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EVIDENCE OF INCOMPLETE NEURAL CONTROL OF MOTOR UNIT PROPERTIES IN CAT TIBIALIS ANTERIOR AFTER SELF-REINNERVATION

BY GRACIELA A. UNGUEZ*, SUE BODINE-FOWLER†, ROLAND R. ROY‡, DAVID J. PIEROTTI* AND V. REGGIE EDGERTON*‡

*From the *Department of Physiological Science and the ‡Brain Research Institute, University of California at Los Angeles, CA 90024-1527, USA and the †Department of Orthopaedics University of California at San Diego School of Medicine, La Jolla, CA 92093-9151, USA*

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SUMMARY

1. The mechanical, morphological and biochemical properties of single motor units from the anterior compartment of the tibialis anterior muscle in adult cats were studied six months after the nerve branches to that compartment were cut and resutured in close proximity to the muscle.

2. In these self-reinnervated muscles, the maximum tetanic tensions were lower in slow than fast units, a relationship similar to that observed among motor units from control adult muscles. The maximum tetanic tensions produced by the fast units were larger than those produced by the same motor unit types in control muscles. Direct counts of muscle fibres belonging to a motor unit showed that factors controlling the number of muscle fibres innervated by a motoneurone type persist during the reinnervation process in that fast motoneurones reinnervated more muscle fibres than slow motoneurones. Thus, the number of muscle fibres reinnervated by a motoneurone principally accounted for the difference in the maximum tension outputs among motor unit types, a relationship similar to that observed in control tibialis anterior muscles.

3. Monoclonal antibodies for specific myosin heavy chains were used to differentiate fibre types. By this criterion, motor units from control muscles were found to contain a homogeneous fibre type composition. In contrast, a heterogeneous, yet markedly biased, fibre type composition was observed in each unit analysed from self-reinnervated muscles.

4. Although not all of the muscle fibres of a motor unit developed the same type-associated parameters after reinnervation, the relationships among myosin heavy chain profile, succinate dehydrogenase activity and the fibre size were similar in fibres of control and self-reinnervated muscles.

5. The processes which dictate both motor unit size and the matching between motoneurone and muscle fibre type during the reinnervation process must be interdependent and result from a hierarchy of decisions which reflects their relative importance. The mechanisms responsible for these two processes may be a

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combination of: (1) selective innervation which may or may not incorporate a pruning process if multiple synaptic connections are initially formed and/or (2) conversion of enough fibres of a motor unit to form a predominant type.

INTRODUCTION

When the original motoneurone–muscle fibre connections are disrupted, the electrical properties of those motoneurones that make functional reconnections with the muscle and the mechanical properties of the muscle unit, i.e. the twitch speed-related properties and maximum force generating capabilities, are for the most part re-established following long-term self-reinnervation in adult muscles (Gordon & Stein, 1982; Foehring, Sypert & Munson, 1986). For example, the time course of the isometric twitch and fatigue properties of motor unit types recover to control levels (Foehring *et al.* 1986). Furthermore, the proportion of each motor unit type in self-reinnervated muscle has been reported to be similar to that found in normal control animals (Gordon & Stein, 1982; Foehring *et al.* 1986). Although some studies have reported an increased incidence of motor units that produce larger (Bagust & Lewis, 1974) and smaller (Bagust & Lewis, 1974; Chan, Edgerton, Goslow, Kurata, Rasmussen & Spector, 1982) than normal tensions after reinnervation, a preponderance of motor units within the normal range in tension production has been shown after a muscle is reinnervated by axons from its own muscle nerve (Chan *et al.* 1982; Gordon & Stein, 1982; Foehring *et al.* 1986).

The wide range of force-producing capabilities among motor unit types in control cat (Bodine, Roy, Eldred & Edgerton, 1987) and rat (Chamberlain & Lewis, 1989) hindlimb muscles is directly proportional and closely correlated to the innervation ratio, i.e. the number of fibres innervated by a motoneurone. However, the extent to which the increased variability in maximum force among motor units after self-reinnervation in cat hindlimb muscles can be attributed to changes in the innervation ratio, or other variables such as fibre size and specific tension, has not been examined directly. Only estimates and indirect measurements of these variables have been reported and it is known that, even in normally innervated muscles, these methodological approaches can be misleading in evaluating the relative contribution of fibre cross-sectional area, fibre number and specific tension to the maximum tension output of motor units (Bodine *et al.* 1987).

The restoration of normal mechanical properties among motor units of adult reinnervated muscles has been attributed to a mechanism in which regenerating motoneurones form functional contacts with muscle fibres at random and subsequently convert this heterogeneous population of fibres into a homogeneous population that matches the properties of the motoneurone (Karpati & Engel, 1968; Kugelberg, Edstrom & Abbruzzese, 1970; Nemeth & Turk, 1984). However, this hypothesis of neural determination of muscle properties is based primarily on indirect measurements of biochemical and morphological properties of reinnervated muscle fibres. Qualitative histochemical observations have been made on a combined total of fourteen glycogen-depleted motor units from the rat (Kugelberg *et al.* 1970) and cat (Foehring *et al.* 1986) following self-reinnervation. Based on these limited data, the degree to which a motoneurone can respecify the size of the motor unit and the biochemical properties of muscle fibres remains unclear.

The present study was designed to assess directly the degree to which normal motor unit size (as defined by innervation ratio and maximum tetanic tension) and biochemical properties (as reflected by succinate dehydrogenase (SDH) activity and myosin heavy chain (MHC) composition) among fibres of a motor unit can be re-established after self-reinnervation. To address these issues, motor units in the tibialis anterior (TA) muscle of the adult cat were functionally isolated using ventral root isolation techniques 6 months after the nerve branches to the anterior compartment had been cut and resutured in close proximity to the muscle. The TA was studied because: (1) it is composed of a heterogeneous population of motor unit and fibre types (Dum & Kennedy, 1980; Iliya & Dum, 1984; West, Roy & Edgerton, 1986) and (2) its anatomical determinants of maximum force in control muscles have been measured directly (Bodine *et al.* 1987). Preliminary results have been reported in abstract form (Roy, Unguez, Pierotti, Bodine-Fowler & Edgerton, 1989; Unguez, Bodine-Fowler, Roy, Pierotti & Edgerton, 1992).

METHODS

Experimental design

Experiments were performed on seven adult female cats (body weight at surgery, 2·6–3·7 kg). The cats were initially given an injection of atropine sulphate (0·10 mg/kg, I.P.) and sedated with a mixture of ketamine (13 mg/kg, I.M.) and acepromazine (0·01 mg/kg, I.M.). The cats were then anaesthetized with sodium pentobarbitone intravenously to effect, i.e. to suppress withdrawal and eye-blink responses. Under aseptic conditions, an incision was made through the skin and fascia on the lateral side of the leg to expose the TA muscle. Care was taken to minimize tissue trauma and bleeding.

The TA receives innervation from two major branches from the common peroneal nerve (Iliya & Dum, 1984). Muscle fibres innervated by each of the TA major nerve branches are localized within different compartments of the muscle (Iliya & Dum, 1984). The distal nerve branch innervates the posterior compartment, i.e. the portion of the muscle closest to the bone and artery. The proximal nerve branch subdivided into two or three smaller branches which innervate the anterior compartment, i.e. the superficial portion of the muscle. The nerve branches to the TA were dissected free near their points of insertion into the muscle. Stimulation of individual nerve branches helped positively to distinguish those branches innervating the anterior muscle compartment from those innervating the posterior compartment. Each branch supplying the anterior compartment was sectioned close (approximately 2 mm) to its entry into the muscle. The proximal and distal stumps of each branch were reattached immediately with 9-0 silk sutures through the epineurium. By cutting the motor nerve near the muscle and studying reinnervation within a single muscle, the opportunity was maximized for selective regeneration of axons to their original target muscle, yet ensured a disruption of the axonal pathways during self-reinnervation. The skin and fascia were closed with 4-0 nylon sutures. This procedure was performed bilaterally in all experimental cats. Upon completion of the surgery, animals recovered in an incubator and were then transferred to large cages (3 m^2 of floor space). The cats were maintained in excellent health for 6 months before physiological measurements were obtained and the muscles removed for analyses. All procedures used in this study followed the American Physiological Society Animal Care Guidelines and were approved by the Animal Use Committee at the University of California at Los Angeles (UCLA), CA, USA.

