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INDUCTION OF ACTION POTENTIALS BY DENERVATION OF TONIC FIBRES IN RAT EXTRAOCULAR MUSCLES

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SUMMARY

1. The effects of denervation by nerve section on the electrical properties of tonic and twitch fibres of rat extraocular muscles were examined.

2. Normally innervated tonic fibres lack action potentials. Upon direct stimulation they generate graded, voltage-dependent responses or slow peak potentials (s.p.p.s). However, one week after denervation the s.p.p.s are transformed into action potentials which are slower and broader than those of twitch fibres. The action potentials are Na dependent and partially resistant to blockade with 10^{-5} M-tetrodotoxin and 10^{-6} M-saxitoxin.

3. Changing the holding potential of the fibres from −80 mV to more negative levels increases the maximal rate of rise of the action potential. This effect is not observed on the s.p.p.s of normally innervated fibres.

4. Following denervation the resting potential of tonic and twitch fibres becomes about 10–15 mV less negative.

5. In denervated muscles stimulation with pulses of hyperpolarizing current evokes graded responses in tonic fibres and action potentials in twitch fibres. In normally innervated muscles, these anodal break responses are never observed in tonic fibres and are very rare in twitch fibres.

6. By two weeks after nerve section, reinnervation is present. The action potentials of tonic fibres are still present but stronger stimulation is needed to evoke anodal break responses. By three weeks, direct stimulation of tonic fibres evokes normal s.p.p.s in about 25% of the studied fibres and action potentials in the rest. By four weeks, most tonic fibres have lost the action potential but small anodal break responses can be evoked in most.

7. It is suggested that following denervation a new population of Na channels appears in tonic fibres. The properties of these channels are different from those of the channels normally present in innervated tonic fibres but they are in some ways similar to those of the channels which appear in twitch fibres following denervation.

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INTRODUCTION

Rat extraocular muscles (e.o.m.s) comprise a mixture of two types of muscle fibres: singly innervated and multiply innervated fibres (Mayr, 1971; Peachey, 1971; Chiarandini & Davidowitz, 1979) which exist intermingled in two layers of the muscle: the orbital, which faces the bony orbit, and the global which faces the eyeball. In normally innervated e.o.m.s the physiological properties of singly and multiply innervated fibres of the global layer are markedly different. Upon depolarization global singly innervated fibres respond as typical twitch fibres and generate a phasic tension while multiply innervated fibres generate a sustained or tonic tension. This contractile property of global multiply innervated fibres led us to classify them as tonic fibres (Chiarandini, 1976; Chiarandini & Davidowitz, 1979). Electrophysiologically the two types of global fibres are also different. Twitch fibres respond to nerve stimulation with a unitary end-plate potential (e.p.p.) which evokes an action potential, and to intracellular depolarizing pulses with an action potential. In contrast, tonic fibres respond to nerve stimulation with a compound junction or end-plate potential (Hess & Pilar, 1963; Chiarandini & Stefani, 1979) and to intracellular depolarization with a graded, voltage-dependent response or slow peak potential (s.p.p.) (Chiarandini & Stefani, 1979; Bondi & Chiarandini, 1979). Moreover, the membrane time constant and effective resistance of tonic fibres are several times larger than those of twitch fibres (Chiarandini & Stefani, 1979; Bondi & Chiarandini, 1979).

Although in rat e.o.m.s the membrane properties of twitch fibres are similar to those of other twitch muscles, the properties of tonic fibres most closely resemble those of the slow tonic fibres of amphibians which also lack action potentials and have a large membrane time constant and effective resistance (Kuffler & Vaughan Williams, 1953; Burke & Ginsborg, 1956; Stefani & Steinbach, 1969). Changes in the electrical properties of muscle fibres following denervation have been well documented. In twitch fibres there is a reduction of the resting potential, an increase of the membrane resistance and time constant and a decrease in the rate of rise of the action potential (Nicholls, 1956; Albuquerque & Thesleff, 1968; Albuquerque & McIsaac, 1970; Redfern & Thesleff, 1971*a*; Robbins, 1977). In frog slow tonic fibres the membrane acquires the ability to generate action potentials (Miledi, Stefani & Steinbach, 1971) which are ultimately suppressed when reinnervation takes place (Schmidt & Stefani, 1977). The current study showed that tonic fibres of rat e.o.m.s also acquire the ability to produce action potentials following denervation. These were subsequently suppressed during the course of reinnervation. Preliminary accounts of these results have appeared in abstracts (Bondi, Chiarandini & Jacoby, 1984; Bondi, Jacoby & Chiarandini, 1984).

