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SLOW-TO-FAST TRANSFORMATION OF DENERVATED SOLEUS MUSCLES BY CHRONIC HIGH-FREQUENCY STIMULATION IN THE RAT

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SUMMARY

1. Adult soleus muscles were denervated and stimulated directly for 2–130 days with 'fast' (short pulse trains at 100 Hz) or 'slow' (continuously at 10 Hz, or long pulse trains at 15 Hz) stimulus patterns.

2. At the end of the period of stimulation isometric twitches and tetani and isotonic shortening velocities were measured. Frozen cross-sections were later examined with antibodies against myosin heavy chains specific for adult fast, adult slow and fetal myosin.

3. Isometric twitch duration (twitch time-to-peak and half-relaxation time) decreased during intermittent 100 Hz stimulation to values that were almost as fast as in the normal extensor digitorum longus (EDL)(95 and 94% transformation). The major part of the decrease occurred between 2 and 21 days after the onset of stimulation, and was accompanied by post-tetanic potentiation of the twitch, 'sag' in tension during an unfused tetanus, lower twitch/tetanus ratio and marked shifts to the right (higher frequencies) of the tension–frequency curve of the muscle. In contrast, during 10 or 15 Hz stimulation the isometric twitch duration remained slow, the twitch continued to show post-tetanic depression and absence of 'sag', while the twitch/tetanus ratio increased.

4. Denervation *per se* led to a slight increase and, then, after about a month, to a moderate and gradual decrease in twitch duration. The twitch/tetanus ratio increased markedly and post-tetanic depression became less pronounced or disappeared. Muscle weight and particularly tetanic tension were markedly reduced and these reductions were to a large extent counteracted by electrical stimulation.

5. Implantation of sham electrodes had no effect on twitch duration of denervated or innervated control muscles, but reduced tetanic tension in the innervated control muscles.

6. Maximum isotonic shortening velocity of the whole muscle (mm/s) increased during intermittent 100 Hz stimulation to a value as fast as in the normal EDL (110% transformation). Since the muscle fibres also increased in length (35%) maximum intrinsic shortening velocity (fibre lengths/s) was only incompletely transformed (55%). The increase in V_{max} occurred between 7 and 14 days after the onset of stimulation.

7. All the fibres stimulated intermittently at 100 Hz were strongly labelled with anti-fast myosin and more than 90% were in addition weakly labelled by anti-slow myosin. Weak and variable labelling with anti-fast myosin was first detected 7 days after the onset of stimulation. In contrast, essentially all the fibres stimulated at 10 or 15 Hz showed no binding of anti-fast but strong binding of anti-slow myosin. Denervation *per se* increased the fraction of fibres labelled by anti-fast myosin but even after 2 months more than half the fibres (57%) reacted only with anti-slow myosin.

8. From the beginning to the end of intermittent stimulation at 100 Hz the bulk of the muscle consisted of large fibres unreactive with anti-fetal myosin which stains regenerating fibres. This indicated that the slow-to-fast transformation occurred in pre-existing adult fibres and was not the result of muscle injury followed by formation of new fibres of a fast type.

INTRODUCTION

It is well known that slow-twitch muscles contract faster than normal after reinnervation by fast motoneurones, and, conversely, that fast-twitch muscles contract slower than normal after reinnervation by slow motoneurones (Buller, Eccles & Eccles, 1960; Close, 1969). Fast and slow motoneurones generate different patterns of impulses (Fischbach & Robbins, 1969: Hennig & Lømo, 1985) and this may account for the effects of cross-reinnervation on contractile speed. Evidence supporting this is that fast muscles become slower when their nerves are stimulated chronically by large numbers of stimuli at 10 Hz (slow pattern, Salmons & Vrbová, 1969; Pette, Smith, Staudte & Vrbová, 1973; Salmons & Sréter, 1976; see Pette & Vrbová, 1985 for review) and that slow muscles become faster when stimulated with brief trains of stimuli at 100 Hz (fast pattern, Smith, 1978; Hennig & Lømo, 1987). In these experiments, however, the muscles are influenced not only by the stimulation but also by activity and chemical signals supplied by the motoneurones. More direct evidence for an effect of activity *per se* may therefore be obtained by first removing all neural influences by denervation and then stimulating the denervated muscles directly with known patterns of stimuli. Using this approach it has been shown that fast-pattern stimulation of denervated rat soleus muscles results in isometric twitches that are almost as fast as those seen in normal extensor digitorum longus (EDL) muscles, while slow-pattern stimulation maintains the slow twitches seen in normal soleus muscles (Lømo, Westgaard & Dahl, 1974; Lømo, Westgaard & Engebretsen, 1980). Since reinnervation by fast EDL and slow soleus motoneurones have similar effects (Close, 1969; Gutmann & Carlson, 1975), it seems likely that patterns of evoked muscle activity play a major role in regulating the contractile speed of skeletal muscle.

However, the effects of stimulation on denervated slow-twitch muscles have been given other interpretations. Al-Amood, Finol & Lewis (1986) and Al-Amood & Lewis (1987) report less pronounced slow-to-fast transformation than Lømo *et al.* (1974, 1980), little or no dependence on frequencies of stimulation ranging from 10 to 100 Hz and some slow-to-fast transformation in denervated unstimulated muscles. Hence they conclude that slow-to-fast transformation is a response to denervation

per se rather than a specific effect of intermittent stimulation. According to Jolesz & Sréter (1981) the transformation may be due to fibre damage followed by regeneration of new fibres of a fast type.

In this work we study further the effects of stimulation on denervated rat soleus muscles. We confirm previous reports (Lømo *et al.* 1974, 1980; Hennig & Lømo, 1987) that intermittent 100 Hz stimulation results in a marked increase in twitch speed and conclude that altered activity rather than denervation *per se* is responsible for the slow-to-fast transformation. Using antibodies against fetal myosin, which stain regenerating fibres (Sartore, Gorza & Schiaffino, 1982), we report evidence that the transformation occurs in pre-existing adult slow fibres and is not the result of fibre regeneration. Finally, we examine isotonic shortening velocities and binding of antibodies against adult fast and slow myosin and report that the effects of intermittent 100 Hz stimulation on these parameters are slower in onset and less pronounced than the effects on several parameters of the isometric twitch.

Some of the present results have been reported at meetings (Gundersen, Westgaard, Schiaffino & Lømo, 1983; Lømo, Gundersen, Hennig & Westgaard, 1985*a*; Lømo, Westgaard, Hennig & Gundersen, 1985*b*; Lømo, 1986).

METHODS

Chronic procedures. In adult male Wistar rats, weighing 250–350 g when the experiment started, Teflon-coated, multistranded steel wires (AS 632, Cooner Sales, Chatsworth, CA, U.S.A.) were implanted into the right and left leg under sodium pentobarbitone anaesthesia (Mebumal, 60 mg/kg I.P.). In each leg the distal ends of two wires with the insulation removed were placed across the soleus, one end proximally and anteriorly and one end distally and posteriorly. The two wires from the right leg were run under the skin to the head and into a flexible silicon tube (outer diameter 6 mm) which was fixed to the skull with screws and dental cement. The wires and the protecting tube were then connected to a rotating contact approximately 0·5 m above the rat, which moved freely in a wide bucket. On the left side the two wires ran from the muscle to the thigh where they were cut. On the day of implantation, or 7 days later, the sciatic nerve was cut in the thigh on the right side, and in some animals also on the left side. The proximal stump of the sciatic nerve was pulled out through the musculature and sutured under the skin to prevent reinnervation. Stimulation started 1 day after denervation and lasted for 2–130 days. The stimulus patterns were: (1) 60 pulses at 100 Hz every 60 s (86400 pulses per 24 h), (2) 150 pulses at 15 Hz every 15 s (864000 pulses per 24 h, and (3) continuous 10 Hz stimulation (864000 pulses per 24 h). The 15 and 10 Hz patterns gave similar results and these groups were combined. Each pulse was bipolar with a duration of 0·2 ms and an intensity of 5–10 mA in each direction. After denervation and during stimulation the rats did not appear to suffer any pain and appeared to eat and sleep normally.

