J. Physiol. (1979), **297**, *pp.* 479–491
With 4 text-figures
Printed in Great Britain

THE TROPHIC INFLUENCE OF TETRODOTOXIN-INACTIVE NERVES ON NORMAL AND REINNERVATED RAT SKELETAL MUSCLES

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(*Received 31 January 1979*)

SUMMARY

1. Nerve impulses in the rat sciatic nerve were blocked for long periods by tetrodotoxin (TTX) released from capillary implants. The TTX capillaries did not block axonal transport, nor did they cause any sign of nerve degeneration.

2. A comparison of the effects of TTX paralysis and denervation was made on both extensor digitorum longus (e.d.l.) and soleus muscles over 21 days, a time when the products of nerve degeneration were unlikely to contribute to the changes associated with denervation. The resting membrane potential of TTX-paralysed muscles was significantly different ($P < 0.005$) from that of the denervated muscles at all periods and at 21 days the decrease that can be attributed to inactivity was 61 % (e.d.l.) and 49 % (soleus) of that which follows denervation. This disparity was even more pronounced for the ACh receptor density where the increase in receptors due to inactivity was only 34 % (e.d.l.) and 21 % (soleus) of that due to denervation.

3. A similar comparison was made on muscles which had been reinnervated by TTX-inactive nerves. These muscles were found to have a significantly higher resting membrane potential and lower ACh receptor density than the denervated muscles ($P < 0.05$).

4. The experiments on reinnervated muscles preclude the possibility that nerve degeneration products are solely responsible for the difference between the TTX-paralysed and denervated muscles and suggest that the difference can be attributed to the trophic influence of the nerve.

5. An observed increase in the m.e.p.p. frequency of the TTX-paralysed muscles indicated that nerve action potentials play a role in regulating the spontaneous release from nerve terminals.

INTRODUCTION

The trophic influence of nerves on skeletal muscle is generally considered to be a long-term action not mediated by nerve impulses (see reviews, Harris, 1974; Gutmann, 1976). However, the arguments presented in support of a neurotrophic influence on muscle properties, such as the resting membrane potential and extrajunctional acetylcholine (ACh) sensitivity, have not been universally accepted. For example, it has been argued that because the onset of the changes in these properties that follow denervation is dependent on nerve-stump length (Luco &

0022-3751/79/5980-0007 $01.50

Ezyaguirre, 1955; Albuquerque, Schuh & Kauffman, 1971), the muscle is subject to regulation by substances supplied by axonal transport. However, this interpretation has been criticized (Lømo & Westgaard, 1976) because the onset of nerve terminal degeneration is also dependent on nerve-stump length (Miledi & Slater, 1970) and it is equally plausible that the products of nerve degeneration might influence muscle membrane properties (Vrbová, 1967; Jones & Vrbová, 1974; Lømo & Westgaard, 1976; Cangiano & Lutzemberger, 1977; Brown, Holland & Ironton, 1978). Other studies in support of trophic regulation have shown that blockade of axonal transport by colchicine precipitates changes similar to denervation (Albuquerque, Warnick, Tasse & Sansone, 1972; Fernandez & Inestrosa, 1976). These studies are again controversial because colchicine has a direct effect on extrajunctional ACh sensitivity in muscle (Lømo, 1974; Cangiano & Fried, 1977).

There is little doubt that nerve-impulse-related activity plays an important role in the maintenance of the intrinsic properties of skeletal muscle, as direct stimulation can reverse many of the changes associated with denervation (Lømo & Rosenthal, 1972; Lømo & Westgaard, 1975). In fact, muscle activity *per se* is so important that the very existence of trophic regulation of extrajunctional ACh sensitivity has been questioned (Lømo & Westgaard, 1976). However, in experiments in which nerve impulses were blocked by tetrodotoxin (TTX), it was demonstrated that denervation caused a larger increase in extrajunctional ACh receptors than did TTX-induced paralysis (Lavoie, Collier & Tenenhouse, 1976; Pestronk, Drachman & Griffin, 1976). Unfortunately, the duration of these experiments of 5–7 days coincided with the period during which degenerating nerve products were likely to contribute to the increase in ACh sensitivity associated with denervation (Lømo & Westgaard, 1976; Cangiano & Lutzemberger, 1977; Brown *et al.* 1978).