METHODS

Preparation and denervation procedure

Inferior rectus muscles from male and female Wistar rats weighing 200–350 g were used. The animals were anaesthetized with sodium pentobarbitone (50 mg/kg) for both denervation and muscle removal. To denervate the muscle, a piece of the zygomatic arch was excised and the intraorbital lacrimal and Harderian glands were removed to expose the inferior rectus muscle and

its nerve. The exorbital lacrimal gland remained intact. The nerve was sectioned and approximately 2 mm of it was removed just before its entrance into the muscle at the global surface to assure a complete denervation. Gentamycin ophthalmic ointment (Garamycin, 3 mg/gm, Schering Corporation, Kenilworth, NJ) was applied internally and externally to the surgical wounds and successfully prevented infection. Wounds were closed with 5–0 silk sutures. Plastic collars made from dram cups prevented the animals from scratching open the wounds during recovery from anaesthesia. Animals ate and groomed normally within a day of surgery. No loss of weight was observed. In two animals a sham-denervation operation was performed. A week later the electrical properties of the muscle fibres in these animals were found to be normal. For recording, all muscles were isolated with a piece of presphenoid bone at the proximal end and of sclera at the distal end and pinned in a Sylgard coated bath.

Solutions

The saline contained (mM): NaCl, 136; KCl, 5; $CaCl_2$, 10; $MgSO_4$, 1·2; glucose, 11; and imidazole sulphate, 5; with a pH 7·35. It was oxygenated continuously, and during recording, was perfused at a rate of about 2 ml/min. A high Ca concentration was used to reduce impalement damage by the double-barrelled micro-electrodes. Bath temperature was 21–22 °C. Tetrodotoxin (TTX; Sankyo Co. Ltd., Japan) and saxitoxin (STX; a generous gift from Dr C. Y. Kao) were diluted in saline. Sodium-free solutions were prepared by replacing sodium with an equivalent concentration of Tris(hydroxymethyl)aminomethane (Tris).

Electrical recording and cell labelling

Standard techniques for intracellular recording were used. All signals were recorded on an FM tape recorder. Because global tonic fibres have small diameters, between 15 and 25 μm (Mayr, 1971), double-barrelled micro-electrodes were used to allow for recording and current injection with only one impalement. The recording barrel was filled with 3 M-KCl or, when fibres were labelled with Lucifer Yellow, with 1 M-LiCl; the other barrel was filled with 1·5 M-K citrate. Micro-electrodes had tip resistances of between 30 and 90 MΩ, and coupling resistances of less than 300 kΩ. Because global tonic fibres have a large effective resistance (R_{eff}) (Chiarandini & Stefani, 1979; Bondi & Chiarandini, 1979), micro-electrode penetration could easily result in a reduction of the resting potential (E_{rp}) (Stefani & Steinbach, 1969). To eliminate the possibility that responses were depressed by such a depolarization, unless otherwise stated, fibres were held at a membrane potential of about −80 mV with d.c. current for at least 30 s before test pulses were delivered. The R_{eff} was determined by dividing the voltage deflexion produced by injecting small pulses of hyperpolarizing current (5–15 nA) by the intensity of the current. The derivative of voltage with respect to time of some signals was obtained using an RC circuit with a time constant of 110 μs.

After electrophysiological characterization, the innervation of some fibres was morphologically identified, following a protocol previously described (Bondi & Chiarandini, 1983). Briefly, it consisted of labelling the fibres with Lucifer Yellow, staining for cholinesterase (ChE) activity, and counting end-plates visualized in sequential, serial 20 μm Epon sections. At 6–9 days denervation there appeared to be minimal reduction of ChE activity. Several of the denervated muscles were treated with 1 % OsO_4 for 1·5 h, embedded in Epon and similarly sectioned. Myelin sheaths appeared to have deteriorated and the nerve bundles appeared disorganized, clearly indicating nerve interruption.

Results are expressed as mean ± S.E. of mean with the number of samples in parentheses. Records included in Figs. 2*B* and 3*B* were retouched to reduce tape noise superimposed on the signals.

RESULTS

Following denervation tonic fibres produce action potentials

In normally innervated rat e.o.m.s, a majority of the fibres of the global layer are twitch fibres that produce action potentials, while a minority are tonic fibres that generate graded s.p.p.s (Chiarandini & Stefani, 1979; Bondi & Chiarandini, 1979). Fig. 1 illustrates a s.p.p. (*A*) and an action potential (*C*) generated by intracellular depolarization of a tonic and a twitch fibre, respectively, and the response of the same

two fibres to nerve stimulation: a compound e.p.p. (B) in the tonic fibre and an action potential (D) in the twitch fibre. After 6–9 days of denervation, it was observed that fibres responding to intracellular stimulation with s.p.p.s had virtually disappeared. Most of the fibres generated action potentials (Fig. 2C) essentially similar to those of normal twitch fibres but a minority of fibres displayed action potentials that were