Acute experiments

Isolation of single motor units in each TA muscle was performed on seven female cats (body weights of 3·0–4·3 kg) following a 6-month self-reinnervation period, and on three control female cats (body weights of 3·3–5·1 kg). The terminal experiment used the techniques and protocols described in detail by Bodine *et al.* (1987). For identification of the motor unit fibres via glycogen depletion procedures, it was essential that only those fibres belonging to the stimulated axon were depleted of glycogen. Therefore, to enhance muscle glycogen stores, each cat was given a daily subcutaneous injection of a 5% dextrose solution (120 ml) for 4 days prior to the terminal

experiment. In addition, the cats were housed in smaller cages ($46 \times 61 \times 76$ cm^2) during this period to minimize spontaneous activity.

The cats were initially given an injection of atropine sulphate (0·10 mg/kg, I.P.) and sedated with a mixture of ketamine (13 mg/kg, I.M) and acepromazine (0·01 mg/kg, I.M.). Experiments were performed under pentobarbitone anaesthesia supplemented intravenously as needed to keep withdrawal and eye blink reflexes suppressed. A laminectomy was performed from L5 to S1 to expose the dorsal and ventral roots supplying the TA. All nerves innervating muscles in the tail, hip, thigh and lower limb were cut except for the branches innervating the anterior compartment of the TA. The distal nerve branches supplying the posterior (deep) compartment of the TA were cut to ensure isolation of motor units located in the reinnervated anterior (superficial) compartment. The TA was carefully isolated from surrounding tissues while preserving the blood supply and nerve branches to the muscle compartment. The experimental leg was clamped securely at the knee and at the ankle. The distal tendon of the TA was attached in series to a force transducer (Cambridge Instruments, Model 305, Watertown, MA, USA) for tension recording. Mineral oil pools were constructed over the spinal cord and muscle by elevating the skin around the incisions of the cord and leg. The leg and body temperatures were maintained at 36 ± 1 °C with radiant heat and a heating pad. For stimulation of the anterior compartment of the TA, the major nerve branch was placed on a bipolar silver electrode. Ventral root filaments were stimulated via a bipolar silver electrode positioned above the cord in the oil bath. A bipolar silver-ball electrode was placed on the muscle surface to record the electromyographic signals (EMG) from the motor unit. Experiments were performed bilaterally.

Motor unit recording and isolation

A single motor unit in each control and self-reinnervated TA muscle was characterized physiologically and glycogen depleted by stimulating its functionally isolated axon (teased from either the L6 or L7 ventral root). Confirmation of functional isolation of a single axon was provided by: (1) an all-or-none twitch response following filament stimulation at voltages ranging from threshold to $10 \times$ threshold; (2) a corresponding all-or-none EMG recorded from the muscle demonstrating a consistent waveform; and (3) an all-or-none action potential in the root filament following graded antidromic stimulation of the muscle nerve.

Motor unit mechanical properties were measured under isometric conditions at a muscle length that produced the largest twitch tension of the unit. The properties measured included single unpotentiated twitches, the presence or absence of 'sag' in unfused isometric contractions, frequency–tension response at frequencies of 5, 10, 15, 20, 25, 30, 40, 50, 75, 100 and 200 Hz which produced the maximum tetanic tension, and fatigue. Fatigue was tested using 40 Hz trains of 330 ms duration delivered 1/s and continued for 2 min (Burke, Levine, Tsairis & Zajac, 1973). A fatigue index was calculated as the ratio of tension at 2 min to the maximum tension recorded during the fatigue test. Motor units were classified as either slow, fatigue resistant (S); fast, fatigue resistant (FR); fast, fatigue intermediate (FI); or fast, fatigable (FF), based on contraction time, fatigability and the presence or absence of sag (Burke *et al.* 1973).

Following the fatigue test, the muscle fibres belonging to the motor unit were depleted of glycogen using a stimulus protocol designed to stress optimally the glycolytic pathways and to use the intracellular glycogen stores (Bodine *et al.* 1987). The train rate, stimulus frequency and train duration were adjusted to give a work-to-rest ratio greater than or equal to 1:1. The procedure generally took between 30 min and 2 h depending on the unit type. Stimulation parameters were adjusted throughout the depletion protocol to minimize any decrement in the EMG recorded from the unit, and thus to avoid neuromuscular fatigue.

Upon cessation of the glycogen depletion protocol, the cat was given an overdose of sodium pentobarbitone. The length of the TA muscle plus tendon was measured *in situ*. The muscle was fixed at this length by clamping the distal tendon to the tibia and keeping the proximal attachment intact. The tibia and TA were excised, and the TA cleaned of excess connective tissues, blotted dry and rapidly frozen in isopentane cooled in liquid nitrogen. This freezing procedure resulted in no detectable length change of the muscle. The frozen muscle was removed carefully from the tibia and cut into 5 mm blocks from the proximal to the distal extent of the muscle. The tissue blocks were subsequently mounted on cork and stored at -70 °C.

Histochemical procedures

Cross-sections (20 μm thick) were cut from each tissue block and stained for glycogen using a periodic acid–Schiff (PAS) stain (Pearse, 1961) to assess the glycogen content of the fibres.

Glycogen levels within individual fibres were quantified by using an image processing computer system (Bodine *et al.* 1987) to determine the optical density (OD) of glycogen staining. The muscle fibres belonging to a stimulated motor unit were identified by their lack of glycogen. A frequency distribution of the OD displayed by the outlined fibres was plotted and a cut-off value that distinguished depleted and non-depleted fibres was determined. Confirmation of the assigned threshold level differentiating depleted from non-depleted fibres was also based on OD measurements of fibres in the non-reinnervated (deep) compartment of the TA muscles.

In ten of the fourteen motor units from self-reinnervated muscles, the cut-off value was very consistent, ranging from 0·180 to 0·215 OD units. In general, only a small percentage (2–8%) of the fibres that were outlined were considered questionable (i.e. within 0·010–0·025 OD units of the cut-off) as to whether they were depleted or not. These questionable fibres were examined closely in PAS-stained sections using a light microscope and were subsequently classified as non-depleted. Four motor units from self-reinnervated muscles were excluded from morphological analyses since a clear cut-off could not be determined from the frequency histograms. For similar reasons, data are presented for only four to six motor units isolated from control TA muscles.

The number of fibres belonging to a motor unit, i.e. the innervation ratio, was determined by counting depleted fibres in PAS-stained sections taken from each tissue block of the muscle. Since the number of depleted fibres often varied between serial sections, individual depleted fibres were identified and followed across sections to ensure that every depleted fibre was included and that each fibre was counted only once. All motor unit fibres in the muscle cross-section containing the largest number of glycogen-depleted fibres were outlined manually and measurements of their cross-sectional area were calculated using an image-processing system (Bodine *et al.* 1987). The mean fibre cross-sectional area of the unit was calculated from glycogen-depleted fibres analysed in this muscle cross-section stained with PAS. The average number of fibres analysed for fibre size was approximately 82% of the innervation ratio of each unit (range, 58–100%). The total cross-sectional area of the motor unit, i.e. the motor unit area, was then calculated by multiplying the mean fibre cross-sectional area of the motor unit fibres by the innervation ratio. Specific tension was calculated as the ratio of maximum tetanic tension to the total cross-sectional area of the unit (Bodine *et al.* 1987). In the cross-section used for determining motor unit fibre sizes, the areas of 150–400 non-depleted fibres from both the anterior and posterior compartments of the muscle were also determined.

Serial cross-sections (10 μm thick) were cut and mounted on either coverslips or gelatin-covered slides to be used for enzyme histochemistry or immunohistochemistry, respectively. The SDH activity, a commonly used marker for activity of mitochondrial enzymes, was determined in a sample of motor unit and non-motor unit fibres using the quantitative histochemical techniques described by Martin, Bodine-Fowler, Roy, Eldred & Edgerton (1988).