Acute experiments. At the end of the stimulation period the soleus was dissected free from most of the surrounding tissue under sodium pentobarbitone anaesthesia (Mebumal, 60 mg/kg I.P.), while care was taken not to damage the main blood supply or the remains of the soleus nerve trunk. The nerve trunk was stimulated electrically just outside the soleus and failure to observe muscle contraction with the microscope was taken as evidence that reinnervation had not occurred. After removing the skin the leg was inserted into a chamber and clamped at the knee and ankle for isometric and isotonic recordings of muscle contraction. The chamber was then sealed by the skin around the thigh and filled with Ringer solution, which was kept oxygenated at 35 °C. A thread was tied to the distal end of the soleus and connected to transducers for isometric and isotonic recording. A pair of platinum plates, insulated by silicone rubber on the outside, were moved close to the soleus for direct stimulation by 0·5–1 ms long and up to 30 mA strong pulses. Under isometric conditions the intensity of stimulus was gradually increased until a plateau of maximum twitch tension was obtained and this intensity was then used for the rest of the experiment. A plateau tension was easily obtained in the denervated and chronically stimulated muscles. In the

denervated non-stimulated muscles, on the other hand, a clear plateau was sometimes not reached at 30 mA. It was then difficult to decide whether this was due to continued recruitment of denervated soleus fibres, double firing in individual fibres, or co-contractions in neighbouring muscles (see also Spector, 1985). In such cases we subsequently stimulated at 30 mA, accepting some uncertainties in the tension measurements obtained.

Isotonic conditions were obtained by letting the stimulated muscle move a vertical lever against different loads provided by air pressure from a large pressure tank against a movable piston (Gundersen, 1985). Shortening velocity was measured by a displacement transducer in series with the piston and muscle tension was measured by a strain gauge glued onto the lever while the muscle was stimulated at frequencies giving maximal tetanic tension (100–300 Hz, see Fig. 4). All measurements were done at optimum length for twitch tension. At the end of the acute experiments with the muscle at optimum length the distance between the proximal and distal myotendinous junctions for a narrow bundle of surface fibres was measured and taken as the average muscle fibre length since all the muscle fibres in the soleus have approximately the same length (Close, 1964). Force–velocity curves like those in Fig. 5*A* were calculated by fitting the measurements to the Hill (1938) equation: $(P+a)\,V = b(P_0-P)$ as described by Katz (1939). The velocity at zero load (V_{max}), given in Fig. 5*B*, was calculated according to Edman, Mulieri & Scubon-Mulieri (1976). This calculation represents a fitting of all the three constants (a, b and P_0) in the Hill (1938) equation and is thus independent of the measured P_0. The maximal shortening velocities obtained in this way were similar to those obtained by the procedure described by Katz (1939), but resulted in less variability among muscles in the same group. The isotonic shortening velocity reflects the sliding velocity between myosin and actin and can be compared in different muscles provided the sarcomere length is the same and that differences in fibre length are corrected for (Close, 1964). Whole-muscle shortening velocities were therefore divided by fibre length and expressed as intrinsic shortening velocity (fibre lengths/s). Finally, the muscles were weighed and frozen in liquid nitrogen for subsequent immunohistochemistry.

Immunohistochemistry. Two monoclonal antibodies, designated BA-D5 and BF-13, reacting specifically with type I ('slow') and type II ('fast') myosin heavy chains respectively, were used in these studies. The preparation and characterization of these antibodies will be described in detail elsewhere. In indirect immunofluorescence BA-D5 stained type I fibres selectively whereas BF-13 stained both type IIA and type IIB fibres, as identified by histochemical staining for myosin ATPase. In addition, a polyclonal antibody raised against bovine fetal myosin (anti-BFM) that reacts with the embryonic and neonatal myosin heavy chains expressed in developing, but not in adult, rat skeletal muscle (Whalen, Sell, Butler-Browne, Schwartz, Bouveret & Pinset-Hardstrom, 1981), was used to identify regenerating fibres. The specificity of this antibody has already been described (Sartore *et al.* 1982).

Transverse sections, about 10 μm thick, of control and experimental muscles were cut in a cryostat, mounted on slides and exposed to the appropriate dilution of unlabelled antibodies. Monoclonal antibodies were used as hybridoma culture supernatants, while anti-BFM consisted of specific immunoglobulins (IgGs) separated by affinity chromatography. Incubation was carried out in a humidified chamber at 37 °C for 30 min; after two washings in phosphate-buffered saline, the sections were treated with fluorescein-labelled second antibodies (anti-mouse IgG for the monoclonals, anti-rabbit IgG for the polyclonal) at 37 °C for 30 min. The sections were mounted in Elvanol and examined with a fluorescence microscope equipped with epi-illumination. The specificity of the reactions was tested by use of mouse or rabbit non-immune IgGs in the first step.

RESULTS

Physiological measurements

Twitch speed. Intermittent stimulation at 100 Hz induced several properties typical of fast muscles in the denervated soleus, including a fast isometric twitch (Fig. 1*D*), post-tetanic potentiation of the twitch (Fig. 1*E*) and 'sag' in tension during an unfused tetanus (Fig. 1*F*). In contrast, stimulation at 10–15 Hz maintained a slow twitch (Fig. 1*G*), post-tetanic depression of the twitch (Fig. 1*H*) and lack of sag in an unfused tetanus (Fig. 1*I*), which are features typical of normal soleus

TABLE 1. Properties of soleus and extensor digitorum longus (EDL) muscles

Muscle	Treatment	Time to twitch peak (ms)	Half-relax. time (ms)	Twitch/ tetanus ratio	Post-tetanic potentiation	Max. tetanic tension (N)	Muscle weight (mg)	Rat weight (g)	Fibre length (mm)	V_{max}, whole muscle (mm/s)	V_{max}, intrinsic (fibre lengths/ s)
Soleus	Innervated	40·9 ± 3·7 (20)	56·4 ± 10·2 (20)	0·20 ± 0·03 (19)	0·92 ± 0·04 (17)	1·889 ± 0·26 (20)	179 ± 30 (20)	390 ± 46 (16)	17·7 ± 1·4 (7)	81·7 ± 16·9 (6)	4·85 ± 1·1 (6)
Soleus	Innervated + sham el.	40·7 ± 4·0 (7)	60·3 ± 12·3 (7)	0·24 ± 0·04 (7)	0·90 ± 0·06 (7)	1·34 ± 0·25 (7)	162 ± 25 (7)	390 ± 38 (7)	—	—	—
Soleus	Denervated	37·6 ± 4·1 (11)	43·5 ± 11·7 (11)	0·48 ± 0·1 (10)	0·97 ± 0·03 (9)	0·046 ± 0·04 (11)	33 ± 6·6 (11)	427 ± 46 (3)	—	—	—
Soleus	Den. + stim. 100 Hz	14·3 ± 1·1 (10)	15·6 ± 1·6 (10)	0·16 ± 0·03 (10)	1·26 ± 0·09 (9)	1·017 ± 0·31 (10)	156 ± 31 (10)	397 ± 51 (10)	24·8 ± 2·3 (7)	198·8 ± 19·6 (7)	8·12 ± 1·09 (7)
Soleus	Den. + stim. 10–15 Hz	43·5 ± 5·6 (8)	75·6 ± 15·5 (8)	0·37 ± 0·08 (8)	0·90 ± 0·04 (8)	0·683 ± 0·16 (8)	118 ± 22 (8)	416 ± 77 (5)	—	—	—
EDL	Innervated	12·9 ± 1·0 (12)	12·9 ± 2·2 (12)	0·23 ± 0·05 (8)	1·44 ± 0·12 (8)	2·588 ± 0·31 (8)	167 ± 27 (8)	351 ± 34 (8)	13·6 ± 1·32 (8)	201·2 ± 18·7 (8)	14·95 ± 2·01 (8)

The soleus muscles were examined 9–79 days after implantation of sham electrodes, 42–108 days after denervation, 45–132 days after denervation plus stimulation at 100 Hz (sixty pulses every 60 s), and 45–106 days after stimulation plus stimulation at 10 Hz (continuously) or at 15 Hz (150 pulses every 15 s).