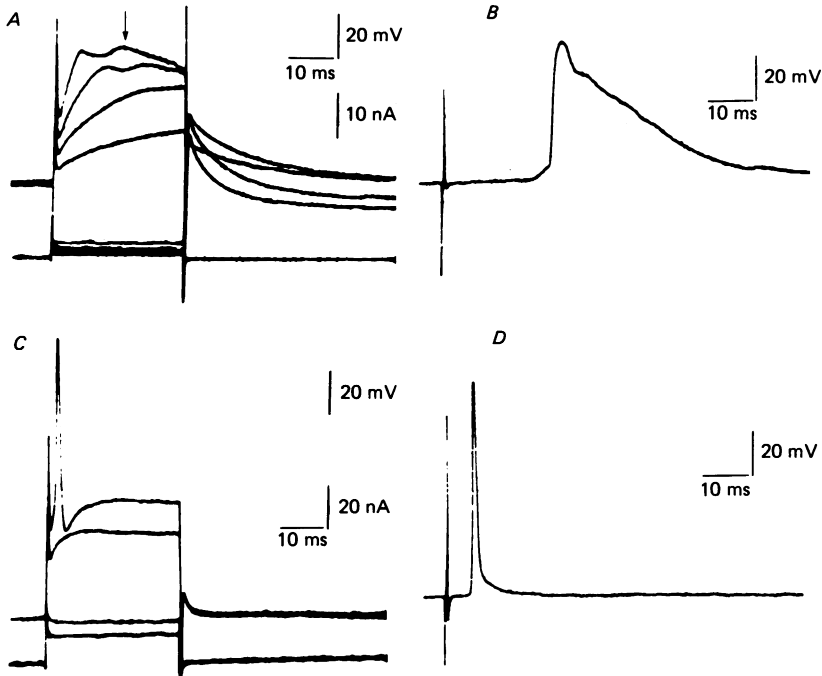

Fig. 1. Responses to intracellular depolarizing stimuli in normally innervated e.o.m.s. Upper traces: voltage. Lower traces: applied current. Records obtained from the same muscle. A and B: tonic fibre ($\tau = 44$ ms). A, the tonic fibre does not respond to depolarization with an action potential, instead it generates a slow, graded response or slow peak potential (s.p.p.). B, compound e.p.p. evoked by nerve stimulation. C and D: twitch fibre ($\tau = 1{\cdot}5$ ms). Action potentials evoked by direct (C) and indirect (D) stimulation. The sharp difference in the latency between stimulation artifact and nerve response in B and D indicates that the two types of fibres are innervated by axons with different speeds of conduction.

wider and had slower rising and falling phases (Fig. 2A). It was hypothesized that these fibres were tonic fibres which had acquired the property of generating action potentials after denervation.

This hypothesis was directly confirmed in eight fibres from five muscles which were denervated for 6–9 days. These fibres were first studied electrophysiologically and found to have slow and wide action potentials. They were subsequently labelled with Lucifer yellow and stained for ChE activity to identify their innervation morphologically. All were recognized as multiply innervated and had an average of $7{\cdot}6 \pm 1{\cdot}1$ end-plates (8) along $0{\cdot}92 \pm 0{\cdot}11$ mm of their length, with a mean distance between end-plates of about 120 μm. For these fibres the mean membrane time constant (τ)

was 23·4 ± 2·9 ms (8; range: 10–33 ms), the mean R_{eff} was 5·29 ± 0·62 MΩ (7; range: 3·3–7·5 MΩ), and all produced slow and wide action potentials with a maximal rate of rise (V_{max}) of 50·8 ± 5·3 V/s (8). A recording from one of these fibres in a muscle denervated for 6 days is shown in Fig. 2*A*. This cell had twelve end-plates along 1·16 mm of its length.