To identify specific fibre types, serial sections were stained immunohistochemically by an indirect immunoperoxidase technique using monoclonal antibodies reacting with specific adult MHCs. Briefly, the tissue sections were incubated with the antibodies overnight at 4 °C. Sections incubated without primary antibody were used as controls to visualize non-specific labelling. A Vectastain ABC kit (Vector Labs, Burlingame, CA, USA) was used to amplify the antigen–antibody complex, which in turn was visualized by treatment with a diaminobenzidine (DAB) peroxidase reaction. The staining profile of muscle fibres with antibodies BA-F8, BF-13 and BF-35 (generously donated by S. Schiaffino, Padova, Italy) was used to type fibres in the cat TA muscle (Table 1). The pattern of reactivity of these antibodies has been previously described for rat skeletal muscle (Bottinelli, Schiaffino & Reggiani, 1991). We found that the relationship of the antibody-binding immunohistochemical reactions and the fibres types I, IIA and IIB based on histochemical staining for myofibrillar ATPase (see below) are not identical in the rat and cat. In the cat, fibres that were specifically labelled by BA-F8 and BF-35 and unreactive with BF-13 were classified as slow MHC fibres. Two subtypes of fast fibres which were unreactive with BA-F8 but reactive with BF-13 could be separated by their reactivity with BF-35. Fast-1 MHC fibres were reactive with BF-13 and BF-35 whereas fast-2 MHC fibres were only reactive with BF-13.

Standard histochemical staining for myofibrillar ATPase after acid (pH, 4·35) and alkaline (pH, 10·4) preincubations was also performed. In general, the antibody-labelled MHC profiles were consistent with the myofibrillar ATPase-based classification scheme for fibre types I, IIA and IIB. However, some inconsistencies in the classification of fibres were observed in both control and experimental muscles using the myofibrillar ATPase method. In contrast, the immunohistochemical technique was less susceptible to inconsistencies in classification and possible

misinterpretation obtained with the ATPase method which may result from co-existence of different MHCs within a single fibre. Thus, for this study, the identification of fibre types was based on distinctive MHC profiles obtained using the three monoclonal antibodies (Table 1). The immunohistochemical analyses were performed at a different muscle level (between 5 and 10 mm) from that which contained the largest number of glycogen-depleted fibres and which was used for fibre size measurements. Thus, a smaller sample of motor unit fibres was available in the cross-sections that were reacted against the three antibodies (Table 1).

Statistical analyses

A stepwise regression analysis (BMDP2R; Dillon & Goldstein, 1984) was used to determine the relative importance of innervation ratio, fibre size and specific tension in determining the maximum tetanic tension of a motor unit. The stepwise regression starts by entering that variable with the largest simple correlation with the dependent measure and continues by entering the variable with the largest squared partial correlation which has an associated F-value greater than the assigned critical F-to-enter value. The F-to-enter value is a measure of the extent to which the summed squares of residuals will be reduced if that variable is entered into the regression equation.

Pearson product correlation coefficients were calculated to determine the relationships between pairs of variables (Dixon & Massey, 1983). A χ^2 test was used to compare the frequency of each fibre type observed within a motor unit to the frequency of occurrence of each fibre type within the territory of the unit. This comparison was deemed appropriate based on the underlying assumption that a higher probability would exist for a reinnervating motoneurone to re-establish functional connections with those muscle fibres which are located within a specified region of the denervated muscle compartment. In addition, this comparison takes into account the existing anatomical gradient of fibre types across the anterior muscle compartment and the relative location of the motor unit fibres within the muscle compartment (Dum & Kennedy, 1980; Iliya & Dum, 1984). The SDH activities of motor unit and non-motor unit fibres from self-reinnervated TA muscles were expressed as OD/min. Differences in mean enzyme activities among motor unit fibres were tested with a one-way analysis of variance (ANOVA). *Post hoc* comparisons were made with Tukey's procedure. Significance was set at $P < 0{\cdot}05$.

The motor unit data from control cats in this study were pooled with data obtained from previous studies using age-matched female cats (Bodine *et al.* 1987; Pierotti, Roy, Bodine-Fowler, Hodgson & Edgerton, 1991). The pooled data were used for statistical comparison with the data from self-reinnervated TA muscles. A t test was used to determine significant ($P < 0{\cdot}05$) differences between the control and self-reinnervated groups (Dixon & Massey, 1983).

RESULTS

Mechanical properties of motor units in self-reinnervated muscles

Single motor units were isolated and the isometric mechanical properties measured to determine the extent to which normal tension and motor unit type characteristics were re-established after axotomy and self-reinnervation. Each of the isolated units was typed according to twitch contraction time, fatigue index and the presence or absence of sag. The population of fourteen motor units studied included types S ($n = 2$), FR ($n = 9$) and FI ($n = 3$) units. No FF motor units were isolated (Figs 1 and 2). Six months after axotomy, the maximum tetanic tensions were significantly lower in slow than fast units in self-reinnervated muscles, a relationship that is similar to that found in motor units from TA muscles in normal adult cats (Fig. 2; Dum & Kennedy, 1980; Bodine *et al.* 1987). In addition, the mean maximum tetanic tensions produced by each of the fast unit types were significantly larger than those produced by the same motor unit types in control TA muscles (Figs 1 and 2).

Morphological features of motor units in self-reinnervated muscles

The maximum tetanic tension of a motor unit is a function of the innervation ratio, fibre cross-sectional area and specific tension. To determine what factor(s) may

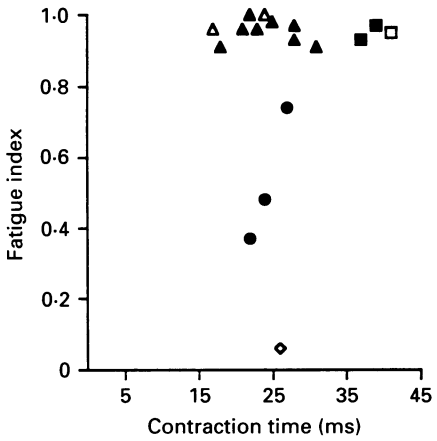

Fig. 1. Relationship between contraction time and fatigue index for the 14 motor units from self-reinnervated tibialis anterior muscles (filled symbols) and 4 motor units from control tibialis anterior muscles (open symbols). Motor units were typed physiologically as slow, fatigue-resistant (□, ■); fast, fatigue-resistant (△, ▲); fast, fatigue-intermediate (●); and fast, fatigable (◇), based on contraction time, fatigability, and the presence or absence of sag (see Methods).

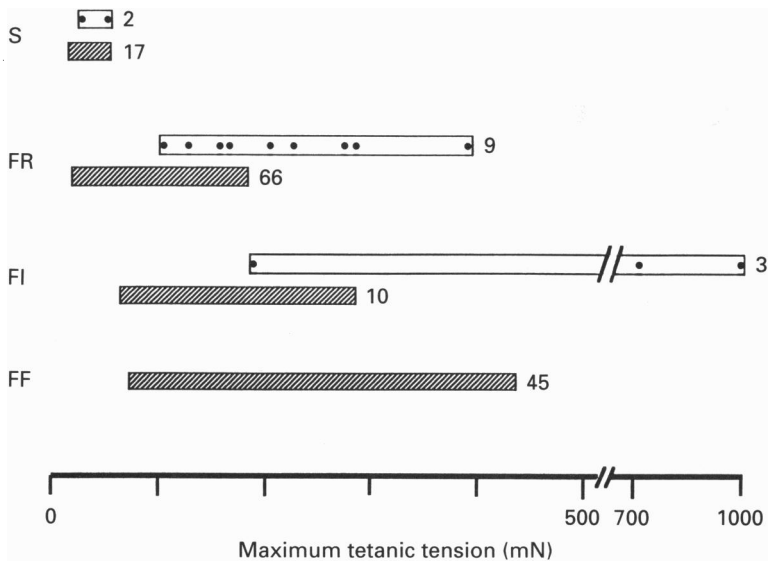

Fig. 2. Ranges in maximum tetanic tension for motor units of self-reinnervated (open bars) and control (hatched bars) cat tibialis anterior muscles. The control data from this study were pooled with control data from Dum & Kennedy (1980), Bodine *et al.* (1987) and Pierotti *et al.* (1991). The individual values for motor units in self-reinnervated muscles are represented by the dots within the open bars. The sample size is indicated to the right of each bar. Motor units were classified physiologically as slow, fatigue-resistant (S); fast, fatigue-resistant (FR); fast, fatigue-intermediate (FI); or fast, fatigable (FF). Note the scale difference on the abscissa after the broken bars.

be responsible for the increase in tension of the fast motor units after self-reinnervation, these morphological characteristics were measured in ten of the fourteen units from self-reinnervated TA muscles.