In this paper we report on the use of a simple method for maintaining TTX inactivity in the rat sciatic nerve for periods of up to 21 days, when the process of nerve degeneration is probably complete (Lømo & Westgaard, 1976). This technique was used to examine the effects of inactivity on the resting membrane potential and extrajunctional ACh receptors of both normal and reinnervated extensor digitorum longus (e.d.l.) and soleus muscles. Preliminary reports of this work have been published (Mills, Bray & Hubbard, 1978*a*, *b*).

METHODS

Preparation. Male and female albino rats of the Wistar strain, weighing 180–250 g, were usod for all experiments. All measurements were made on the extensor digitorum longus and soleus muscles. In terminal experiments animals were maintained under ether anaesthesia while the treated muscles and then the contralateral control muscles were removed for recording. Measurements were made on control muscles, muscles paralysed by TTX-induced nerve inactivity, denervated muscles and muscles reinnervated after a close nerve crush.

TTX inactivity. Paralysis of the lower hind limbs was induced and maintained by implanting a glass capillary containing 8–10 μg TTX (Sigma Chemical Co.) in the sciatic nerve. The 7–8 mm long capillaries were made from 0·75 mm o.d. glass tubing (Drummond 2 μl. Microcaps) by heating one end in a coil element of a De Fonbrune Microforge until the end was restricted to a pore 25–27 μm in diameter. The capillaries were filled with the TTX solution (25 mg/ml. citrate buffer) and the other end plugged with plasticine. The rats were anaesthetized with ether and the capillaries implanted under the epineurium of the sciatic nerve in the mid-thigh region. The capillaries were oriented along the longitudinal axis of the nerve with the pore end closer

to the spinal cord. Paralysis of the limb was tested by lifting the rats by the tail and observing the toe-spreading reflex. After implanting a TTX capillary, paralysis was evident on recovery from the anaesthetic and lasted for 6–9 days. Animals were tested twice daily, and any that showed signs of recovery were discarded. When TTX paralysis was to be maintained for prolonged periods the capillaries were replaced on the sixth day and every 6 days thereafter. During the replacement and prior to recording the resting membrane potential, the sciatic nerve was stimulated electrically above the capillary at a strength five times greater than needed to produce movement when applied below the capillary. Any animals that displayed contractile activity in the lower limbs were discarded. In some experiments control capillaries containing citrate buffer (125 mg/ml.) were implanted in the contralateral leg.

Denervation. The e.d.l. muscle was denervated by cutting the peroneal nerve as it descended below the peroneal muscles just below the knee. The soleus was denervated by cutting the nerve 1–2 mm from the muscle. When cutting the nerve care was taken not to damage the muscles, and in fact it was not necessary to expose the e.d.l. when cutting the peroneal nerve. To prevent reinnervation a 5 mm segment was cut from the sciatic nerve in the mid-thigh region and for long periods of deneration the nerve was cut again at 15 days.

Reinnervation. To promote rapid reinnervation the nerve to the e.d.l. and soleus were crushed with forceps, the peroneal just below the knee and the nerve to the soleus 1–2 mm from the muscle. After crushing, the nerves were allowed to reinnervate muscles in the normal way or while exposed to TTX as described above. These animals were also tested by observing the toe-spreading reflex and by direct stimulation of the sciatic nerve.

Resting membrane potential. Muscles were removed and placed in Liley solution (Liley, 1956) which was equilibrated with 95 % O_2 – 5 % CO_2 and maintained at room temperature. The muscles were pinned in a 4 ml. Silastic trough (Sylgard 184, Dow Corning) through which the solution flowed at 3–4 ml./min. The resting membrane potential and miniature end-plate potentials (m.e.p.p.s) were recorded using conventional micro-electrode techniques. Fibre-filled micro-electrodes, filled with 3 M-KCl and having resistances of 25–35 MΩ, were inserted into muscle fibres. The penetrations were made in a track adjacent to intramuscular nerve branches within 3 mm of the point of insertion of the nerve into the muscle. Permanent records of the resting membrane potential and m.e.p.p.s were obtained on a rectilinear pen recorder and film respectively.