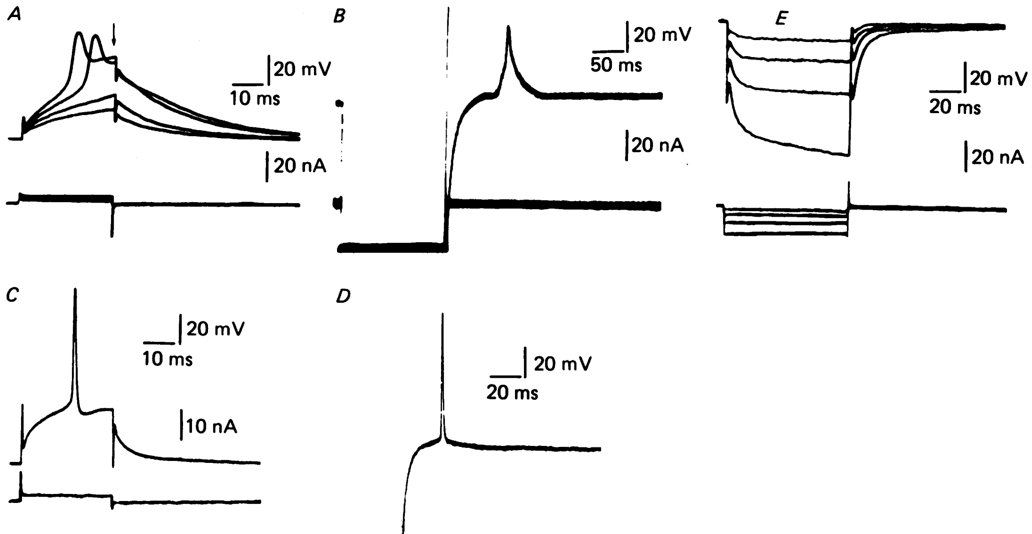

Fig. 2. Depolarizing and anodal break responses evoked in denervated muscles. *A* and *B*: tonic fibre (τ = 31 ms; V_{max} = 40 V/s; 6 day denervation). This cell was morphologically identified as a tonic fibre and twelve end-plates were counted along 1·16 mm of its length. Depolarizing pulses elicited an action potential which was slower and broader than that shown in *C* and *D*, recorded from a twitch fibre (τ = 9 ms) in a muscle denervated for 7 days. The action potential of denervated tonic fibres consistently displayed a 'hump' (arrow) following the early repolarizing phase. Only the initial portion of this hump is seen in *A*, but it is clearly observed in other records (Fig. 4*A*, *B* and *D*). When large hyperpolarizing pulses were applied (*B* and *D*) the fibres responded at the break of the pulse with a depolarizing signal which was usually graded with the strength of stimulation in tonic fibres (*B*) and an all-or-none action potential in twitch fibres (*D*). Note the slower time base in *B*. In normally innervated muscles, anodal break responses were never observed in tonic fibres (*E*) and were very unusual in twitch fibres.

Cell labelling is a very time-consuming procedure and therefore it was considered more practical for the rest of the study to base the recognition of fibre type only on electrophysiological criteria. In innervated e.o.m.s we previously used three criteria for this purpose (Chiarandini & Stefani, 1979; Bondi & Chiarandini, 1979). Tonic fibres were recognized by the absence of action potentials, the presence of multifocal miniature end-plate potentials (m.e.p.p.s) and their high τ value. The first two criteria cannot be used in denervated muscles. The third criterion is applicable, although with some restrictions because following denervation τ increases in twitch fibres of extensor digitorum longus (e.d.l.) muscle (Albuquerque & McIsaac, 1970), and the same was expected to occur in e.o.m.s. Considering that in most twitch fibres of innervated e.o.m.s, τ is about 1–3 ms with an average of 2·4 ms (Table 1) and that

in e.d.l. muscle fibres τ increases about 3·4 times after 7–10 days of denervation (Albuquerque & McIsaac, 1970) it was decided as a first distinguishing criterion to consider as twitch fibres those with a $\tau <= 9$ ms and as tonic fibres those with $\tau >= 20$ ms. Fibres with τ between 10 and 20 ms were excluded because some overlap between the two fibre types most likely exists in that range. Two of the morphologically identified denervated tonic fibres had a τ as low as 10 and 13 ms and innervated twitch fibres occasionally had a τ of 4–6 ms, which after denervation might increase to about 12–18 ms.

A second criterion was the presence of a hump in the action potential of tonic fibres. In morphologically identified, denervated tonic fibres their wide and slow action potentials consistently had a slight oscillation or 'hump' following the early portion of their falling phase (arrows in Figs. 2*A*, 4*A*, *B* and *D*). This hump was also present in the s.p.p. of normally innervated tonic fibres (arrow in Fig. 1*A*) but it was never observed in the faster action potential of normal, denervated and reinnervated twitch fibres. Thus, fibre types were recognized by the value of τ and the presence or absence of a 'hump' in the action potential.

TABLE 1. Membrane properties of innervated and denervated fibres

	Innervated	*P*	Denervated*
		Tonic fibres	
E_{rp} (mV)	−53·6 ± 3·4 (20)	< 0·02	−44·1 ± 1·4 (50)
R_{eff} (MΩ)	5·96 ± 0·4 (18)	n.s.	6·07 ± 0·34 (39)
τ: mean (ms)	20·8 ± 1·9 (20)	< 0·005	28·7 ± 1·3 (50)
Range	10–80		20–60
		Twitch fibres	
E_{rp} (mV)	−62·4 ± 1·8 (34)	< 0·001	−48·8 ± 1·1 (57)
R_{eff} (MΩ)	1·49 ± 0·1 (33)	< 0·001	2·06 ± 0·11 (54)
τ: mean (ms)	2·4 ± 0·2 (34)	< 0·001	5·0 ± 0·3 (67)
Range	1–6		1–9

* The values presented for denervated fibres might be biased as a consequence of the selection process. The time constants of the tonic fibres may be slightly high and of the twitch fibres may be slightly low (see text).

Modification of electrical properties following denervation

It is well known that denervation has profound effects on the electrical properties of twitch fibres of limb muscles in mammals (for review see McArdle, 1983). In e.o.m.s the initial E_{rp} of both tonic and twitch fibres was significantly reduced following 6–9 days of denervation but the reduction in twitch fibres was more pronounced (Table 1). A similar change has been reported for rat twitch fibres (Albuquerque & Thesleff, 1968).

The R_{eff} of tonic fibres remained unchanged while that of twitch fibres increased by about one-third (Table 1). In twitch fibres of rat limb muscle, a similar effect on R_{eff} has been reported (Albuquerque & McIsaac, 1970). Despite these changes in R_{eff} the marked difference in the value of this parameter between normally innervated tonic and twitch fibres was still present after denervation (Table 1). The increase of

R_{eff} in twitch fibres corresponded with a significant increase in the value of τ. An increase in the τ value was also observed in tonic fibres (Table 1).