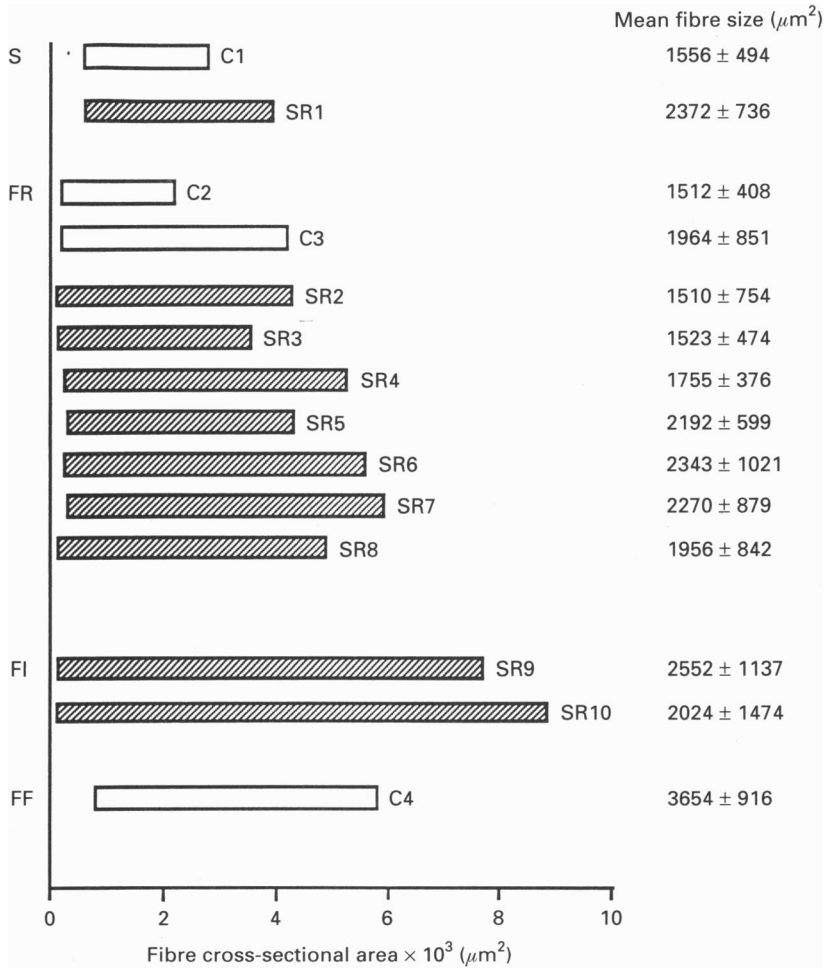

Fig. 3. Ranges in fibre cross-sectional area for 10 motor units from self-reinnervated (SR) (hatched bars) and for 4 motor units from control (C) (open bars) tibialis anterior muscles. The mean (±S.D.) fibre cross-sectional area is given for each motor unit. Motor unit numbers and types correspond to those in Table 1.

Fibre cross-sectional area

The cross-sectional area of every glycogen-depleted fibre in that cross-section containing the largest number of motor unit fibres was measured. The mean cross-sectional areas of fibres in each of the motor unit types in self-reinnervated muscles were similar, i.e. S, 2372 μm^2; FR, 1936 μm^2; and FI, 2288 μm^2. Although the means were similar across motor unit types, fibres that were larger than 6000 μm^2 were found only in the FI units (Fig. 3). In general, the variability in fibre size, as

determined by the standard deviation and range, was greater in motor units from self-reinnervated than control muscles (Bodine *et al.* 1987; Pierotti *et al.* 1991) (Fig. 3). As in control TA muscles, S and FR units of self-reinnervated muscles had similar ranges in fibre size, while the FI units had a much greater range of fibre sizes. The

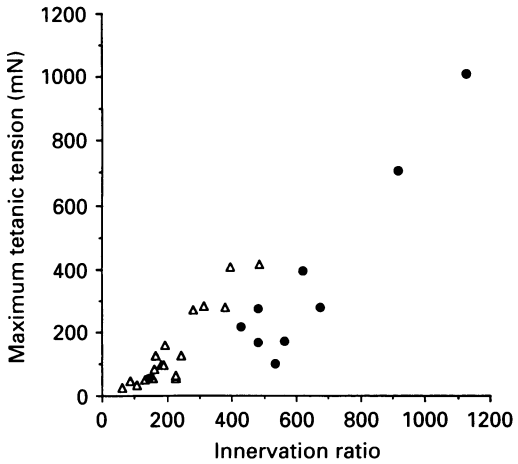

Fig. 4. Relationship between maximum tetanic tension and innervation ratio for motor units in self-reinnervated (●, $n = 10$, $r = 0{\cdot}92$) and control (△, $n = 19$, $r = 0{\cdot}93$) tibialis anterior muscles. Pooled control data are taken from the present study, Bodine *et al.* (1987) and Pierotti *et al.* (1991).

correlation coefficient between the mean cross-sectional area of the fibres within the unit and the maximum tetanic tension produced by the motor unit was not significant ($r = 0{\cdot}29$, $P > 0{\cdot}05$).

To determine whether denervation and reinnervation of the anterior compartment affected the cross-sectional area of the fibres within that muscle compartment, the mean fibre cross-sectional area for a sample (150–400) of fibres from the non-denervated (posterior) compartment was compared to the mean fibre size of a sample of fibres in the self-reinnervated (anterior) compartment. This comparison was deemed appropriate since in the control TA muscles there is no significant difference between the mean fibre size of the anterior ($2181 \pm 540\ \mu m^2$) and posterior compartments ($2019 \pm 229\ \mu m^2$). Mean fibre sizes of the self-reinnervated ($2061 \pm 236\ \mu m^2$) and the non-denervated ($2144 \pm 256\ \mu m^2$) compartment were not significantly different. Consequently, there was no evidence of fibre atrophy in the anterior compartment of the TA muscle 6 months after self-reinnervation.

Specific tension

The specific tension, i.e. the maximum tension developed per unit cross-sectional area, of the S unit ($15{\cdot}3\ N/cm^2$) was comparable to the mean specific tension reported for S units in control TA ($17{\cdot}2\ N/cm^2$) (present study; Bodine *et al.* 1987; Pierotti *et al.* 1991). The mean specific tension of the nine fast units ($25{\cdot}0\ N/cm^2$) was comparable to that reported for control fast units ($24{\cdot}0\ N/cm^2$) (present study; Bodine *et al.* 1987; Pierotti *et al.* 1991), although the range in specific tension was larger in motor units from self-reinnervated muscles compared to motor units from

TABLE 1. Myosin heavy chain (MHC) profiles of motor units in control and self-reinnervated cat tibialis anterior

Motor unit	Type	Innervation ratio	Number of fibres typed	MHC antibody profile: Slow (+F8/−13/+35) (%)	Fast-1 (−F8/+13/+35) (%)	Fast-2 (−F8/+13/−35) (%)	Slow-fast-1 (+F8/+13/+35) (%)
SR1	S	144	90	90	10	0	0
SR2	FR	536	—	—	—	—	—
SR3	FR	482	286	0	84	16	0
SR4	FR	565	400	1	98	1	0
SR5	FR	427	265	16	84	0	0
SR6	FR	480	298	0	35	65	0
SR7	FR	673	562	16	61	23	0
SR8	FR	619	218	18	76	6	0
SR9	FI	917	694	1	3	96	0
SR10	FI	1126	770	5	30	62	3
C1	S	106	47	100	0	0	0
C2	FR	180	150	0	100	0	0
C3	FR	156	110	0	0	100	0
C4	FF	395	330	0	0	100	0

A sample of glycogen-depleted fibres from each motor unit was typed according to the profile obtained after staining with monoclonal antibodies BA-F8 (F8), BF-13 (13) and BF-35 (35) which react with specific myosin heavy chains. Fibres were classified as slow, fast (fast-1 and fast-2) or slow-fast-1 MHC fibres; +, positive label; −, no label (see Methods). The numbers assigned to the motor units correspond to those presented in Bodine-Fowler *et al.* (1993).