ACh receptors. Immediately after recording the r.m.p. the muscles were frozen and stored at −20 °C. Later the muscles were assayed for ACh receptors by measuring the binding of ^{125}I-labelled α-neurotoxin to membrane preparations. The α-neurotoxin was prepared from the venom of *Naja naja atra* (Sigma Chemical Co.) according to the method of Lee, Chang, Chin, Chin, Treng & Lee (1968). The toxin was labelled with ^{125}I by the method of Greenwood, Hunter & Glover (1963) using a lower concentration of chloramine T (20 mM) to a specific activity of 250–350 c/m-mole. To ensure that any comparison between the denervated and TTX-paralysed muscles was not affected by differences in batches of ^{125}I-labelled α-neurotoxin, they were assayed together.

The muscles were homogenized using a Potter-Elvehjem homogenizer with a Teflon pestle in 25 volumes ice-cold 100 mM-NaCl, 5 mM-EDTA, 50 mM-Tris buffer, pH 7·2. Samples (300 μl.) were incubated with 1·0 nM-[^{125}I]α-neurotoxin in a final volume of 400 μl. of buffer containing 0·1 % bovine serum albumin, for 2 hr at 25 °C. The toxin bound to muscle membranes was separated from free toxin by adding 10 ml. ice-cold BSA buffer and centrifuging at 50,000 *g* for 15 min. The washing and centrifugation step was repeated. The pellet was resuspended in 1·5 ml. bovine serum albumin buffer and filtered through a Whatman GF/C glass fibre paper. The filter paper was washed with 10 ml. bovine serum albumin buffer and counted in a gamma scintillation spectrometer. Preliminary studies of the assay conditions with 15-day denervated muscles showed that toxin binding reached saturation at 2 hr, was linear within the protein range studied and was inhibited 89 % by 10^{-3} M and 96 % by 10^{-2} M-carbachol.

The estimation of protein content was performed on the original muscle homogenate by the method of Lowry, Rosebrough, Farr & Randall (1951).

Axonal transport. Studies were made on animals with TTX capillaries implanted in the right sciatic nerve for 7–15 days. The motoneurones which supply the sciatic nerve were labelled by injection of radioactive amino acid as described by Lasek (1968). Under ether anaesthesia the spinal cord was exposed by laminectomy of the L_1 vertebra and L-[5-^{3}H]proline (specific activity

24 c/m-mole) was injected into the ventral horn with a micropipette fashioned from glass capillary tubing (Drummond 1 μl. Microcaps). A series of four pairs of 0·1 μl. injections were made symmetrically into both sides of the cord at 1 mm intervals. A total of 20 μc of [^{3}H]proline in a volume of 0·8 μl. 0·9 % saline was injected per animal. In some experiments, just prior to injecting the isotope the left sciatic nerve was ligated with a silk thread in the mid-thigh region. The wound was sutured and the animal was allowed to recover. At periods of 3–24 hr after injection the animals were sacrificed and the sciatic nerves were removed beginning at the level where the L_5 ventral root leaves the vertebral column. The nerves were cut into 2·5 mm segments and each segment was placed in 0·8 ml. 0·5 M-NaOH for 48 hr. Each sample was sonicated, neutralized with 2 M-HCl and 10 ml. of scintillation fluid (30 % Triton X-100; 0·3 % 2,5-diphenyloxazole; 0·01 % 1,4-bis-(2-(5 phenyloxazolyl))-benzene in toluene) was added. The samples were counted in a Packard Tri-carb liquid scintillation spectrometer at an efficiency of 33–35 %.

Histology. Segments of the sciatic nerve (proximal, adjacent and distal to the capillary), peroneal, tibial and sural nerves were fixed in 2 % glutaraldehyde and then 1 % osmium. Transverse sections and teased fibres were examined by light microscopy.

Contractile studies. The peak isometric tensions of unoperated control and reinnervated e.d.l. and soleus muscles were recorded *in vivo*. The knee joint of each limb was rigidly clamped and the distal tendon attached to a Grass FTO3 tension transducer. The sciatic nerve in the mid-thigh region was stimulated by 0·2 msec duration pulses at a voltage five times that required to give the peak tension. A micrometer was used to adjust the length of each muscle to its peak twitch tension. The peak tetanic tensions of the e.d.l. and soleus muscles were recorded in response to 1 sec bursts of stimuli at 125 and 60 Hz respectively. To assess the contribution of other muscles to the tension record, the sciatic was stimulated after the nerves to the e.d.l. and soleus had been cut. On no occasion did movement of the lower limb contribute more than 2 % to the peak tetanic tension of control muscles.