Mean values with ± S.D. and number of muscles in parenthesis underneath.

muscles (Fig. 1*A–C*). These effects are further documented in Table 1. The twitch time-to-peak started to decrease rapidly after 2 days of intermittent 100 Hz stimulation and reached a stable fast value after about a month (Fig. 2*A*). These data include three muscles from Lømo *et al.* (1980) which were stimulated for 35, 64 and 70 days with sixty pulses at 100 Hz every 6000 s rather than every 60 s. These

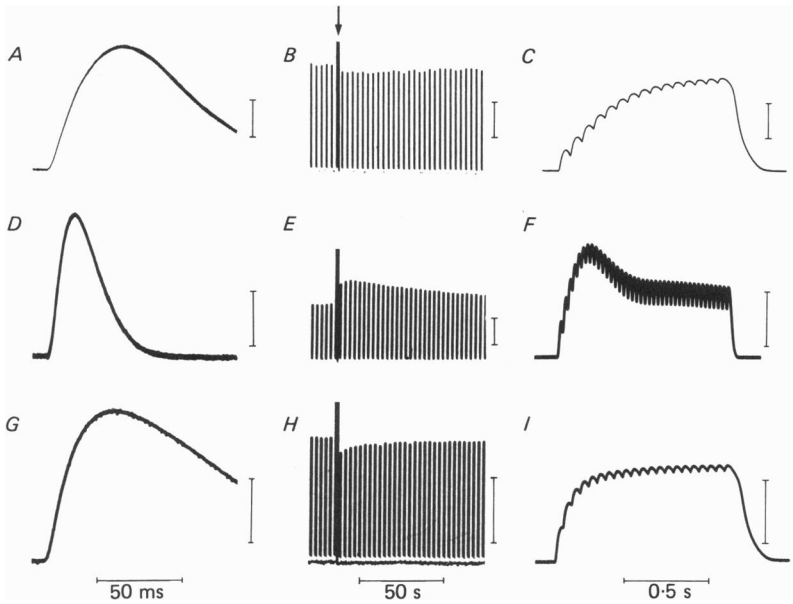

Fig. 1. Isometric tension responses of a normal soleus muscle (*A*, *B* and *C*), a soleus that had been denervated and stimulated intermittently at 100 Hz for 56 days (*D*, *E* and *F*) and a soleus that had been denervated and stimulated intermittently at 15 Hz for 50 days (*G*, *H* and *I*). *A*, *D* and *G* show three superimposed single twitches. *B*, *E* and *H* show single twitches evoked every 3 s before and after a 1 s train of stimuli at 200 Hz (arrow). *C*, *F* and *I* show unfused tetanic contractions during 15, 40 and 20 Hz stimulation respectively. Vertical bars represent 0·1 N for *A*, *B*, *D*, *E*, *G* and *H* and 0·4 N for *C*, *F* and *I*.

muscles developed the same fast twitch time-to-peak as the others. During continuous stimulation at 10 Hz or intermittent stimulation at 15 Hz, on the other hand, the twitch time-to-peak remained slow (Fig. 2*A*). Similarly, the twitch half-relaxation time became almost as fast as in the normal EDL during intermittent stimulation at 100 Hz but remained slow during stimulation at 10–15 Hz (Table 1). Denervation alone also decreased the twitch time-to-peak (Fig. 2*B*) and twitch half-relaxation time (Table 1) but the decrease started much later (after about a month) and was much less pronounced than in the stimulated muscles. Implantation of sham electrodes on innervated and denervated control muscles had no clear effect on twitch time-to-peak (Fig. 2*B*), twitch half-relaxation time, post-tetanic depression or twitch/tetanus ratio (shown only for the innervated muscles in Table 1).

Post-tetanic potentiation. During intermittent stimulation at 100 Hz the twitch changed from being depressed to being potentiated after a tetanus (Fig. 3*A*, see also Fig. 1*E*). This change could be detected already after 2 days of stimulation. Post-tetanic potentiation then increased in magnitude along approximately the same time

course as the decrease in twitch time-to-peak (Fig. 2*A*). During continuous stimulation at 10 Hz or intermittent stimulation at 15 Hz, on the other hand, the post-tetanic twitch remained depressed (Table 1). After denervation alone the post-tetanic twitch continued to be depressed for about 1 month. After this time the depression disappeared in some muscles and in one case was replaced by potentiation (Fig. 3*A*)

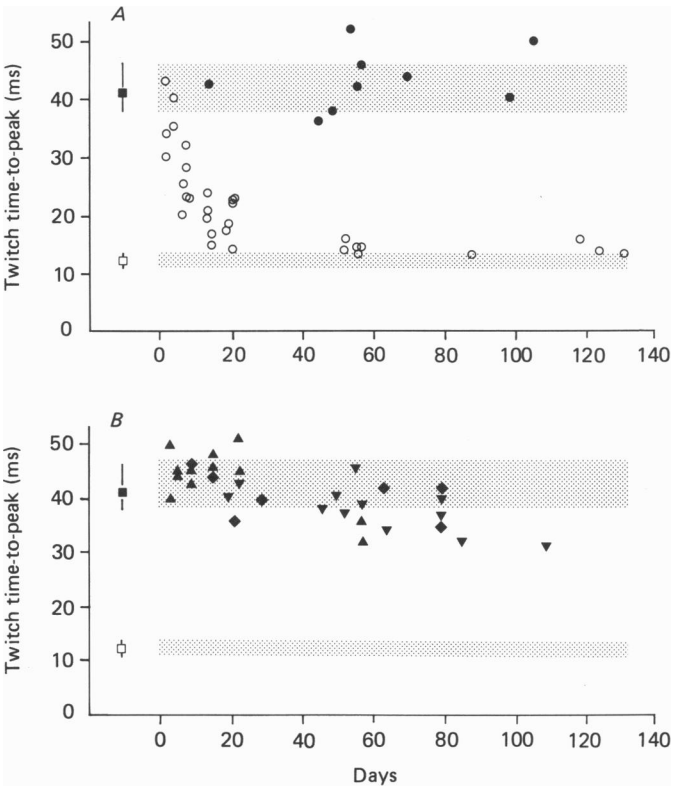

Fig. 2. Twitch time-to-peak after denervation plus stimulation (*A*) or denervation without stimulation (*B*). Stimulation started on day 0 (1 day after denervation) and was applied either as brief pulse trains at 100 Hz (○), or continuously at 10 Hz, or as long pulse trains at 15 Hz (●). Denervation alone was either accompanied (▲) or not accompanied (▼) by implantation of sham electrodes. Sham electrodes were also implanted in some innervated control muscles (◆). Mean values from normal soleus (n = 10) are indicated by ■, and from normal EDL muscles (n = 8) by □, while the total range of values are indicated by vertical bars and dotted areas.

Twitch/tetanus ratio. Denervation alone caused a rapid and marked increase in twitch/tetanus ratio (Fig. 3*B*). Intermittent stimulation at 100 Hz not only prevented this increase but resulted in a slightly lower than normal twitch/tetanus ratio for soleus muscles. Normal EDL muscles have been reported to have a lower mean twitch/tetanus ratio than normal soleus muscles (0·17 *vs.* 0·25, Bárány & Close, 1971), but this is not always the case (Close, 1969; Finol, Lewis & Owens, 1981). In the present experiments the ratios in normal EDL and soleus muscles were similar (0·23 *vs.* 0·20). During continuous 10 Hz stimulation or intermittent 15 Hz stimulation the twitch/tetanus ratio became larger than normal (Table 1).

Tension–frequency relation. Figure 4 shows that intermittent stimulation at 100 Hz caused the steep part of the tension–frequency curve of the soleus to move to the right, i.e. towards higher frequencies, as expected from the decrease in twitch duration (Fig. 1 *D*). The effect could be detected already after 2 days of stimulation; it proceeded rapidly during tne next 1–2 weeks, and then more slowly. Between 50

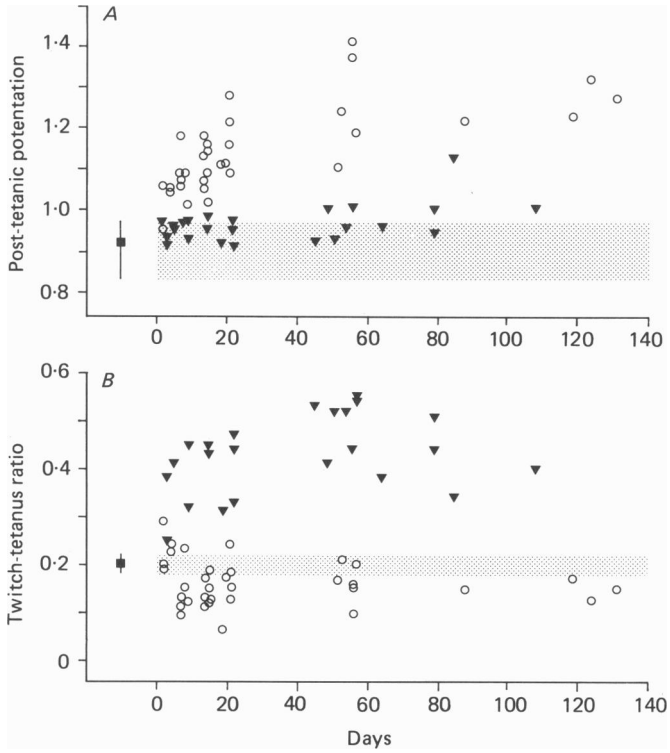

Fig. 3. Post-tetanic potentiation (*A*) and twitch/tetanus ratio (*B*) after denervation alone (▼) and denervation plus intermittent stimulation at 100 Hz (○). Stimulation started on day 0 (1 day after denervation). Post-tetanic potentiation is given as the ratio between maximum twitch tension after and before a tetanus at 200 Hz lasting 1 s. Twitch/tetanus ratio is given as the ratio between twitch and tetanic tensions, using supramaximal stimulation at 100–200 Hz to obtain maximum tetanic tension (see Fig. 4). Both the post-tetanic potentiation and the twitch/tetanus ratio were measured at the beginning of the acute experiments when the twitch was unpotentiated. Mean values from normal soleus ($n = 10$) are indicated by ■, and total range of values by vertical bars and dotted areas.

days (not shown) and 130 days no further movement occurred. In contrast, denervation alone moved the tension–frequency curve towards the left, mainly because of the marked increase in twitch/tetanus ratio (Fig. 4).