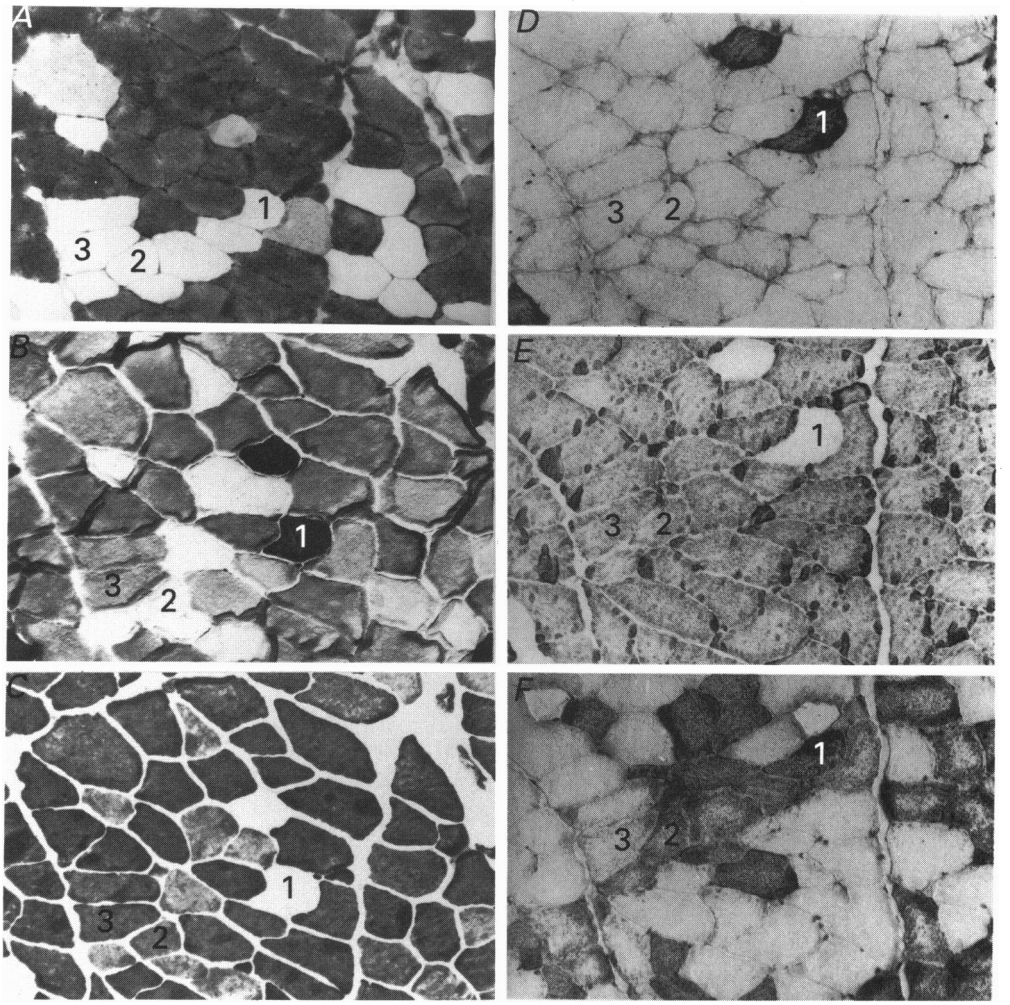

Fig. 5. Fibre-type composition of a portion of a single, glycogen-depleted motor unit (SR7) in a self-reinnervated tibialis anterior muscle. Glycogen-depleted fibres stained negatively for the periodic acid–Schiff (PAS) stain (*A*). Serial sections were stained for myosin ATPase at an acid pre-incubation (pH = 4·35) (*B*), alkaline pre-incubation (pH = 10·4) (*C*) and reacted with monoclonal antibodies BA-F8 (*D*), BF-13 (*E*) and BF-35 (*F*) which react with specific myosin heavy chains (see Methods and Table 1). The fibres corresponding to the depleted fibres in *A* have been marked with numbers: fibre 1, slow MHC; fibre 2, fast-1 MHC; and fibre 3, fast-2 MHC.

control muscles (12·4–44·4 and 17·9–34·9 N/cm^2, respectively). A significant correlation was found between specific tension and maximum tension for all motor units studied from self-reinnervated TA muscles ($r = 0{\cdot}91$, $n = 10$).

Innervation ratio

Calculation of the innervation ratio included all of the fibres belonging to the motor unit (see Methods). A significant correlation was found between maximum tetanic tension and innervation ratio ($r = 0{\cdot}92$, $n = 10$) (Fig. 4) for the motor units