RESULTS

Resting membrane potentials. All recordings of the resting membrane potential were made from regions close to the end-plate. In control and TTX-paralysed muscles the resting membrane potentials were only accepted if m.e.p.p.s could be recorded and in denervated preparations penetrations were made close to the remains of nerve branches. TTX-paralysed and denervated muscles exhibited strong fibrillations which declined during the initial 10–15 min in the recording bath. Fibrillations were evident in both TTX-paralysed and denervated muscles after 5 days.

Denervation of both e.d.l. and soleus muscles produced a dramatic fall in r.m.p. As shown in Fig. 1 by 3 days the resting membrane potential of the e.d.l. had fallen from 79·8 $\pm$ 0·6 to 62·2 $\pm$ 0·9 mV and that of the soleus from 76·1 $\pm$ 0·7 to 64·0 $\pm$ 0·6 mV. After a slight rise in potential at 10 days the resting membrane potentials of the denervated muscles appear to have stabilized at 61–63 mV. In contrast to the abrupt fall in the resting membrane potential of the denervated muscles, those of the TTX-paralysed muscles fell gradually over a period of 15 days and then stabilized, and were significantly different from denervated muscles (Student's t test, $P < 0{\cdot}005$) at all time periods. At 21 days the TTX-paralysed e.d.l. and soleus muscles had resting membrane potentials of 69·5 $\pm$ 1·5 and 69·0 $\pm$ 0·8 mV respectively; in contrast to the denervated e.d.l. and soleus which were 62·8 $\pm$ 1·2 and 61·6 $\pm$ 1·0 mV respectively. Thus at 21 days the fall in resting membrane potential that can be attributed to inactivity was 61 % (e.d.l.) and 49 % (soleus) of that which follows denervation.

Control capillaries containing citrate buffer implanted for 3, 10 and 21 days had no significant effect on the resting membrane potential (Fig. 1).

ACh receptors. As shown in Fig. 2, denervation caused a marked increase in the

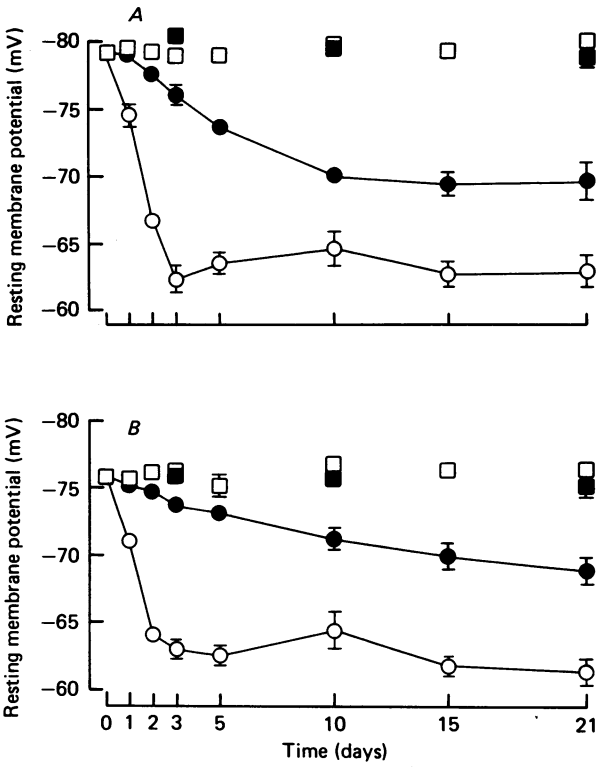

Fig. 1. Effect of TTX paralysis and denervation on the resting membrane potential of the e.d.l. (*A*) and soleus (*B*) muscles. The open squares indicate results from unoperated control muscles, the filled squares the results from muscles in which a control capillary was implanted in the sciatic nerve. Open circles indicate the results from denervated muscles, filled circles the results from TTX-paralysed muscles. Each symbol indicates the mean resting membrane potential of six to eleven muscles; a minimum of fifteen impalements were made in each muscle. Each bar indicates the S.E. of the mean; in some cases the bars are obscured by the symbols. The difference between the TTX-paralysed and denervated muscles was significant at all times (Student's t test, $P < 0{\cdot}005$).