As mentioned above, following denervation the response of tonic fibres to depolarization was transformed from a s.p.p. into an action potential. This change in excitability involved an approximately 5-fold increase in the V_{max} of the responses. In addition, the V_{max} of the action potential of denervated tonic fibres could be increased by hyperpolarizing the holding potential of the fibre. In normally innervated fibres this was never observed.

Development of anodal break responses

Marshall & Ward (1974) have demonstrated that following denervation twitch fibres of rat e.d.l. muscle respond with action potentials to anodal break excitation, i.e. stimulation with a brief pulse of anodal (hyperpolarizing) current. This property is absent in virtually all normally innervated twitch fibres. Miledi *et al.* (1971) also found that denervated slow tonic fibres of frog could generate action potentials at the break of a strong hyperpolarizing pulse.

In normally innervated rat e.o.m.s, we found that twitch fibres were very resistant to anodal break stimulation, as expected. In many fibres graded stimulation with hyperpolarizing pulses to a membrane potential of -200 mV or, sometimes, even more negative levels failed to produce action potentials. In a few fibres several of such exaggerated stimuli were required to induce a response. Following denervation, twitch fibres responded to anodal break stimulation with action potentials. In twelve out of seventeen twitch fibres, action potentials were elicited from a mean hyperpolarization of about 50 mV, the remaining five requiring much larger pulses. No indication of membrane breakdown was seen (cf. Marshall & Ward, 1974).

In normally innervated tonic fibres hyperpolarizing pulses of up to -200 mV never elicited any response (Fig. 2*E*). In contrast, in muscles denervated 6–9 days, tonic fibres responded to anodal break stimulation usually with responses that were slow and graded with stimulation strength (Fig. 2*B*). The responses often appeared with a long delay of 50–150 ms following the end of the pulse. In four denervated muscles, in nine out of twelve tonic fibres tested the mean hyperpolarization required to obtain a response was about 50 mV. One fibre required a hyperpolarization of -100 mV, and two fibres gave no response at all. In no instance was membrane breakdown observed during the stimulation.

The records in Fig. 3 suggest that following denervation the onset of the capacity to produce anodal break responses is gradual and can precede the ability to produce action potentials by depolarization. For this fibre, and one other in another muscle, both denervated for 6 days, the response to depolarizing pulses still appeared normal, but a small and very slow graded response to hyperpolarizing pulses already had developed.

As reported for rat e.d.l. muscle (Marshall & Ward, 1974), in denervated e.o.m.s the threshold for firing anodal break responses in twitch and tonic fibres was always more negative than the threshold for action potentials evoked by depolarizing pulses. In several muscles denervated 6–9 days, the thresholds for twitch and tonic fibres for responses evoked by hyperpolarizing pulses were $-71{\cdot}3 \pm 2{\cdot}9$ mV (9) and

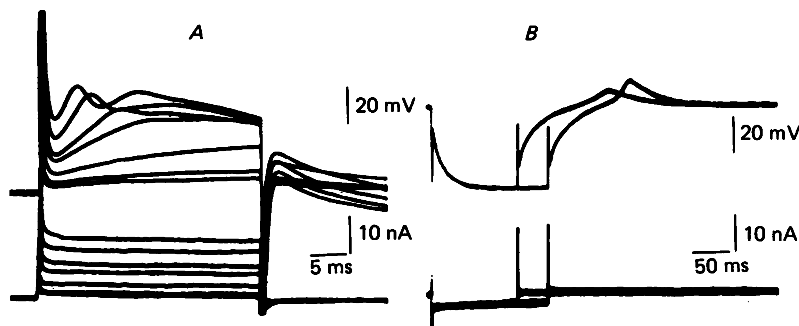

Fig. 3. A tonic fibre (τ = 30 ms) denervated for 6 days. When stimulated with depolarizing pulses (A) it had graded responses or s.p.p.s similar to those of innervated fibres (Fig. 1 A) but responded to anodal break stimulation (B) with transient depolarizations that were graded with current duration and that were only found in denervated fibres.

$-73 \cdot 0 \pm 1 \cdot 9$ mV (9) and for action potentials evoked by depolarizing pulses, they were $-58 \cdot 7 \pm 1 \cdot 2$ mV (15) and $-56 \cdot 4 \pm 1 \cdot 9$ mV (10). The holding potential was -80 mV in all instances.

The tonic fibre action potential is Na dependent and resistant to TTX and STX

Previously, we demonstrated that in normally innervated tonic fibres the s.p.p. evoked with pulses of moderate intensity (10–15 nA) is eliminated by removing Na from the external solution or by adding $1 \cdot 25 \times 10^{-6}$ or 10^{-5} M-TTX (Bondi & Chiarandini, 1979). When 6–9 day denervated e.o.m.s were exposed to saline containing 25 or 0 % Na, the V_{max} of both twitch fibre and tonic fibre action potentials and anodal break responses were reduced by about 50 % or abolished, respectively. The effect was observed within 6 min after reducing external Na; the responses returned to normal within 10 min after restoration of control saline.