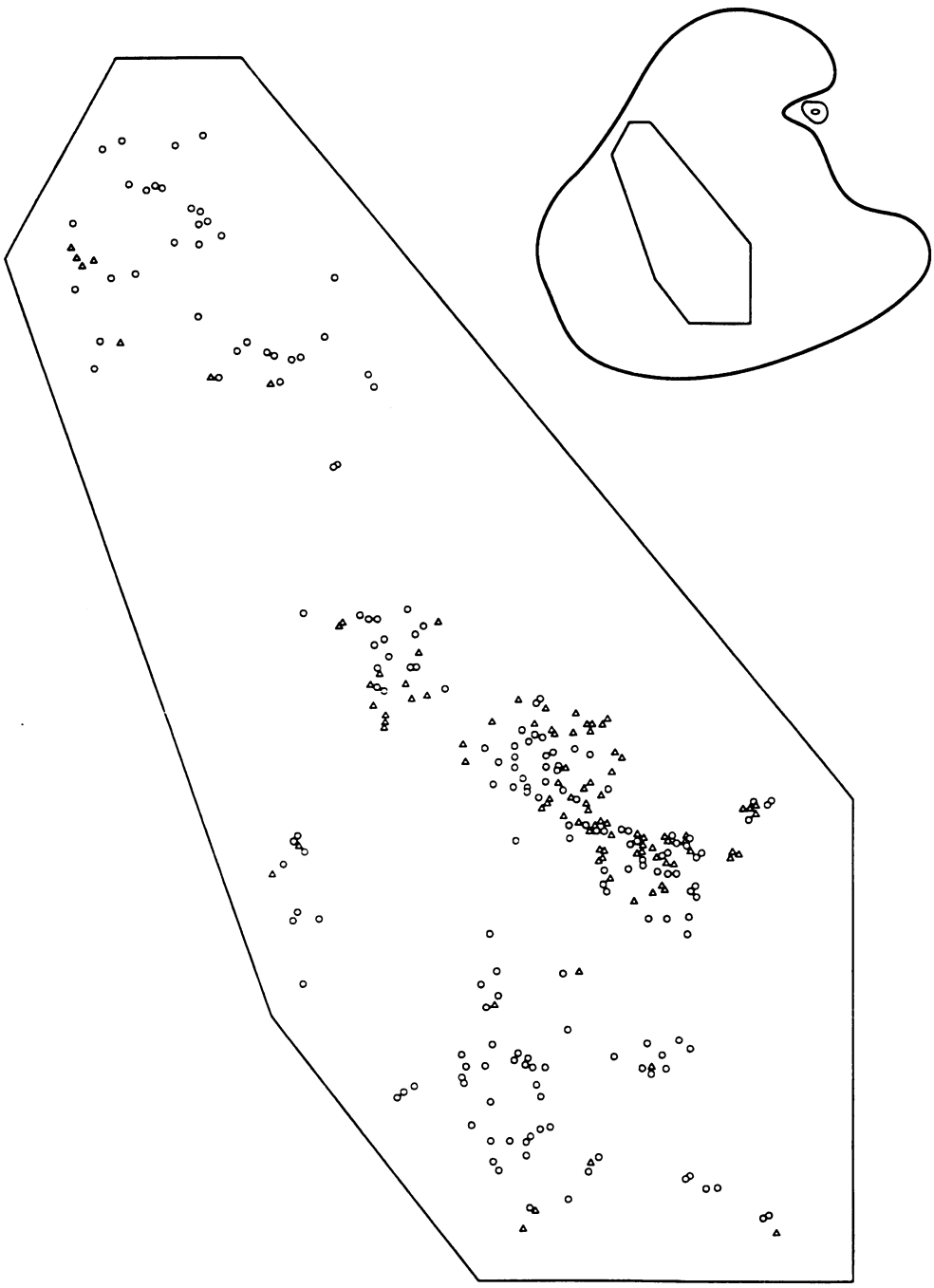

Fig. 6. Schematic representation of the location of the motor unit territory within the whole muscle cross-section, and the distribution of depleted motor unit fibres of different myosin heavy chain (MHC) profiles within the territory of motor unit SR6. The motor unit territory was estimated by connecting the outermost fibres by straight lines to form the smallest convex area containing all of the motor unit fibres identified in the muscle cross-section stained with PAS (see Methods). The fibre-type composition for this motor

in self-reinnervated muscles. The relationships among innervation ratio, maximum tetanic tension capability and motor unit type for motor units in self-reinnervated muscles were similar to those found in control hindlimb cat TA muscle (Bodine *et al.* 1987). For example, fast motor units produced greater maximum tetanic tensions than slow motor units and this was closely related to the innervation ratio. The innervation ratio of the slow unit was comparable to that reported for control slow units whereas the innervation ratio of the fast units exceeded that reported for control fast units (Table 1; Bodine *et al.* 1987).

A stepwise regression analysis was used to determine the relative importance of each of the morphological properties (i.e. innervation ratio, fibre cross-sectional area and specific tension) in determining the maximum tetanic tension of a motor unit. The innervation ratio accounted for 84% of the variability in maximum tetanic tension. Thus, innervation ratio was the primary determinant of maximum force output in motor units of self-reinnervated TA muscles and the increase in innervation ratio observed in the fast motor units from self-reinnervated muscles was the predominant factor in determining the increased maximum tetanic tension observed in those units.

Fibre type composition of individual motor units

To determine the extent to which motor units are composed of a homogeneous population of fibres after reinnervation, the fibres belonging to a motor unit were examined for their MHC composition. The total number of fibres analysed for MHC composition and the percentage of each fibre type in motor units from control ($n = 4$) and self-reinnervated ($n = 9$) TA muscles are summarized in Table 1. The control motor units were homogeneous with regard to fibre type composition. In contrast, at least two fibre types were present in the sample of fibres analysed from each of the nine motor units from self-reinnervated TA muscles (Fig. 5). However, there was a marked bias towards one of the three defined MHC profiles in each motor unit.

The S unit from self-reinnervated muscle contained predominantly slow MHC fibres (90%), with the fast fibres (10%) being of the fast-1 MHC profile. One fast unit (SR5) contained fibres of slow MHC (16%) and fast-1 MHC (84%). Two subtypes of fast MHC fibres were observed in the other seven fast motor units. Five of six FR units had a range of 61–99% fibres of fast-1 MHC. In the two FI units, 62 and 96% of the fibres were of the fast-2 MHC type.

The location of the motor unit territory within the whole muscle cross-section and the distribution pattern of different fibre types belonging to two single motor units in self-reinnervated muscles (Table 1; SR6 and SR7) are shown schematically in Figs 6 and 7. The distribution of motor unit fibres by MHC profile within the territory of the unit was similar to that of non-motor unit fibres across the muscle compartment, i.e. the highest proportions of fast-2 MHC fibres were observed in the

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unit was obtained from a sample of motor unit fibres (62% of the innervation ratio, see Table 1), some of which were on the boundary. Note that the boundaries of the territory have been expanded for illustrative purposes. Fast-1 MHC fibres, △; fast-2 MHC fibres, ○ (see Table 1).

Fig. 7. Schematic representation of the location of the motor unit territory within the whole muscle cross-section and the distribution of depleted motor unit fibres of different myosin heavy chain (MHC) profiles within the territory of unit SR7. The motor unit territory was estimated by connecting the outermost fibres by straight lines to form the smallest convex area containing all of the motor unit fibres identified in the muscle cross-section stained with PAS (see Methods). The fibre-type composition for this unit was

superficial region and the highest proportions of slow MHC fibres in the deep region of the territory (Fig. 7). Fast-1 MHC fibres appeared to be evenly distributed throughout the motor unit territory. This pattern of fibre type distribution is similar to that observed in control TA muscles (present study; Iliya & Dum, 1984) (see below).

A random innervation would result in motor units whose fibre type composition mirrored that of the territory of the unit. Based on a χ^2 test, the observed frequencies of fibres of distinct MHC profile within each of the units analysed from nine self-reinnervated muscles differed significantly from the expected frequencies based on the proportions of each fibre type within the motor unit territory (Fig. 8). Thus, the biased fibre type composition of motor units in self-reinnervated muscles did not reflect a process in which there was a random selection of fibre types innervated by a motoneurone.

Fibre type distribution of self-reinnervated muscles

To investigate the hypothesis that fibre type conversion may have produced the fibre type compositions observed within the motor units of self-reinnervated muscles, the fibre type distribution within the muscle was calculated from samples of 400–900 fibres taken from each compartment of the muscle. The mean percentage of slow (10 ± 3) and fast (90 ± 3) fibres in the anterior compartment of the self-reinnervated TA muscles was similar to that of control TA muscles (slow, 9 ± 3; fast, 91 ± 3) (Iliya & Dum, 1984; West *et al.* 1986). Further, based on visual inspection, the uneven distribution of fibre types within the cross-section of self-reinnervated TA muscles was similar to that observed in control muscles. For example, the proportions of slow fibres decreased and fast fibres increased from the deep to the superficial regions of the muscle compartment in both groups. No obvious fibre type grouping, i.e. an unusually large number of fibres of the same type appearing adjacent to one another, was observed based on the visual inspection of sections stained for myofibrillar ATPase or SDH or reacted with the three MHC monoclonal antibodies. Furthermore, the percentage of slow fibres found on the boundaries of fascicles within the anterior compartment was similar in self-reinnervated and control TA muscles (Bodine-Fowler, Unguez, Roy, Armstrong & Edgerton, 1993). These results suggest that the relative proportions of different fibre types and their distribution across the anterior compartment of the TA muscle did not change upon denervation and subsequent self-reinnervation.