binding of ^{125}I-labelled α-neurotoxin to muscle membranes. Presumably this represented an increase in extrajunctional receptors as has been previously demonstrated by Hartzell & Fambrough (1972). The ACh receptor density of TTX-paralysed muscles reached a maximum at 10 days and was significantly less (Student's t test, $P < 0{\cdot}001$, except 10-day e.d.l. values $P < 0{\cdot}02$) than that of denervated muscles at all time periods examined (Fig. 2). Control capillaries containing citrate buffer only had no effect. At 21 days the mean binding of α-neurotoxin in the denervated e.d.l. and soleus muscles was $109{\cdot}2 \pm 14{\cdot}1$ and $143{\cdot}7 \pm 14{\cdot}1$ f-mole/mg protein respectively compared to $42{\cdot}9 \pm 9{\cdot}3$ and $35{\cdot}0 \pm 6{\cdot}4$ f-mole/mg protein respectively in the TTX-paralysed muscles. When the initial level of binding of the e.d.l. ($8{\cdot}8 \pm 0{\cdot}5$ f-mole/mg protein) and soleus, ($6{\cdot}9 \pm 0{\cdot}5$ f-mole/mg protein) is subtracted from these values, then the increase in ACh receptors due to inactivity was only 34 % and 21 % respectively of that due to denervation. This comparison is made on the basis of muscle

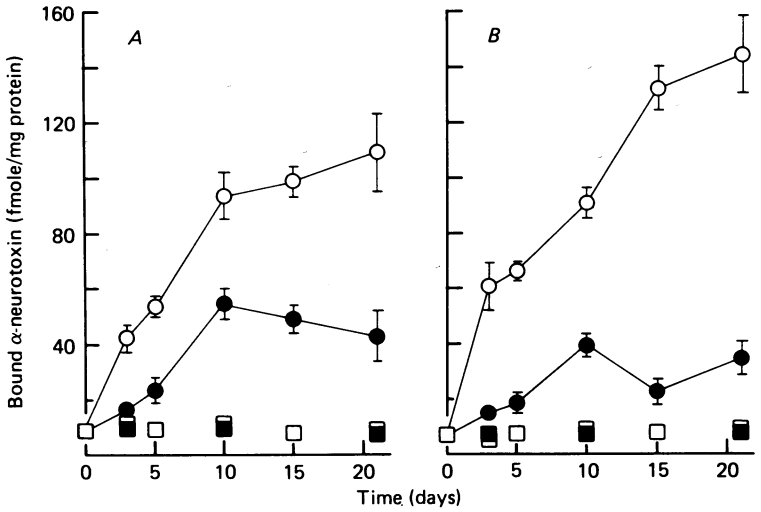

Fig. 2. Effect of TTX paralysis and denervation on the ACh receptor density of e.d.l. (*A*) and soleus (*B*) muscles. Symbols are as in Fig. 1. Each symbol indicates the mean binding of ^{125}I-labelled α-neurotoxin (f-mole/mg protein) of six to eleven muscles. Each bar indicates the S.E. of the mean. The difference between TTX-paralysed and denervated muscles was significant (Student's *t* test) at the $P < 0{\cdot}001$ level in all cases, except the 10-day e.d.l. values, $P < 0{\cdot}02$.

protein although the ratio is not substantially altered if α-neurotoxin binding is calculated as f-mole per muscle, because the protein content of TTX-paralysed and denervated muscles were similar (Fig. 3).

Muscle protein content. As shown in Fig. 3, apart from an initial hypertrophy in the denervated e.d.l., the protein contents of both denervated and TTX-paralysed