Force–velocity relation. Intermittent stimulation at 100 Hz increased the isotonic shortening velocity of whole muscles measured in mm/s (Table 1) and of individual sarcomeres, as indicated by the changes in 'intrinsic' shortening velocity measured in fibre lengths per second (Fig. 5, see Methods). Intrinsic V_{max} started to increase between 1 and 2 weeks after the onset of stimulation and reached an apparently

stable intermediate value within 2 months, which amounted to about 40% of the normal difference between soleus and EDL (Table 1). Cross-reinnervation with EDL nerve results in a similarly incomplete (55%) slow-to-fast transformation of intrinsic V_{max} (Close, 1969).

Intermittent stimulation at 100 Hz resulted in longer than normal muscle fibres

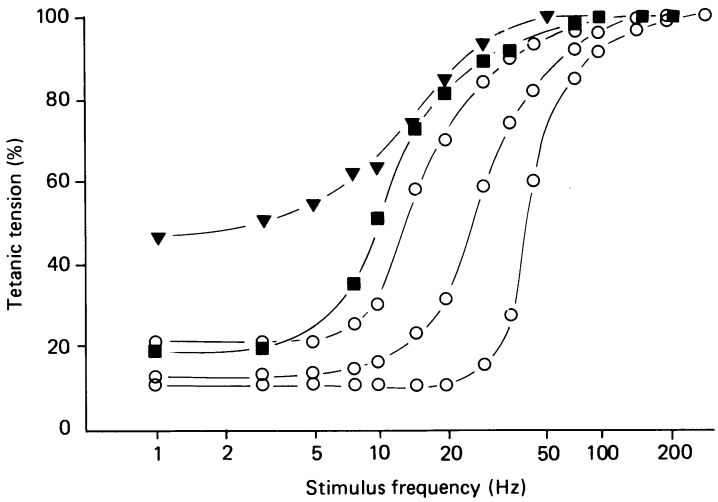

Fig. 4. Tension–frequency curves from denervated soleus muscles stimulated intermittently at 100 Hz (○), and innervated (■) and denervated (▼) control soleus muscles. The tension–frequency curve for the stimulated muscles moved progressively to the right as the duration of the stimulation increased from 7 to 14 to 130 days. The unstimulated muscles had been denervated for 56 days. Each symbol is the mean isometric tension obtained from two to six muscles and represents the highest tension produced regardless of when this occurred during a 1 s stimulus train at the frequencies indicated.

(35%, Table 1). As a result whole-muscle V_{max} (mm/s) became more completely transformed (116%) than intrinsic V_{max} (fibre lengths/s). Similar effects are obtained by cross-reinnervating the soleus with the EDL nerve. Thus, cross-reinnervated soleus fibres became 15 and 41% longer than normal in the experiments of Close (1969) and Bárány & Close (1971), respectively, and whole-muscle V_{max} became about as fast as in control EDL muscles (110% transformation, Close, 1969). Why stimulation and cross-reinnervation result in longer muscle fibres is not known. Chronic stretch is known to increase the length of muscle fibres through addition of sarcomeres (Tabary, Tabary, Tardieu, Tardieu & Goldspink, 1972) but this is not obviously relevant here. The type of activity imposed may be relevant since self-reinnervation by the soleus nerve results in less increase in fibre length (1% in Close, 1969, and 9% in Bárány & Close, 1971). In the present experiments we did not measure the fibre length of muscles stimulated with the slow pattern (10–15 Hz). In other experiments, however, a similar slow pattern (200 stimuli at 20 Hz every 30 s) increased fibre length by 15%, while maintaining a normally slow intrinsic V_{max} (Eken & Gundersen, 1988).

Tetanic tension. Denervation reduced tetanic tension rapidly during the first two

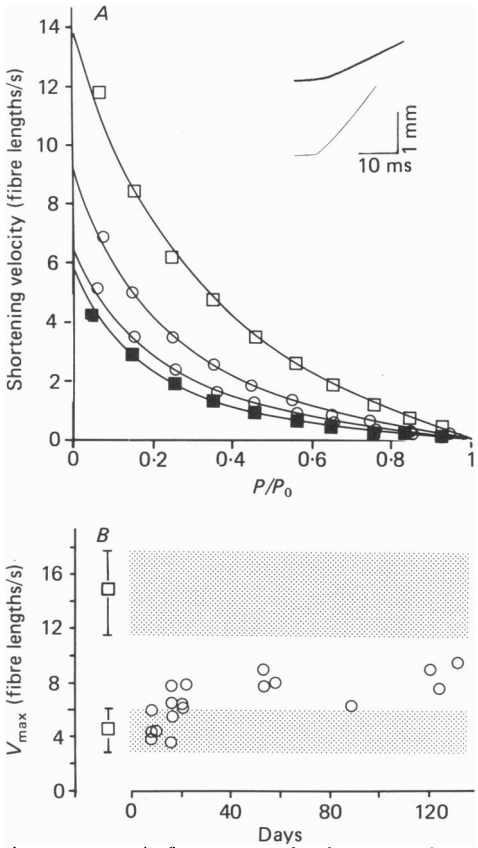

Fig. 5. Force–velocity curves (*A*) from normal soleus muscles (■), soleus muscles which had been denervated and stimulated directly at 100 Hz for 3 weeks (○, lower curve) or 4 months (○, upper curve), and normal EDL muscles (□). Each symbol is mean of single velocity measurements from three to six muscles at different loads, after division by estimated mean muscle fibre length (see Methods), and after grouping the data into bins of 0·1 P_0. The curves were obtained by fitting the data to Hill's equation (1938) as described by Katz (1939). Records in the upper right-hand corner show isotonic shortenings of a normal soleus (above) and a soleus denervated and stimulated at 100 Hz for 4 months (below) at loads corresponding to 0·13 and 0·15 P_0. In *B* the maximum 'intrinsic' isotonic shortening velocity (V_{max}) is plotted against time after denervation and onset of stimulation at 100 Hz. Upper and lower dotted areas with symbols and bars to the left represent mean values and total range of observations from normal EDL ($n = 8$) and normal soleus ($n = 5$) muscles.

weeks, then more slowly until very low stable values were reached after about 2 months (Fig. 6*A*). Intermittent stimulation at 100 Hz counteracted, but did not completely prevent, the fall in tetanic tension. Soon after denervation and onset of stimulation mean tetanic tension fell to between 30 and 70% of normal and then either remained at this level or slightly increased. A similar reduction in tetanic tension occurred in innervated control muscles with implanted sham electrodes (Fig. 6*A*, Table 1). Implantation of electrodes may therefore contribute to the lower than normal tetanic tension seen in stimulated muscles.

