}$ wet wt. ($n = 15$) for the denervated muscle. The SR yield was 2-fold higher ($P < 0.05$) for the denervated muscle ($0.40 \pm 0.06\text{ mg}$ of protein/g wet wt. compared with $0.18 \pm 0.04\text{ mg}$ of protein/g wet wt. for the contralateral muscle). However, the estimated SR content (product of SR yield and muscle weight before trimming) was not significantly different between the two muscles ($13.1 \pm 2\text{ mg}$ of protein/contralateral muscle; $17.6 \pm 2\text{ mg}$ of protein/denervated muscle).

The protein profiles of the SR isolated from both muscles were qualitatively similar with the quantitative exceptions as noted (Fig. 1). The amount of contaminating sarcolemma and mitochondria was less than 1% in the SR isolated from both muscles. The Ca^{2+} -ATPase represented $37.7 \pm 3\%$ of the total area under the densitometric curve in the contralateral muscle and $28.2 \pm 3\%$ in the denervated muscle. Thus, on a per mg of SR protein basis, the Ca^{2+} -ATPase content of the SR isolated from denervated muscle was reduced by $25.2 \pm 5.5\%$ ($P < 0.05$).

As indicated in Fig. 1, there was an apparent 2.4-fold increase in a 60000-mol.wt. protein of the SR isolated from denervated muscle. To determine whether this protein was calsequestrin or the M_{55} intrinsic Ca^{2+} -binding protein, the Stains-All

Fig. 1. Densitometric traces of SR isolated from chicken muscle

Peak 1 is the 100000-mol.wt. Ca^{2+} -ATPase ($37.7 \pm 3\%$, contralateral; $28.2 \pm 3\%$, denervated); peak 2 is the 60000-mol.wt. M_{55} protein ($6.3 \pm 0.3\%$, contralateral; $15.3 \pm 1.3\%$, denervated); and peak 3 is a 30000-mol.wt. component ($2.4 \pm 0.4\%$, contralateral; $9.3 \pm 1\%$, denervated). Upper trace, contralateral; lower trace, denervated. Cathode (top) is to the left of the trace. See the Experimental section for details.

procedure was used (see the Experimental section) with a comparison made between a 'heavy' SR fraction isolated from rabbit skeletal muscle, which is enriched in calsequestrin (Van Winkle *et al.*, 1982), and the two chicken SR fractions. Calsequestrin, an extrinsic Ca^{2+} -binding protein of the SR, is found almost exclusively in the terminal cistern region of rabbit skeletal-muscle SR, both *in situ* and *in vitro* (Meissner, 1975; Campbell *et al.*, 1980), and is stained a dark blue with Stains-All (Jones & Cala, 1981). Calsequestrin, however, is virtually absent in the 'light' SR from rabbit muscle, which is thought to be derived from the longitudinal SR. With Stains-All, calsequestrin of the rabbit 'heavy' SR stained dark blue, whereas the 60000-mol.wt. protein of the chicken muscle SR stained purple and migrated to a position below calsequestrin, where the M_{55} protein is localized on sodium dodecyl sulphate/polyacrylamide gels run by the method of Laemmli (1970). Therefore, we believe that this protein of the chicken muscle SR represents the M_{55} protein. Thus the isolated chicken muscle SR probably represents the longitudinal SR.

In addition to the apparent increase in the M_{55} protein as a consequence of denervation, there was also a 4-fold increase in a 30000-mol.wt. component of unknown properties (Fig. 1), but noted in other SR preparations as well. Although we have at present no explanation for the apparent increase in the M_{55} protein and the 30000-mol.wt. component of SR isolated from denervated muscle, it is noteworthy that the ratio of Ca^{2+} -ATPase to either protein was markedly reduced; i.e. Ca^{2+} -ATPase/ M_{55} protein was approx. 6 for the contralateral SR and about 2 for the denervated SR. Furthermore, the Ca^{2+} -ATPase/30000-mol.wt. protein ratio was 16 for the contralateral muscle SR compared with 3 for the denervated muscle SR.

ATPase activity of isolated SR

Ca^{2+} -dependent ATPase activity of the SR isolated from denervated muscle was lower at all concentrations of free $[\text{Ca}^{2+}]$. Analyses of the data yielded the kinetic parameters presented in Table 1. The SR from denervated muscle had a $64 \pm 5\%$ apparent lower affinity for Ca^{2+} as well as a

$42.6 \pm 0.8\%$ lower maximal velocity with respect to the contralateral muscle SR. In addition, maximal phosphoenzyme formation was $56.1 \pm 8\%$ lower in the SR isolated from denervated muscle (Table 1). Since the V_{max} was also lower, the calculated enzyme turnover ($V_{\text{max}}/[\text{phosphoenzyme}]$) was not significantly affected by denervation.