In contrast to the results above, the addition of 10^{-6} M- and 10^{-5} M-TTX for 20 min to denervated e.o.m.s reduced the V_{max} of the tonic fibre action potentials only to, respectively, about 67 and 55 % of the responses of untreated fibres in the same muscles, demonstrating that the action potentials are rather resistant to TTX. Harris & Thesleff (1971) found that rat e.d.l. muscles denervated 6–7 days were more resistant to TTX than to STX blockade at the same concentrations. Similarly, we found that 10^{-6} M-STX reduced the tonic fibre response to approximately 50 % of control, suggesting that STX is about ten times more potent than TTX in its capacity to depress action potentials in denervated e.o.m.s. E.o.m.s appear to be somewhat more resistant to both toxins than rat e.d.l.

Reinnervation of muscle fibres

When frog pyriformis muscles were given time to reinnervate following denervation by nerve crush, action potentials still were observed in tonic fibres during the early period of reinnervation. As reinnervation progressed, the action potentials finally were suppressed and the responses to depolarization returned to normal (Schmidt & Stefani, 1977). Since rat e.o.m. tonic fibres have been shown to have many similarities

to frog slow tonic fibres, it followed that a similar phenomenon might occur in those fibres during the course of reinnervation. Several denervated inferior rectus muscles were allowed to reinnervate for two, three or four weeks. Reinnervation took place quite accurately along the pathway of the distal stump. Identification of fibre types in reinnervated muscles was no longer ambiguous because the overlap of τ values of the two fibre types disappeared. Fibres with fast action potentials and no 'hump' had a τ of $< = 11$ ms by two weeks reinnervation and of $< = 7$ ms by four weeks reinnervation.

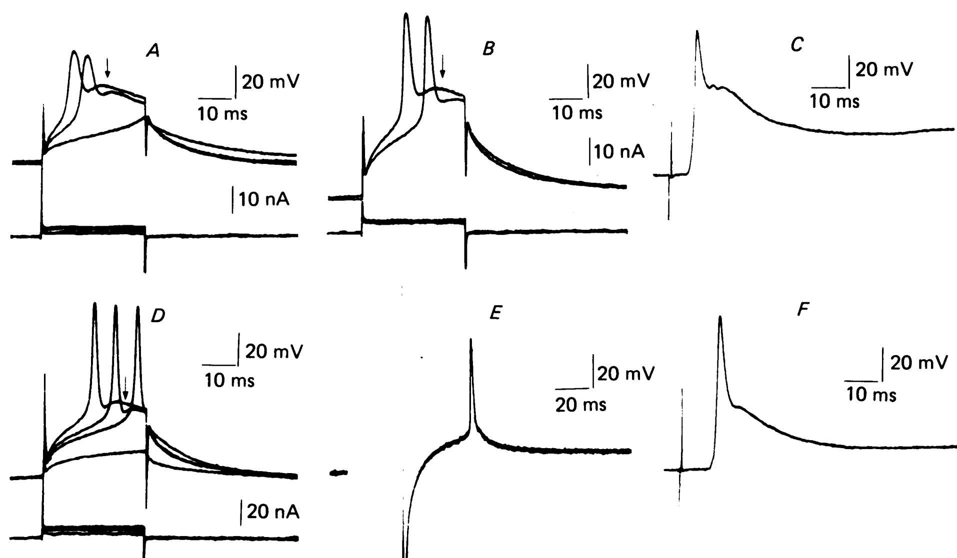

Fig. 4. Responses from two tonic fibres in the same muscle which had been allowed to reinnervate for three weeks. *A–C*, fibre with $\tau = 30$ ms. Increasing the holding potential from -80 mV (*A*) to -100 mV (*B*) augmented the V_{max} of the directly evoked action potentials from 18 and 24 V/s to 64 V/s. A hump (arrow) follows the early repolarization phase of the action potential. *C*, action potential evoked by nerve stimulation. *D–F*, fibre with a $\tau = 26$ ms; holding potential of -80 mV. *D*, action potential evoked by intracellular stimulation; V_{max} is 63 V/s. *E*, comparable action potential evoked by anodal break stimulation; V_{max} is 64 V/s. *F*, action potential evoked by nerve stimulation.

By two weeks reinnervation (i.e. two weeks after nerve section) the action potentials in tonic fibres were present and unchanged. The only apparent change from fibres denervated 6–9 days was that anodal break responses required a larger number of more negative hyperpolarizing pulses to be evoked. The responses remained graded. In several experiments responses to nerve stimulation were obtained. The nerve enters at the proximal end of the global surface, and with the progression of reinnervation larger groups of muscle fibres responded to nerve stimulation, beginning with those at the proximal end. In the muscles reinnervated for two weeks, scattered fascicles responded with twitches to nerve stimulation, but no m.e.p.p.s were recorded from any fibres, presumably because the recording electrode was placed in the distal third of the muscle, far from the region under reinnervation. Of fourteen twitch fibres impaled, two responded with action potentials to nerve stimulation while none of the five tonic fibres responded at all.