Muscle weight decreased to about 18% of normal after denervation, while tetanic

tension decreased to about 2·5 % (Fig. 6*B*, Table 1), presumably because denervation affects force-generating structures to a greater degree than other structures of the muscle such as connective tissue (Finol *et al.* 1981). Tetanic tension was reduced to a greater extent than muscle weight also in denervated and stimulated muscles and in innervated control muscles with sham electrodes (Fig. 6), suggesting that

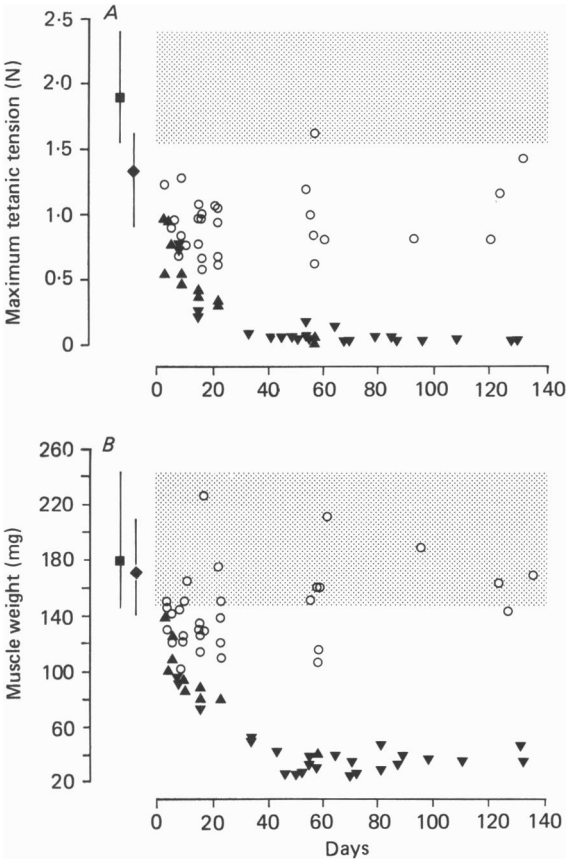

Fig. 6. Maximum isometric tetanic tension (*A*) and weight (*B*) of soleus muscles at different times after denervation and onset of intermittent stimulation at 100 Hz. Stimulation started on day 0 (1 day after denervation). Denervated plus stimulated muscles are indicated by ○, denervated muscles with sham electrodes by ▼, and without sham electrodes by ▲. Mean values from normal muscles ($n = 10$) are indicated by ■, and from innervated muscles with sham electrodes ($n = 7$) by ◆. Vertical bars indicate total range of values. Total range of values for normal muscles are also indicated by dotted areas.

connective tissue or other non-contractile structures were relatively more abundant. An increase in the amount of connective tissue could occur because denervation is accompanied by marked proliferation of cells, particularly fibroblasts, in the connective tissue between muscle fibres (Murray & Robbins, 1982; Connor, McMahan & Marshall, 1987).

Specific tetanic tension (mN/mm^2) was estimated by multiplying measured tetanic tension by the ratio between fibre length and muscle mass (Close, 1964). In

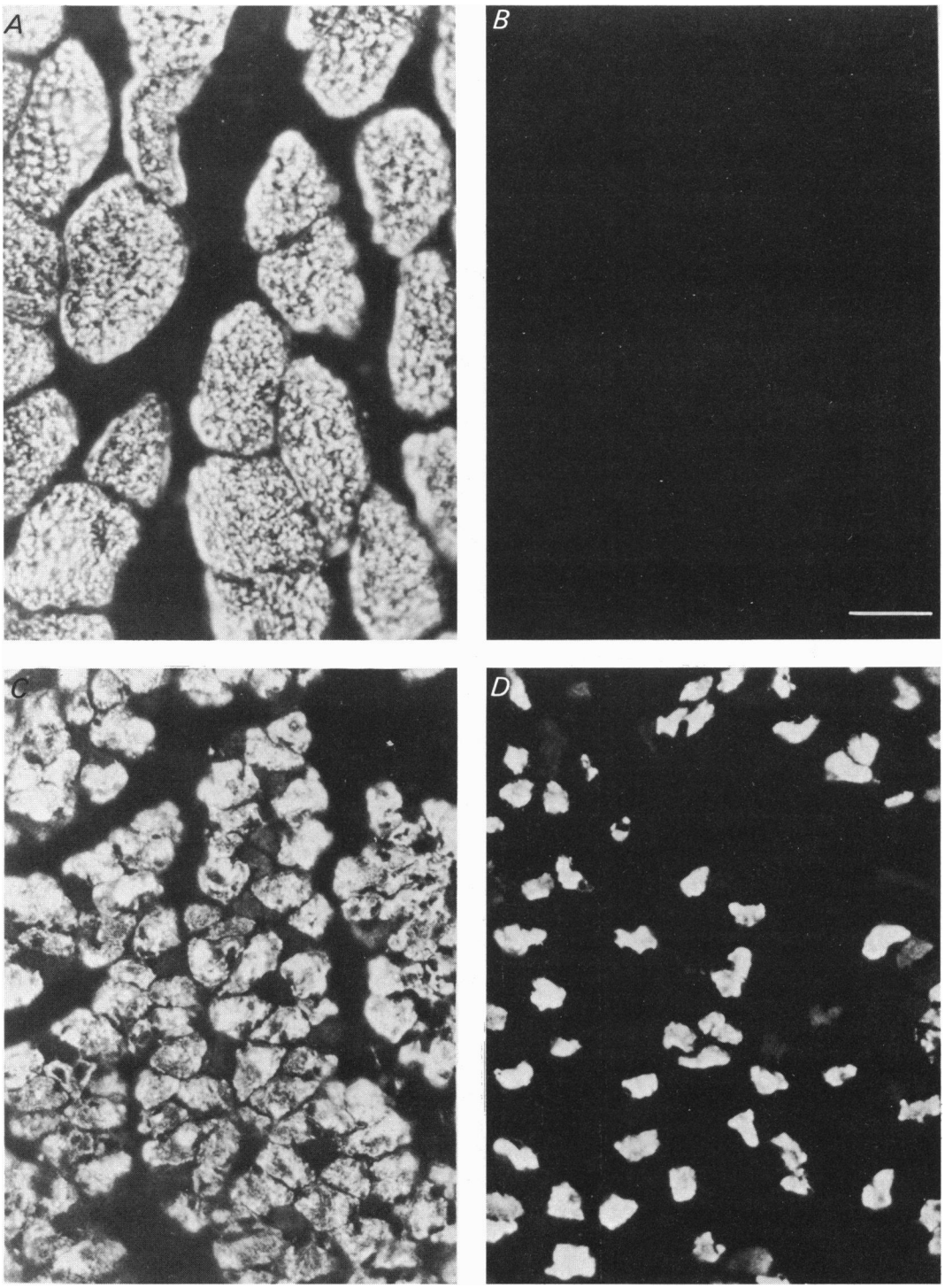

Fig. 7. Distribution of type I and type II myosins in a normal soleus (*A* and *B*) and in a soleus denervated for 56 days (*C* and *D*) based on immunofluorescence staining with anti-type I (*A* and *C*) and anti-type II (*B* and *D*) myosin antibodies in serial sections from each muscle. Scale bar is 30 μm.

normal soleus muscles the specific tension was 160 ± 41 mN/mm^2 (mean $\pm$ S.D., $n = 7$) and in denervated soleus muscles stimulated intermittently at 100 Hz for more than 45 days, 153 ± 48 mN/mm^2 ($n = 7$). For the denervated soleus muscles we did not measure the fibre length but in an earlier work we found a mean increase of 6% (unpublished results from Lømo *et al.* 1974). If a similar increase in fibre length occurred in the present experiments, the mean specific tension of the soleus muscles denervated for 42–108 days would have been about 26 mN/mm^2. Although this value is somewhat uncertain, it seems clear that long-term denervation results in a marked decrease in specific tetanic tension which can be almost completely prevented by intermittent stimulation at 100 Hz. Finally, it is worth noting that tetanic tension may easily become artificially low in denervated and stimulated muscles if some of the fibres escape stimulation due to misplaced electrodes or a significant number of muscle fibres become damaged by the electrodes (see below). Therefore the 'true' ability of stimulation to substitute for innervation may not be accurately reflected by comparing mean values from denervated plus stimulated and innervated control muscles.

Immunohistochemistry

The fibre type composition of 2- to 4-month-denervated stimulated muscles was compared to that of denervated or normal muscles using indirect immunofluorescence procedures with antibodies specific for fast (type II) and slow (type I) myosin types. Regardless of the presence or absence of sham electrodes normally innervated soleus muscles consisted of a major proportion of type I fibres which were labelled by anti-type I and unlabelled by anti-type II (Fig. 7*A* and *B*) and a minor proportion (less than 10%) of type II fibres showing the reverse reaction pattern. Denervated muscles showed a significant proportion of fibres labelled by anti-type II myosin antibodies (Fig. 7*C* and *D*). Counts were performed on selected areas from serial sections of 2-month-denervated muscles (three different muscles, 739 fibres examined). Fibres labelled only by anti-type II myosin were $22{\cdot}1 \pm 4{\cdot}3$% (mean for three muscles $\pm$ S.D.), fibres labelled only by anti-type I myosin were $56{\cdot}6 \pm 4{\cdot}1$%, and fibres stained by both antibodies were $21{\cdot}3 \pm 0{\cdot}4$%. These data are consistent with previous histochemical results based on myosin ATPase staining (Jaweed, Herbison & Ditunno, 1975) and show that a partial transformation of type I into type II fibres takes place in denervated muscles; however, even in long-term-denervated soleus muscles most muscle fibres were labelled exclusively by anti-type I myosin.