SDH activity of motor units fibres from self-reinnervated muscles

The SDH activity of a sample of motor unit and non-motor unit fibres from self-reinnervated muscles was measured to determine the level of influence that a motoneurone exerts on the oxidative properties of the fibres that it innervates after 6 months of self-reinnervation compared to control. The relationship between SDH

obtained from a sample of unit fibres (83 % of the innervation ratio, see Table 1). This unit contained slow MHC (□), fast-1 MHC (△), and fast-2 MHC (○) fibres (see Table 1). The superficial (S) and deep (D) regions of the muscle are indicated by the arrows.

activity and MHC profile across fibres of different MHC profiles within a motor unit after self-reinnervation was consistent with that found between fibres of different MHC profiles in control muscles. In control TA muscles, the mean SDH activity was similar in slow (0·020 OD/min) and fast-1 (0·017 OD/min) MHC fibres and lower in

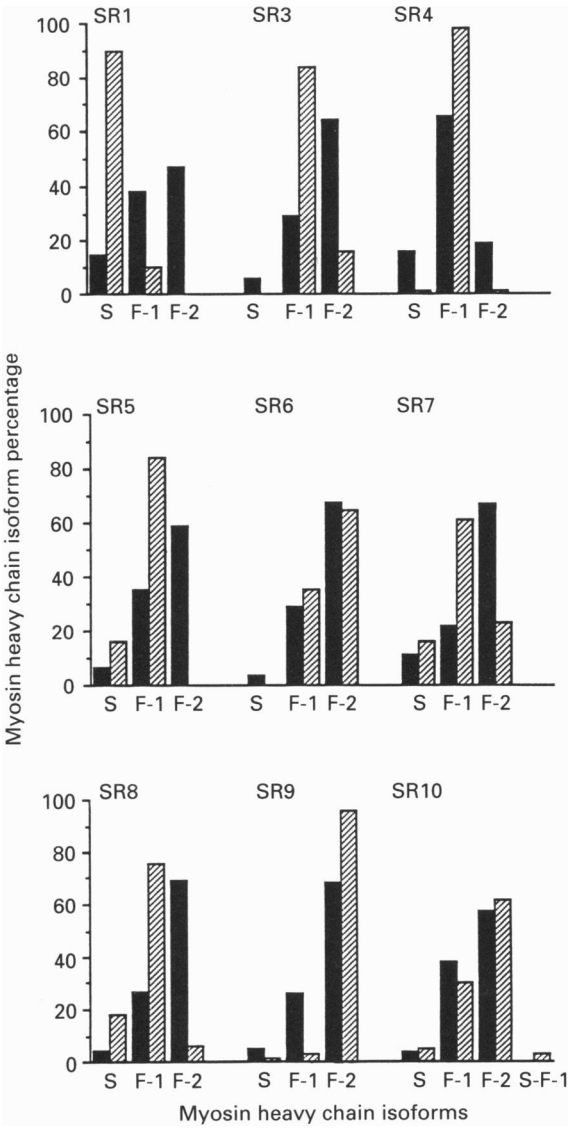

Fig. 8. Fibre type composition of a sample of fibres in 9 glycogen-depleted motor units (hatched bars) and of a sample of fibres within the territory of the motor unit (solid bars) in self-reinnervated (SR) tibialis anterior muscles. Based on a χ^2 test, the observed frequencies of fibres of a distinct myosin heavy chain (MHC) type within each of the 9 motor units analysed were found to differ significantly from the expected frequencies based on the proportions of each fibre type within the motor unit territory. Motor unit numbers correspond to those in Table 1. Abbreviations: S, slow MHC; F-1, fast-1 MHC; F-2, fast-2 MHC; S-F-1, slow-fast-1 MHC fibres.

fast-2 (0·007 OD/min) MHC fibres. Similarly, slow (0·021 OD/min) and fast-1 (0·019 OD/min) MHC fibres had a higher mean SDH activity than fast-2 MHC fibres (0·011 OD/min) in self-reinnervated TA muscles. Across units from self-reinnervated muscles, the coefficients of variation ranged from 10 to 20% for fibres of slow MHC,

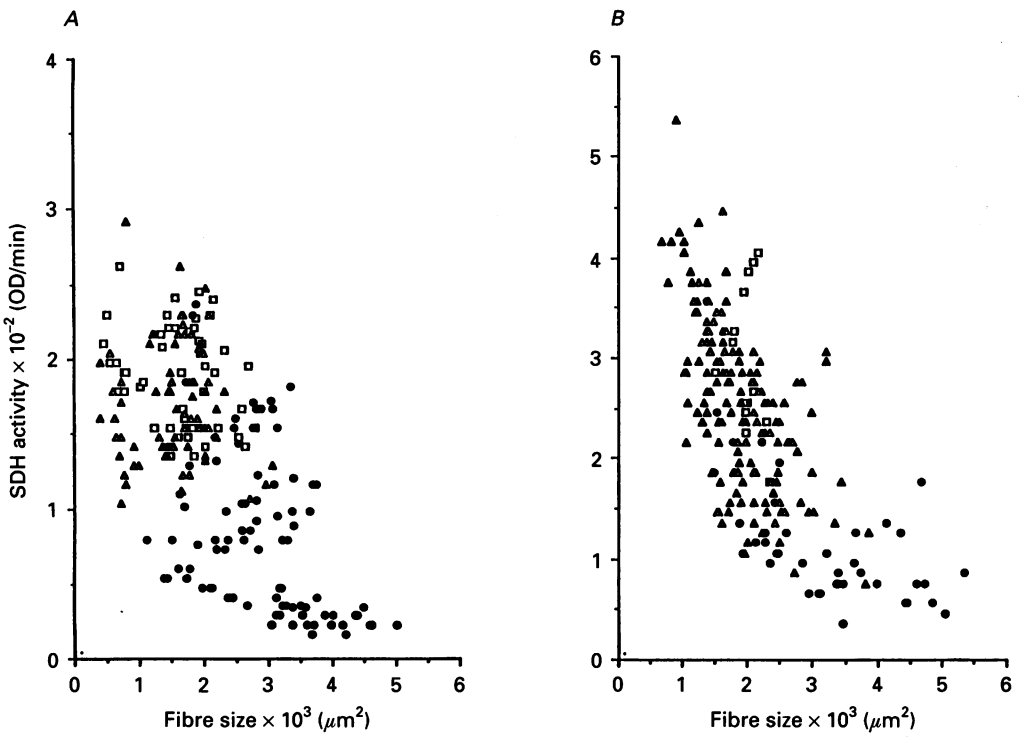

Fig. 9. Relationship between fibre size and succinate dehydrogenase (SDH) activity of a sample of fibres of different myosin heavy chain profiles from 4 control tibialis anterior muscles (*A*) and from a glycogen-depleted motor unit (Table 1: SR7, containing a mixture of fibres of different myosin heavy chain profiles) from a self-reinnervated tibialis anterior muscle (*B*). Slow MHC (□), fast-1 MHC (△), and fast-2 MHC (●) fibres.

from 13 to 33% for fibres of fast-1 MHC, and from 29 to 65% for fibres of fast-2 MHC. The ranges in the coefficients of variation for SDH activity for each fibre type across control muscles were: slow MHC, 6–16%, fast-1 MHC, 17–25%, and fast-2 MHC, 29–43%.

The similarity in the interrelationships among the SDH activity, the MHC profile and the cross-sectional area of fibres across motor units in control muscles and within a unit in a self-reinnervated muscle containing a mixture of fibre types is illustrated in Fig. 9. In this unit, fibres of fast-2 MHC had a significantly lower mean SDH activity than fibres of fast-1 or slow MHC. There was no significant difference between the mean SDH activity of fibres of fast-1 and slow MHC. Together, these data are consistent with the interpretation that the SDH activity of a fibre and its relationship with fibre size and MHC profile did not change after reinnervation.

DISCUSSION

The results presented above indicate that the normal relationship between the maximum tetanic tension of a motor unit and the motor unit type was re-established within 6 months of self-reinnervation in the adult cat TA. Based on direct counts of the muscle fibres per motor unit, the maximum tetanic tension of a motor unit was determined primarily by its innervation ratio, as has been reported for control motor units in the adult cat TA muscle (present study; Bodine *et al.* 1987) and adult rat soleus muscle (Chamberlain & Lewis, 1989). Furthermore, the finding that motor units producing large forces do so mainly because they reinnervated more muscle fibres has been reported in the adult rat TA following reinnervation (Totosy de Zepetnek, Zung, Erdebil & Gordon, 1992). These results suggest a limitation in the number of muscle fibres that a slow motoneurone can innervate. On the other hand, assuming some selectivity in the reinnervation process, the difference in the number of fibres between slow and fast motor units could be due to a limited number of slow MHC fibres in the denervated muscle compartment available for reinnervation by a slow motoneurone. Regardless of the regulatory mechanism(s), it is clear that the eventual size of motor units after self-reinnervation in the adult was determined by the unit type. Furthermore, since the order of recruitment is tightly coupled to the size of the motor unit (Henneman & Olson, 1965), and this relationship persists after self-reinnervation (Clark, Dacko & Cope, 1992), it would appear that a functionally significant level of synaptic input to motoneurones, i.e. sufficient to maintain order of recruitment, remains undisturbed after a 6-month reinnervation period. Thus, in spite of the disruptions of the afferent inputs from the muscle to the homonymous motoneurones, normal recruitment order, and anatomical and biochemical relationships, although imperfect, are re-established among motor unit types following self-reinnervation.