By three weeks reinnervation, about half of the twich fibres responded to nerve stimulation with normal action potentials. The tonic fibres, in contrast, showed a multiplicity of responses (Fig. 4). Of seventeen studied, in four the action potentials had disappeared and had been replaced by s.p.p. responses, thirteen still produced action potentials, twelve had anodal break responses (Fig. 4*E*) although most required many stimuli before an anodal break response was evoked, twelve had m.e.p.p.s, and

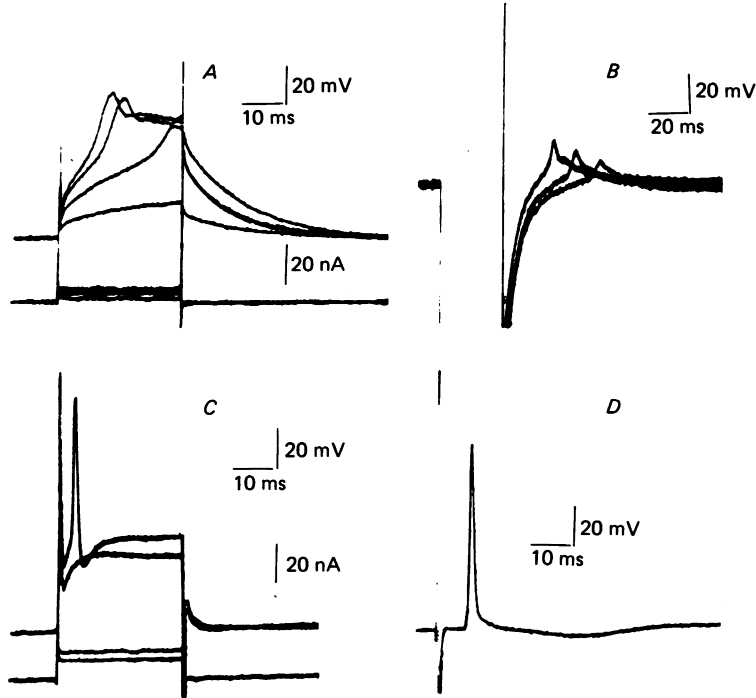

Fig. 5. Responses from a tonic and a twitch fibre in a muscle that had been allowed to reinnervate for four weeks. *A* and *B*, tonic fibre with τ = 18 ms and V_{max} = 7 V/s. *C* and *D*, twitch fibre with τ = 6 ms. *A* and *C*, intracellular cathodal stimulation evokes, in the tonic fibre, a response that is virtually a s.p.p. and, in the twitch fibre, an action potential. *B*, the anodal break response of this tonic fibre is slower and smaller than those seen at three weeks after denervation (Fig. 4*E*). Two tonic fibres out of seven in this muscle no longer responded at all to anodal break stimulation. *D*, response to nerve stimulation.

seven responded to nerve stimulation (Fig. 4*C* and *F*). There was no consistent pattern of correlation among any of the responses. In most fibres reinnervated for three weeks, the V_{max} of the action potential could still be increased by hyperpolarizing the cell (Fig. 4*A* and *B*), a property acquired within a week following denervation.

By four weeks the s.p.p. response had returned in six of the seven fibres recorded (Fig. 5*A*), and one fibre still produced an action potential which could be increased by hyperpolarization. All seven fibres had m.e.p.p.s and five still produced very small graded anodal break responses to maximal stimulation (Fig. 5*B*). In Fig. 5*C* and *D* are shown action potentials evoked in a twitch fibre in the same muscle by depolarization and nerve stimulation, respectively. Very large hyperpolarizing pulses failed to produce an anodal break response in this cell.

These reinnervation experiments were carried out primarily to determine whether the appearance of the action potential mechanism in denervated rat tonic fibres was reversible, which was confirmed. Unfortunately, they do not clarify another interesting aspect of reinnervation: whether or not it is specific. Motor nerves to muscles with a mixed fibre population, such as some amphibian muscles and e.o.m.s, contain fast and slow conducting axons which specifically innervate twitch and tonic fibres (Kuffler & Vaughan Williams, 1953; Chiarandini & Stefani, 1979). The question arises as to whether during reinnervation the two fibre types are reinnervated by the corresponding type of motor axons. In control muscles, latencies between nerve stimulation artifact and muscle responses were, for twitch fibres, between 5 and 8 ms, with a mean of $6{\cdot}7 \pm 0{\cdot}5$ ms (6), and for tonic fibres, two had latencies of 8 ms while seven other fibres had latencies of between 13 and 35 ms, with a mean of $21{\cdot}3 \pm 3{\cdot}3$ ms (9). In muscles reinnervated for three weeks, latencies for twitch fibres were between 4 and 9 ms (5) and for tonic fibres were 4, 6·5, 8·5, 16, 18 and 20 ms.