The reactivity of stimulated muscles with the two monoclonal antibodies was clearly dependent upon the pattern of impulses used, as illustrated in Fig. 8. Muscles stimulated intermittently for 2 months at 15 Hz appeared to be homogeneously composed of fibres reactive with anti-type I and unreactive with anti-type II myosin (Fig. 8*A* and *B*). In contrast, muscles stimulated at 100 Hz (train of sixty pulses), given either frequently (every 60 s) or infrequently (every 6000 s, from Lømo *et al.* 1980), consisted of fibres stained brightly by anti-type II and weakly by anti-type I myosin (Fig. 8*C–F*). Only a minor proportion (less than 10%) of these fibres were unreactive with anti-type I myosin possibly because they were original type II*A* fibres.

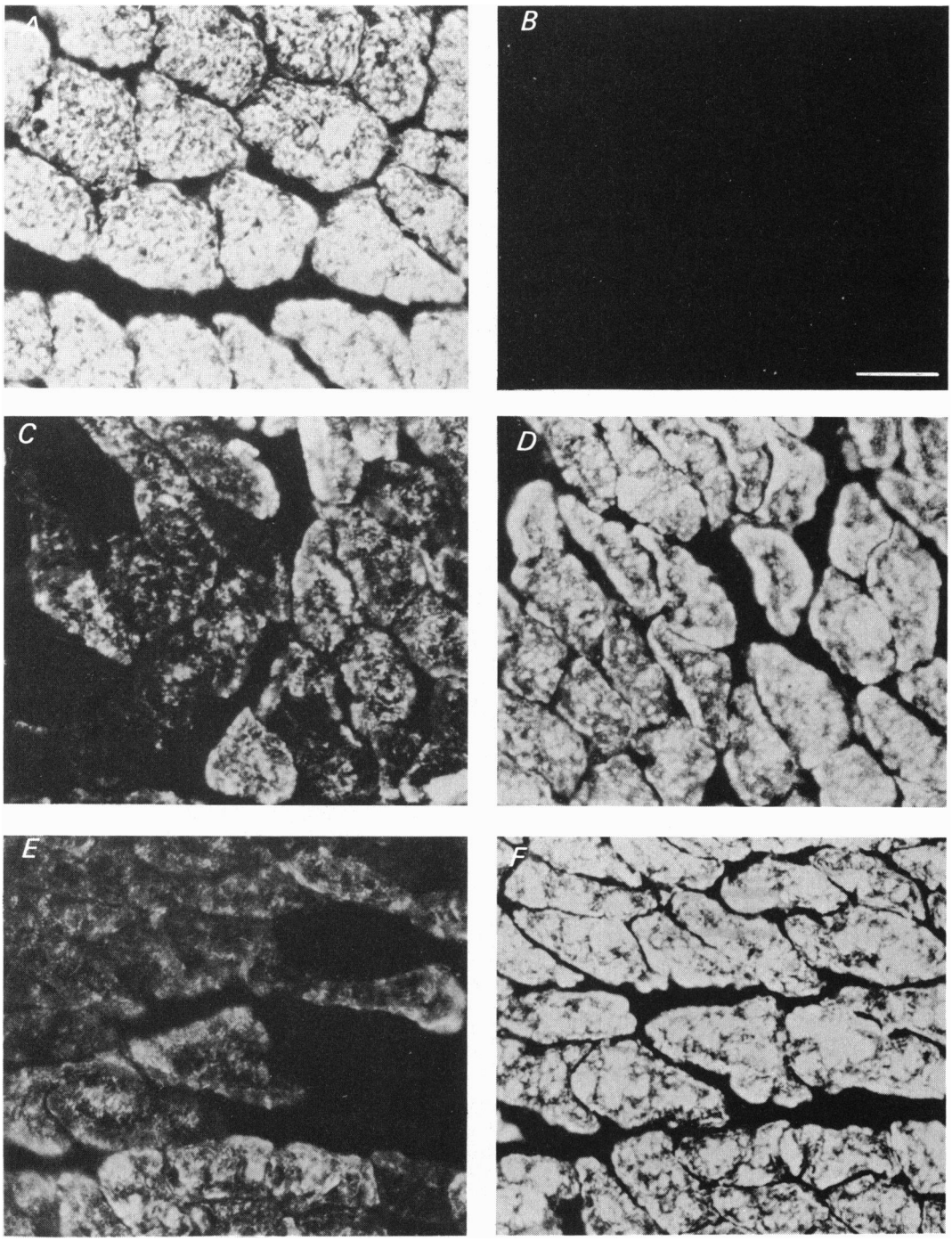

Fig. 8. Distribution of type I (*A*, *C* and *E*) and type II (*B*, *D* and *F*) myosin in soleus muscles that had been denervated and stimulated for 57 or 56 days by long trains at 15 Hz (*A* and *B*) or at 100 Hz (*C*, *D*, *E* and *F*). The muscle in *C* and *D* received sixty pulses at 100 Hz every minute and the muscle in *E* and *F* (from experiments by Lømo *et al.* 1980) sixty pulses at 100 Hz every 1 h and 40 min. Same treatment as in Fig. 7. Scale bar is 30 μm.

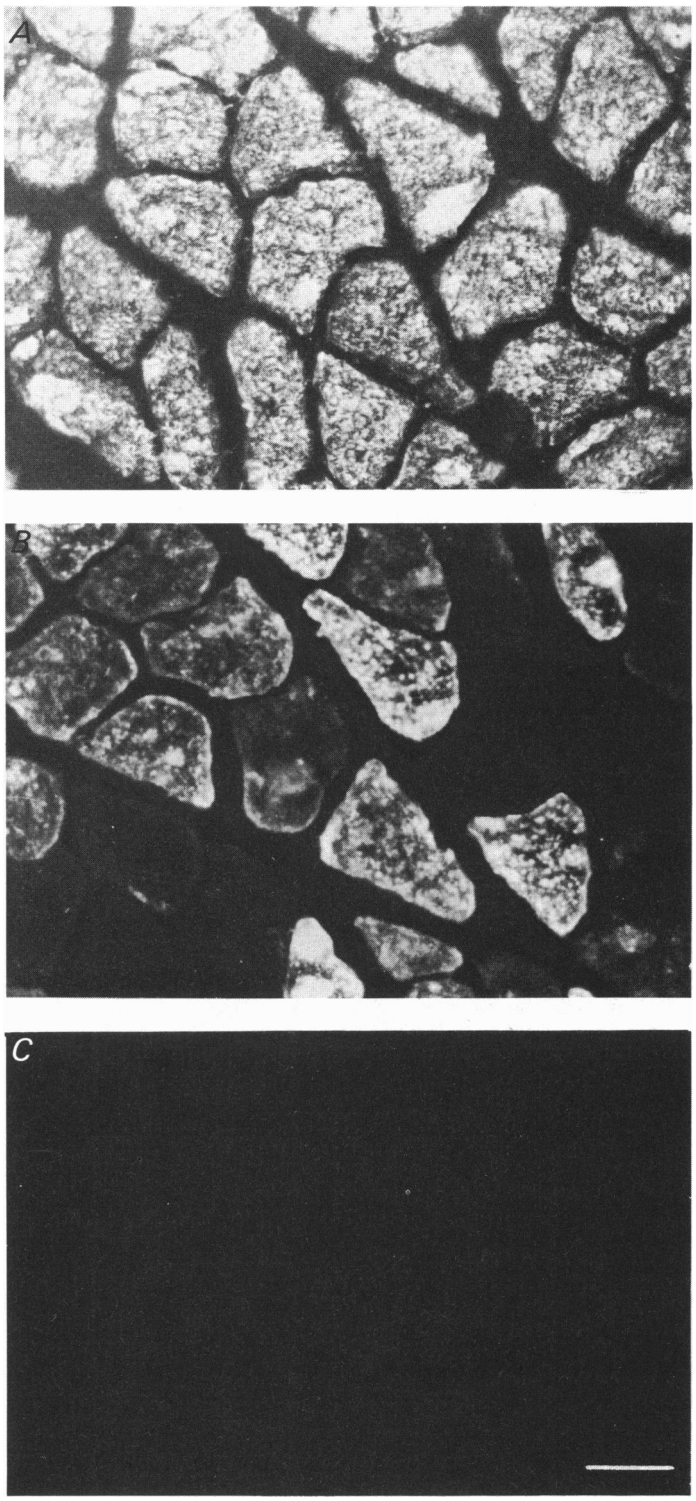

Fig. 9. Serial sections from soleus muscle denervated 9 days and stimulated intermittently at 100 Hz last 8 days. The fibres react with anti-type I (*A*), and anti-type II (*B*), but not with anti-bovine fetal myosin (anti-BFM, *C*), which stains regenerating fibres (Sartore *et al.* 1982). Scale bar is 30 μm.