Hill plots of the Ca^{2+} -ATPase activity data yielded an apparent Hill coefficient of 1.0, which was not altered by denervation ($h = 1.05 \pm 0.06$ for the contralateral SR; $h = 0.95 \pm 0.05$ for the denervated SR). This finding was unexpected, since the apparent Hill coefficient is 2 for SR isolated from rabbit fast-twitch muscle (Ikemoto, 1975). We therefore isolated SR from rabbit fast-twitch muscle under identical conditions and found that the K_m and V_{max} were similar to the SR isolated from the chicken contralateral muscle (Table 1). In contrast with chicken muscle SR, the apparent Hill coefficient was 2.0 for the rabbit muscle SR. Although the physiological relevance of this finding is unknown, it may be related to species differences in the structure of the Ca^{2+} -ATPase from the same muscle type (Ohnoki & Martonosi, 1980; DeFoor *et al.*, 1980).

The Mg^{2+} -dependent ATPase activity was more dramatically affected by denervation than was Ca^{2+} -ATPase activity, with a $73.5 \pm 2\%$ ($P < 0.05$) decrease in activity (614.89 ± 112 nmol/mg of protein per min in the contralateral-muscle SR; 162.76 ± 31 nmol/mg of protein per min in the denervated-muscle SR). The ratio of Ca^{2+} -dependent ATPase activity (V_{max}) to Mg^{2+} -dependent ATPase activity increased from 1.0 to greater than 2.0 after denervation.

ATP-dependent Ca^{2+} accumulation

The reduction of Ca^{2+} -dependent ATPase activity of SR isolated from denervated muscle was accompanied by a significant decrease in ATP-dependent Ca^{2+} binding (Fig. 2; Table 2). This significant attenuation of Ca^{2+} binding by denervated SR was also evident both in the rate of ATP-dependent Ca^{2+} uptake and in the maximum Ca^{2+} uptake determined in the presence of the Ca^{2+} -precipitating agent oxalate (Table 2). The ratio of Ca^{2+} uptake rate to Ca^{2+} -ATPase activity

Table 1. Kinetic parameters for Ca^{2+} -dependent ATPase activity, phosphoenzyme formation and enzyme turnover. Reaction conditions for maximal phosphoenzyme formation (EP) are described in the Experimental section. Enzyme turnover is represented as $V_{\text{max}}/[\text{EP}]$. Abbreviation: ns, not significant.

Muscle SR	K_m (μM)	V_{max} (nmol/mg of protein per min)	[EP] (nmol/mg of protein)	$V_{\text{max}}/[\text{EP}]$ (min^{-1})
Contralateral	0.85 ± 0.03	628.08 ± 59	2.04 ± 0.56	339.54 ± 57
Denervated	1.40 ± 0.03 ($P < 0.05$)	360.01 ± 32 ($P < 0.05$)	0.82 ± 0.12 ($P < 0.05$)	448.19 ± 50 (ns)

Table 2. *ATP-dependent Ca^{2+} accumulation by isolated chicken muscle SR*

Reaction conditions for Ca^{2+} accumulation in the absence and presence of oxalate are described in the Experimental section.

Muscle SR	Peak Ca^{2+} binding* (nmol/mg of protein)	Ca^{2+} uptake rate† (nmol/mg of protein per min)	Maximum Ca^{2+} uptake† ($\mu\text{mol/mg}$ of protein)
Contralateral	84.4 ± 9	739.49 ± 86	1.55 ± 0.03
Denervated	61.9 ± 2 ($P < 0.05$)	492.81 ± 37 ($P < 0.05$)	1.17 ± 0.03 ($P < 0.05$)

* In the absence of oxalate.

† In the presence of 5 mM-oxalate.

Fig. 2. *ATP-dependent Ca^{2+} binding by isolated chicken muscle SR*

ATP-dependent Ca^{2+} binding in the absence of oxalate was determined as described in the Experimental section. Note that the SR isolated from denervated muscle (D) took longer to reach peak Ca^{2+} accumulation than the control (C).

measured under identical conditions was approx. 1.0 for both contralateral- and denervated-muscle SR. This correlated well with the apparent Hill coefficient of 1.0 described above.