DISCUSSION

Following denervation, tonic fibres of rat e.o.m.s develop the capacity for generating action potentials. The appearance of this voltage-dependent Na conductance indicates that in these fibres, as in mammalian twitch fibres, the central nervous system controls the membrane ionic permeability (for review see McArdle, 1983). A similar phenomenon has been observed in frog slow tonic fibres (Miledi *et al.* 1971). The action potential which is Na dependent is partially resistant to blockade by TTX and STX. A similar resistance has been observed in rat twitch muscles after denervation (Harris & Thesleff, 1971; Redfern & Thesleff, 1971*b*). The action potentials of denervated tonic fibres of e.o.m.s, similarly to those of denervated frog slow tonic and rat twitch fibres, have slower rates of rise and fall than the action potentials in innervated twitch fibres. It has been reported that Na channels in denervated rat twitch fibres have slower kinetics than in normal fibres (Pappone, 1980). This and a low density of Na channels could account for the slow time course of the action potential in tonic fibres of e.o.m.s.

In rat twitch fibres it has been reported that modifications of the Na conductance that appear after denervation are inhibited by blocking protein synthesis with actinomycin D (Marshall & Ward, 1974) and that following one week denervation about 25% of the normal Na channels are replaced by new ones with different properties (Hansen Bay & Strichartz, 1980), suggesting that *de novo* synthesis of Na channels might also be involved in the appearance of action potentials in denervated rat tonic fibres.

The Na channels that appear on tonic fibres after denervation have a voltage-dependence similar to those of the Na channels that appear in denervated rat twitch fibres. Pappone (1980) found in 5–7-day-denervated rat e.d.l. muscles that the TTX-insensitive Na channels were activated and inactivated at membrane potentials about 10 mV more negative than the Na channels from normally innervated muscles, and that in contrast to normal muscle, continued hyperpolarization failed to saturate the activation mechanism. A similar lack of saturation of the activation mechanism exists in e.o.m. tonic fibres following denervation and during the first weeks of

reinnervation these fibres produce action potentials with greater V_{max} with increasing conditioning hyperpolarizations (Fig. 4*A* and *B*). This was not seen in innervated fibres. Furthermore, similar to rat denervated e.d.l. twitch fibres (Marshall & Ward, 1974), tonic fibres acquired the ability to fire action potentials with anodal break stimulation, with a threshold more negative than that for action potentials evoked by depolarization. The difference in thresholds most likely is related to the degree of inactivation of the Na channels at the two potentials. The widely variable long latency (50–150 ms) between the end of a hyperpolarizing pulse and the anodal break response in tonic fibres (Fig. 3*B*) suggests that after denervation Na channels may not be evenly distributed in the membrane and sometimes may be located at a considerable distance from the site of stimulation and recording. Evidence for an uneven density of Na channels in normal amphibian twitch fibres has been presented by Almers, Stanfield & Stuhmer (1983).

The mechanism underlying the reduction of the E_{rp} of tonic fibres that follows denervation is unknown but it might involve changes in the resting permeabilities to Na or K, as is the case for twitch fibres (Albuquerque & Thesleff, 1968; Albuquerque & McIsaac, 1970; for review see McArdle, 1983). Apparently denervation did not change R_{eff} but it increased τ significantly. These findings, however, are difficult to interpret because among the denervated tonic fibres only those with a $\tau >= 20$ ms were included (Table 1) while among innervated tonic fibres there were cells with τ as low as 10 ms (Table 1). Consequently, our results may have been biased and skewed towards higher values.

The sequence of changes in electrical properties of rat tonic fibres during reinnervation parallels that observed in frog (Miledi *et al.* 1971). At two weeks after denervation, innervation was again present as demonstrated by the twitch contraction of fascicles of fibres evoked with nerve stimulation in the proximal portion of the muscle. In tonic fibres, however, no m.e.p.p.s or e.p.p.s were recorded, although a partial restoration of their electrical properties was evident because they were less sensitive to anodal break stimulation. E.p.p.s might have been absent because of failure of stimulation. Other possibilities, however, may explain the absence of synaptic potentials. Firstly, the fibres might have been reinnervated only in their proximal region making impossible the recording of synaptic activity from the distal third of the muscle, where the micro-electrode was routinely placed. Secondly, the fibres might have been recently reinnervated and their nerve terminals were not yet producing m.e.p.p.s (Carmignoto, Finesso, Siliprandi & Gorio, 1983). Finally, the fibres might not have been reinnervated but the presence of nerves in surrounding fibres somehow influenced the properties of the fibres.

By three weeks after denervation, it appeared that some tonic fibres had been reinnervated by nerves which had stimulus–response latencies in the range of fast conducting axons (latencies of 4 and 6·5 ms) and that others appeared to be reinnervated by slow conducting nerves (latencies of 16, 18 and 20 ms). However, it is not clear from this study what type of innervation ultimately was associated with the suppression of the action potential observed four weeks after denervation.

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