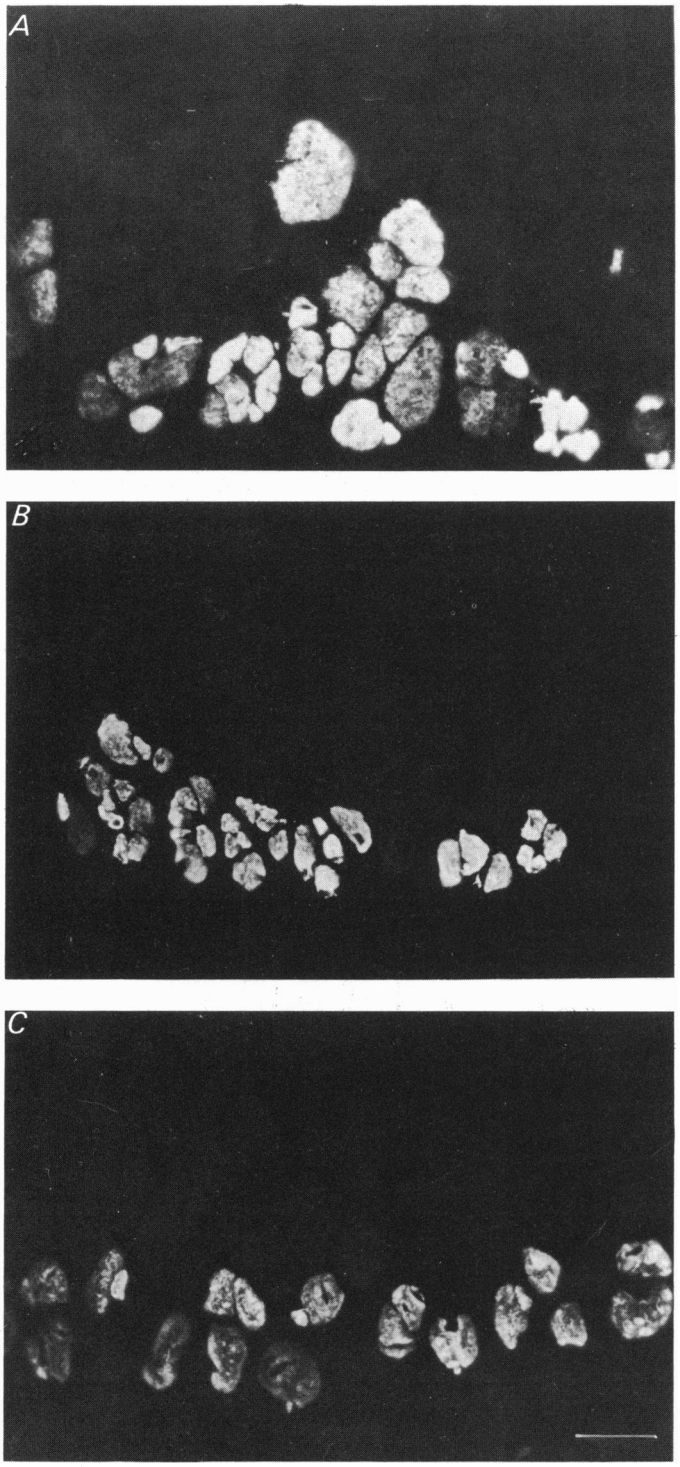

Fig. 10. Soleus muscles containing fibres reacting with anti-bovine fetal myosin (anti-BFM) in a superficial area underlying the implanted electrodes. The muscle in *A* was innervated and examined 8 days after implantation of sham electrodes. The muscle in *B* was examined 21 or 22 days after denervation and implantation of sham electrodes. The muscle in *C* was examined 57 or 56 days after denervation, implantation of electrodes and onset of intermittent stimulation at 100 Hz. Scale bar is 30 μm.

The altered myosin composition of stimulated muscles could either be due to transformation of pre-existing fibres or to appearance of new fibres. In order to distinguish between the two alternatives we examined the myosin composition throughout the entire period of transformation starting 2 days and ending 130 days after the onset of stimulation. To detect regenerating fibres we used an antibody (anti-BFM), which reacts specifically with myosin heavy chains of embryonic and neonatal type, which is expressed during muscle regeneration. This antibody stains regenerating fibres brightly, but does not stain adult soleus type I and type II fibres (Sartore *et al.* 1982). In a first series of experiments we denervated and started stimulation 8 and 9 days after implantation of electrodes to allow some recovery after the surgery. When these muscles were removed, however, new fibres might have regenerated to a stage where labelling with anti-BFM would already have disappeared. To avoid this possibility we did a second series of experiments in which we denervated and implanted stimulating electrodes on the same day and started stimulation 1 day later. In neither series did we find any labelling with anti-BFM throughout the bulk of the muscle during the transformation period. The first clear indications of a change in myosin composition occurred after 8 days of stimulation when numerous large fibres labelled by anti-type I myosin also showed variable reactivity with anti-type II myosin antibodies (Fig. 9*A* and *B*). These fibres were not labelled by anti-BFM and were therefore not regenerating fibres (Fig. 9*C*).

At all times and in all muscles with electrode implants, including unstimulated, normally innervated and denervated control muscles, anti-BFM stained small-sized fibres located at the periphery of the muscle (Fig. 10). These fibres were usually grouped in small clusters or were arranged as thin superficial layers. The area containing labelled fibres varied between muscles but did not exceed 1–2% of the total cross-sectional area. Evidently, tissue injury around the stimulating electrodes was responsible for this local reaction. Similar anti-BFM-labelled fibres were not observed throughout the rest of the muscle at any time after the onset of stimulation.

DISCUSSION

Slow-to-fast transformation occurs in original soleus muscle fibres

The present results confirm earlier reports (Lømo *et al.* 1974, 1980) that denervated rat soleus muscles develop fast isometric twitches during intermittent stimulation at 100 Hz. Compared to the normal EDL the transformation was almost complete with respect to twitch time-to-peak (14·3 *vs.* 12·9 ms, 95% transformation) and twitch half-relaxation time (15·6 *vs.* 12·9 ms, 94% transformation). We can now attribute this slow-to-fast transformation to changes in original fibres rather than to formation of new fibres for the following reasons. The majority of the muscle fibres remained large throughout the period of denervation and stimulation. These fibres did not bind anti-fetal myosin, which labels regenerating fibres (Sartore *et al.* 1982), but continued to bind anti-slow myosin while gradually acquiring fast myosin. These results do not support the suggestion by Jolesz & Sréter (1981) that injury and muscle fibre regeneration caused by electrode implants is responsible for the slow-to-fast transformation. The electrodes did cause some fibre degeneration and regeneration, but only near the electrodes in superficial regions of the muscle. These regions did not

spread into the rest of the muscle. Moreover, comparable signs of degeneration and regeneration were seen in the superficial regions of normal and denervated control muscles that had been implanted with sham electrodes without any noticeable effects on the contractile speed of these muscles.

Slow-to-fast transformation of the twitch

In the present experiments the isometric twitch duration started to decline rapidly after only 2 days of 100 Hz stimulation and reached stable fast values after about 3 weeks. This may be accounted for, at least in part, by faster removal of Ca^{2+} from the cytosol after an impulse. In denervated rat soleus muscles Ca^{2+} uptake by the sarcoplasmic reticulum and parvalbumin content in the cytosol are markedly increased by intermittent, high-frequency stimulation (Gundersen, Leberer, Lømo, Pette & Staron, 1988). Similarly, in the denervated fast EDL of the rat (Gundersen *et al.* 1988) and in the innervated fast tibialis anterior of the rabbit (Leberer, Härtner & Pette, 1987; Klug, Leberer, Leisner, Simoneau & Pette, 1988) these parameters are markedly decreased by 'slow'-pattern stimulation. The effect is particularly rapid on the initial rate and total capacity of Ca^{2+} uptake by sarcoplasmic reticulum which decrease abruptly to 50% of normal after 2 days of stimulation (Leberer *et al.* 1987). Twitch relaxation, however, depends not only on Ca^{2+} movements but also on the properties of the myofibrils since maximally activated skinned soleus fibres relax much more slowly than maximally activated skinned EDL fibres when Ca^{2+} is suddenly removed (Stephenson & Williams, 1981). On the other hand, in the present experiments changes in sliding velocities between actin and myosin and in myosin composition were detected only after 1 week of stimulation, whereas the twitch decreased rapidly in duration after 2 days of stimulation. Therefore, it appears that twitch duration and hence the tension–frequency characteristics of the muscle (Fig. 4) may be quickly adjusted to altered activity through changes in excitation–contraction coupling processes. Such adjustments may contribute to the match that normally exists between twitch speed and motoneurone firing pattern (Kernell, 1979; Hennig & Lømo, 1985).