The fast motor units in self-reinnervated TA muscles generally had higher maximum tetanic tensions than fast motor units in control muscles. Two possibilities, not mutually exclusive, can account for this difference in size among fast units. First, an increase in the number of fibres per unit may have been due to fewer fast motoneurones being available to establish functional contact with the same number of muscle fibres after than before the lesion, resulting in less competition between regenerating motor axons (Foehring *et al.* 1986). Consequently, those motoneurones that successfully reinnervated muscle fibres may have formed and maintained a larger number of synapses than prior to the injury. In effect, this would be analogous to the larger than normal motor units, as has been reported in partial-denervation studies in adult muscles (Brown & Ironton, 1978; Luff, Hatcher & Torkko, 1988). A second possibility is that synapse elimination may not have been complete after 6 months of self-reinnervation of the cat TA muscle. Polyneuronal innervation of endplates by regenerating axons has been observed in soleus (Benoit & Changeux, 1978), extensor digitorum longus (Gorio, Carmignoto, Finesso, Polato & Nunzi, 1983) and lumbrical (Taxt, 1983) muscles in adult rats, and in the sternomastoid in adult mice (Rich & Lichtman, 1989) following nerve crush. As in newborn animals (Balice-Gordon & Thompson, 1988), the period of polyneuronal innervation is transient, and all but one axon per muscle fibre seems to be lost by the elimination of multiple

synapses. In the adult rat, multiple innervation is eliminated within 60 days (Benoit & Changeux, 1978; Gorio *et al.* 1983; Taxt, 1983) to 125 days (Tax, 1983) after nerve injury, whereas in development multiple innervation is eliminated within 20 days after birth (Balice-Gordon & Thompson, 1988). In the cat, single innervation of muscle fibres is completed within 45 days after birth (Westerman, Lewis, Bagust, Edjtehadi & Pallot, 1973). Although the extent of polyneuronal innervation and the time course of synapse elimination following reinnervation in the adult cat is unknown, it seems likely that most, if not all of the muscle fibres in the present study would be singly innervated 6 months after denervation and subsequent reinnervation. Thus, it seems unlikely that the tendency for the fast motor units in self-reinnervated muscles to be larger than control was because of the presence of multiple axons per muscle fibre.

In addition to determining the number of muscle fibres with which to maintain functional synapses, a motoneurone must also decide which fibres to reinnervate. When an axon is transected, the basal lamina persists in the distal portion of a transected nerve bundle, despite the degeneration of the nerve fibre and its associated myelin (Thomas, 1964). Regenerating axons grow from the proximal stump and enter the cut ends of vacant basal lamina tubes at the lesion site. In the present study, it is possible that the distal stump provided sufficient cues to bias the axons to return to the myelin–Schwann cell sheaths that would direct them to reinnervate their original territory. The normal fibre type distribution observed in the self-reinnervated muscles in the present study and that found in the adult cat medial gastrocnemius muscle after 10 and 33 months of self-reinnervation (Nemeth, Cope, Kushner & Nemeth, 1993) could have occurred as a result of such a reinnervation process. However, a normal fibre-type gradient throughout the muscle cross-section has also been reported in TA muscles of adult mice after the peroneal nerve was transected and rotated in an attempt to prevent axons from regrowing into their original myelin sheaths (Parry & Wilkinson, 1989). These results are consistent with a matching between axons and muscle fibres of a compatible type rather than an alteration of the phenotype of muscle fibres after the regenerating axons made functional contact.

The heterogeneous fibre-type composition of the motor units in self-reinnervated muscles with respect to their myosin and metabolic properties indicate that each regenerating motoneurone reinnervated some muscle fibres that it did not innervate prior to nerve transection. Furthermore, the spatial distribution of fibres from all of the fast motor units in the self-innervated muscles (Bodine-Fowler *et al.* 1993) was significantly different from control (Bodine-Fowler, Garfinkel, Roy & Edgerton, 1990), suggesting that the original axon–muscle fibre connections were often not re-established. For example, there was a larger than normal number of adjacent muscle fibres in all fast motor units from self-reinnervated muscles (Bodine-Fowler *et al.* 1993). Thus, the higher incidence of adjacent motor unit fibres in the absence of obvious type grouping indicates that a motoneurone made connections in a number of cases with fibres of a similar type but not with the same fibres that it originally innervated.

If the initial synapse formation during the 6 month self-reinnervation period in our adult muscles had been a random process and neurally induced interconversion of

fibre types had occurred, one might have expected to see an increase in the grouping of fibres of the same type, similar to that reported in other studies of self- and cross-reinnervated muscles (Karpati & Engel, 1968; Kugelberg *et al.* 1970; Gauthier, Burke, Lowey & Hobbs, 1983; Foehring *et al.* 1986). This fibre-type arrangement, however, was not observed in the present study. The combination of an increased incidence of adjacencies between fibres of the same motor unit but not of fibre-type grouping throughout the self-reinnervated muscles is consistent with a highly selective axon–fibre-type matching at the expense of accepting an abnormal spatial distribution of some fibres within a motor unit. Further evidence that the neural induction of fibre-type conversion did not play a primary role in the formation of motor units after self-reinnervation was the presence of more than one phenotype among muscle fibres under the influence of the same motoneurone. Thus, these data question the interpretation in previous reports emphasizing the complete neural determination of fibre types following reinnervation (Karpati & Engel, 1968; Kugelberg *et al.* 1970; Nemeth & Turk, 1984).

Evidence that the motoneurone does not have complete control of the type-related properties of all muscle fibres it innervates is consistent with several other observations. For example, cross-reinnervation experiments in the cat where a nerve to a fast muscle is selectively routed towards a slow muscle, such as the soleus, have shown mismatches between the electrical properties of the motoneurones and contractile properties of the muscle units (Foehring & Munson, 1990). Furthermore, a much higher than expected proportion of S motor units ($\sim 74\%$) is maintained in the cross-reinnervated soleus in spite of the soleus being reinnervated by a nerve containing more than 75% fast motoneurones (Chan *et al.* 1982; Foehring & Munson, 1990) and those motor units which developed 'fast' properties remained fatigue resistant (Chan *et al.* 1982). In concurrence with no change in fatigability of the muscle, the oxidative potential of the reinnervated soleus fibres, as shown by qualitative SDH histochemistry, was also maintained (Chan *et al.* 1982). Thus, even after long postoperative periods, a large proportion of fast motoneurones that reinnervate originally slow fibres fail to convert them to fast. The implication of these results is that the neural control of muscle properties is shared with other regulatory systems such as the humoral, etc. (Edgerton, Martin, Bodine & Roy, 1985). Furthermore, the persistence of the normal interrelationships among the relative cross-sectional area, MHC profile and relative oxidative potential of the muscle fibres in all motor units with mixed fibre types after 6 months of self-reinnervation (see Fig. 9) is consistent with the concept of a neurally independent source of modulation by factors intrinsic to the individual fibres.

The biased but not pure fibre-type composition in the motor units of self-reinnervated adult muscles in this study is similar to that observed in motor units of the neonatal rat after self-reinnervation (Soileau, Silberstein, Blau & Thompson, 1987). As in development, the present results suggest that the motoneurones can recognize fibre types during the reinnervation process in the adult cat. Although the present study cannot determine the relative importance of selective reinnervation of fibres of different phenotypes (Soileau *et al.* 1987) *versus* a systematic exchange of terminals between the different fibres (Gates & Ridge, 1992), these findings suggest that mechanisms leading to the innervation of specific muscle fibres by their own

motoneurones in the neonate may be present under certain conditions in the adult. Thus, it is proposed that the biased fibre-type distributions observed in motor units after self-reinnervation were primarily due to a preferred affinity of a motoneurone to a specific muscle fibre type rather than to a non-specific reinnervation process followed by a conversion of the fibre type to match the motoneurone.

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