Discussion

This study demonstrates that denervation of adult chicken fast-twitch skeletal muscle results in a diminution of SR function *in vitro*. The data suggest that the absence of neural information affects a single class of Ca^{2+} -binding sites of the Ca^{2+} -ATPase resulting in a lower affinity for Ca^{2+} , a decreased maximal velocity and a reduction of Ca^{2+} accumulation. Therefore, the relationship between Ca^{2+} accumulation and Ca^{2+} -ATPase activity is well maintained in the SR from chicken denervated fast-twitch skeletal muscle with a coupling ratio of 1. Although the maximal velocity and phosphoenzyme level are decreased after denervation, the enzyme turnover is similar to the contralateral muscle

SR. Since the 25% loss of Ca^{2+} -ATPase mass in itself cannot account for the noted changes, the Ca^{2+} -ATPase may be functionally modified so that less pump units are turning over, although the units turning over do so in a normal manner (normal turnover in comparison with contralateral-muscle SR).

Additional evidence for a denervation-induced alteration of the Ca^{2+} -ATPase is suggested by the increased Ca^{2+} -ATPase activity/ Mg^{2+} -dependent ATPase activity ratio. In the present study, the ratio increased from 1 in the contralateral-muscle SR to more than 2 in the denervated-muscle SR, resulting from a much greater decrease in Mg^{2+} -dependent ATPase activity relative to the diminished Ca^{2+} -ATPase activity. Recent work from our laboratory (Van Winkle *et al.*, 1981) as well as by Inesi *et al.* (1980) suggests that the Mg^{2+} -dependent ATPase activity may represent a conformational state of the Ca^{2+} -ATPase, whereas Fernandez *et al.* (1980) believe that the Mg^{2+} -dependent ATPase activity comes from contamination of the isolated SR by mitochondrial and surface-membrane fractions. Our SR preparations, however, isolated from both muscles are virtually devoid (<1%) of such contaminants as judged by marker enzymes and have similar qualitative protein profiles. We therefore believe that the Mg^{2+} -dependent ATPase activity evolves from a single enzyme, the Ca^{2+} -ATPase, rather than being the result of non-specific contamination. If the Mg^{2+} -dependent ATPase activity does indeed represent a conformation of the Ca^{2+} -ATPase, then the increased ratio of Ca^{2+} -ATPase activity to Mg^{2+} -dependent ATPase activity in the SR after denervation further suggests that the Ca^{2+} -ATPase has been functionally modified.

In addition to the denervation-induced modification of SR function, there was an apparent quantitative alteration of the protein profile of the SR after denervation. The Ca^{2+} -ATPase was the major protein of the SR isolated from the contralateral muscle, with less of the other two estimated proteins, as suggested by the high Ca^{2+} -ATPase/lower-molecular-weight protein ratios.

These ratios were markedly attenuated in the SR isolated from denervated muscle, demonstrating an enhancement of these minor proteins with respect to the Ca^{2+} -ATPase. A similar apparent shift in the protein profile of the SR membranes was noted by Margreth *et al.* (1972) with SR isolated from rat denervated fast-twitch muscle. Although the role of these lower-molecular-weight proteins in the Ca^{2+} transport process of the Ca^{2+} -ATPase is unknown, Hasselbach *et al.* (1975) previously discussed the probability that there are Ca^{2+} sequestration sites in the SR that function to maintain low levels of free internal Ca^{2+} for optimal organelle function *in vivo*. In this context, it is noteworthy that the Ca^{2+} content of whole muscle increases after denervation (Hines & Knowlton, 1933; Picken & Kirby, 1976).

Although there is considerable debate concerning the mode through which the motor nerve influences the genetic functional expression of skeletal muscle, it is clear that neural information is required for the maintenance of normal function *in vitro* of SR isolated from adult chicken fast-twitch pectoralis muscle. In addition, the molecular composition of the SR membranes, as noted by the altered protein profile after denervation, is also apparently under neural control. Even though the estimated Ca^{2+} -ATPase mass was affected by denervation, the absence of neural input modifies the Ca^{2+} -ATPase as shown by several lines of evidence. The modulation of the Ca^{2+} -ATPase may evolve from a change in the enzyme molecule itself, from an alteration in the lipid-protein interaction or from a variety of other factors, including a redistribution of the SR proteins.

We express our appreciation to Dr. Jim Blosser, Department of Neurology, for demonstrating the denervation technique, to Mrs. K. Scott and Mr. K. Youker for technical assistance during various phases of the study and to Ms. Concepcion Mata and Mrs. Corneille Smith for secretarial assistance. This work was supported in part by grants from the Muscular Dystrophy Association and the N.I.H. (HL 22856 and HL 13870).

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