Twitch speed is determined by the pattern of stimulation

Stimulation delivered as brief trains at 100 Hz resulted in fast twitches, while continuous stimulation at 10 Hz or long pulse trains at 15 Hz resulted in slow twitches. This difference in twitch speed cannot be attributed unequivocally to the difference in frequency because these stimulation patterns also differed in number of pulses as well as in number and duration of pulse trains and intervening rest periods. Elsewhere we have reported marked effects of variations in pulse frequency (as well as in pulse number) on the duration of the soleus twitch (Lømo *et al.* 1985*b*). In contrast, in similar experiments by Al-Amood & Lewis (1987) the twitch duration was almost independent of the frequency of stimulation which ranged from 10 to 100 Hz. In their experiments Al-Amood & Lewis (1987) used pulse trains of the same duration (1 s), whereas we have used pulse trains containing the same number of stimuli (sixty) and this may have contributed to the different results. For the stimulations at 100 Hz, however, the two laboratories have used quite similar patterns (100 pulses at 100 Hz every 90 s in Al-Amood & Lewis (1987) *vs.* 60 pulses

at 100 Hz every 60 s in our experiments) and we find it unlikely that this small difference in pattern can account for the fact that in several independent experiments by us (Lømo *et al.* 1974, 1980, 1985*b*; Hennig & Lømo, 1987; Eken & Gundersen, 1988; this work) the increase in twitch speed occurred much earlier and was considerably more pronounced than in the experiments by Al-Amood & Lewis (1987). We have no satisfactory explanation for these conflicting results, which lead to different views on the possible mechanisms behind the effects of stimulation (see below).

Eerbeek, Kernell & Verhey (1984) and Kernell, Eerbeek, Verhey & Donselaar (1987) stimulated chronically the nerve to the fast peroneus longus muscle of the cat with different combinations of pulse trains at frequencies ranging from 5 to 100 Hz. In each case the twitch became slower than normal and there was no evidence that the pulse rate had specific effects on twitch speed. Instead, other aspects, such as total daily amount and distribution of the stimulation, appeared to be important. Others have observed similar slowing of the twitch after stimulating fast muscles at relatively high frequencies (up to 40 Hz, Hudlická, Tyler, Srihari, Heilig & Pette, 1982; Sréter, Pinter, Jolesz & Mabuchi, 1982). In our view differences between animal species and types of muscles probably account for many of the different results obtained by chronic stimulation of muscle in different laboratories. Such differences may explain why large numbers of stimuli at low frequencies result in only modest fast-to-slow transformation of the twitch and no conversion from type II to type I fibres in the EDL of the rat (Gundersen *et al.* 1988; Eken & Gundersen, 1988), but marked fast-to-slow transformation and apparently complete transformation of type II to type I fibres in fast muscles of the cat and rabbit (Salmons & Sréter, 1976; Eerbeek *et al.* 1984). It may also explain why identical slow-pattern stimulation results in much faster twitches in the EDL than in the soleus of the rat (Gundersen *et al.* 1988) and why pulse frequency appears to play an important role in our experiments on the slow soleus of the rat but not in the experiments by Kernell *et al.* (1987) on the fast peroneus longus muscle of the cat. Also in the rat soleus, however, variations in the amount and other aspects of the stimulation patterns have important effects on twitch speed (Lømo *et al.* 1985*b*; Eken & Gundersen, 1988). Clearly, more experiments are needed to identify the aspects of patterned activity that are particularly important for the regulation of contractile speed in different types of muscles in different species.

Transformation of intrinsic shortening velocity is incomplete

Intrinsic V_{max} (normalized for fibre length), which reflects the sliding velocity between myosin and actin, became faster with intermittent 100 Hz stimulation, but not nearly as fast as in the normal EDL. Incomplete transformation of intrinsic V_{max} may be attributed in part to the co-expression of fast and slow myosin heavy chains in the stimulated fibres (Fig. 8*C–F*) since V_{max} is closely correlated to the relative amount of fast and slow myosin heavy chains in single muscle fibres (Reiser, Moss, Giulian & Greaser, 1985). Another possibility is that stimulated soleus muscle fibres do not express the same fast myosins as most normal EDL fibres. Thus, in preliminary experiments we have found that stimulated soleus fibres express predominantly a recently described IIX myosin heavy chain in contrast to most

normal EDL fibres which express II B myosin heavy chains (Schiaffino, Saggin, Viel, Ausoni, Sartore & Gorza, 1986). Intermittent stimulation at 100 Hz caused fast myosin to appear and V_{max} to start increasing after about 1–2 weeks. These effects are slow compared to the effects on twitch duration, as expected if intrinsic V_{max} changes as a result of replacement of one type of slowly turning over myosin molecule by another. Why stimulated soleus fibres fail to express the same myosins as normal EDL fibres is not known. It could be due to an inappropriate pattern of stimulation. A more interesting possibility, however, is that tissue-specific differences may not allow a particular myosin heavy chain gene that is expressed in one muscle to be expressed in another muscle (see Izumo, Nadal-Ginard & Mahdavi, 1986; Hoh, Hughes, Hale, Chow, Fitzsimons & Schiaffino, 1988).

Denervation per se vs. stimulation

Al-Amood *et al.* (1986) and Al-Amood & Lewis (1987) interpret their experiments to mean that slow-to-fast transformation in rat soleus muscles results from denervation *per se* and not from the intermittent direct stimulation. They suggest that denervation is responsible for the switch in synthesis from slow to fast myosin, while intermittent stimulation, regardless of the frequency, merely enhances protein synthesis. In our view, neither denervation as such nor the accompanying cessation of normal motor unit activity is necessary for slow-to-fast transformation in the rat soleus. This is indicated by the finding that intermittent 100 Hz stimulation results in faster twitches and higher percentages of type II fibres in normally innervated soleus muscles of the rat where normal motor unit activity persists despite the stimulation (Hennig & Lømo, 1987). We also believe that the effects of denervation on twitch speed can be accounted for by altered activity since denervated soleus muscles are probably much less active than normal (Hennig & Lømo, 1985) and therefore no longer exposed to the slowing effect of large amounts of activity (Lømo *et al.* 1985*b*; Al-Amood & Lewis, 1987).

In the present material several findings point to important differences between the effects of denervation *per se* and the effects of stimulation. First, denervation leads initially to an increase and then, after about a month, to a gradual and moderate decrease in twitch contraction speed. In contrast, intermittent stimulation at 100 Hz results in a much more pronounced decrease which develops rapidly after only 2 days of stimulation. The effects on post-tetanic depression–potentiation of the twitch are similarly different. Secondly, the twitch/tetanus ratio increases rapidly and markedly in the denervated muscles, but decreases in the muscles stimulated intermittently at 100 Hz. Thirdly, less than half the fibres in the denervated muscles acquire fast myosin, whereas all the fibres in muscles stimulated intermittently at 100 Hz do so.

The effects of direct muscle stimulation obtained in this study resembled closely the effects of reinnervation by different nerves. Like intermittent 100 Hz stimulation, cross-reinnervation with the EDL nerve leads to an almost complete transformation of the isometric twitch (Gutmann & Carlson, 1975), but only to an incomplete transformation of intrinsic V_{max} (Close, 1969). Like stimulation with large numbers of stimuli at 10 or 15 Hz, self-reinnervation with the soleus nerve restores normal slow properties (Close, 1969; Gutmann & Carlson, 1975). Direct stimulation largely

counteracted the reduction in specific tetanic tension produced by denervation. In long-term-denervated rat soleus muscles stimulation and reinnervation result in similar recovery of tetanic tension (Hennig & Lømo, 1987). For these reasons we believe that the responses of denervated soleus muscles to different patterns of stimulation reflect important mechanisms through which motoneurones normally control the contractile properties of muscle.

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