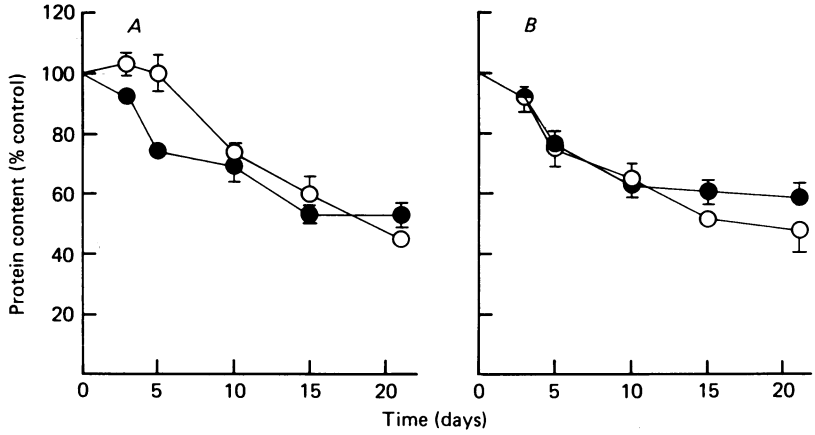

Fig. 3. Effect of TTX paralysis and denervation on the protein content of the e.d.l. (*A*) and soleus (*B*) muscles. Open circles indicate the results from denervated muscles; filled circles the results from TTX-paralysed muscles. Each symbol indicates the mean protein content of six to eleven muscles calculated as a percentage of the contralateral control. Each bar indicates the S.E. of the mean.

muscles fell in parallel. At 21 days the protein content of denervated and TTX-paralysed muscles was less than 60 % of the unoperated controls and there was no significant difference between the two treatments.

Recovery from TTX paralysis. The recovery of the resting membrane potential and ACh receptor density was assessed after removal of TTX capillaries. In each experiment the paralysis was maintained for 10 days. The capillary was then removed and the animal allowed to regain use of its limb. One hour after removal of the capillary the toe-spreading reflex began to recover and 24 hr later there was no visible sign of paralysis. As Table 1 shows, the resting membrane potential and ACh receptor density were restored to normal by 6 days after removal of the capillary. There was no sign of fibrillatory activity in the recovering muscles.

TABLE 1. Recovery of resting membrane potential and ACh receptor density after 10 days TTX paralysis

		Resting membrane potential (mV)		ACh receptors (f-mole α-neurotoxin bound/mg protein)	
Preparation	*n*	e.d.l.	Soleus	e.d.l.	Soleus
Control	6	80·1 ± 0·4	75·3 ± 0·5	9·2 ± 0·6	6·9 ± 1·0
10 days TTX paralysis	11	69·9 ± 0·7	71·4 ± 0·6	54·3 ± 5·4	39·6 ± 3·9
2 days recovery	3	71·2 ± 0·5	70·2 ± 0·6	26·4 ± 2·4	16·1 ± 3·9
4 days recovery	3	73·5 ± 0·5	72·9 ± 0·4	19·2 ± 5·2	9·8 ± 1·4
6 days recovery	3	79·3 ± 0·8	73·9 ± 0·7	9·6 ± 0·8	8·1 ± 0·8

TTX paralysis was maintained for 10 days before the TTX capillaries were removed. At 2, 4 and 6 days after removal of the capillaries the resting membrane potential and ACh receptor density were determined. Unoperated control muscles were taken from the contralateral limb. Each value is the mean ± S.E. of the number of muscles indicated by *n* and the resting membrane potential of each muscle was the mean of at least fifteen to twenty penetrations.

Reinnervated muscles. Contractile studies were carried out to determine the time course of reinnervation following nerve crush. At various intervals the isometric tetanic tensions were recorded from reinnervated muscles and from muscles of the unoperated contralateral limbs. At 6, 8, 14, 16 and 18 days after crushing, the peak tensions (mean of two rats) of the soleus muscles were 26·6, 42·0, 82·2, 86·5 and 96·5 % of the control respectively, while those of the e.d.l. were 0, 7·8, 63·2, 87·5 and 109·8 % respectively. Thus both muscles were completely reinnervated by 18 days following nerve crush.

In another series of experiments the resting membrane potential and ACh receptor density of reinnervated muscles, muscles reinnervated by TTX-inactive nerves (TTX-reinnervated), and denervated muscles were compared (Table 2). The resting membrane potential of TTX-reinnervated muscles, measured close to the point of insertion of the nerve, was significantly different (Student's *t* test, $P < 0{\cdot}05$) from that of the denervated muscles, both e.d.l. and soleus, at 10, 15 and 21 days and had regained the levels found in TTX-paralysed muscles (Fig. 1). The ACh receptor density of whole muscles was measured at 15 and 21 days and TTX-reinnervated

muscles were found to be lower than denervated muscles (Table 2). The TTX-reinnervated muscles had not regained the levels found in TTX-paralysed muscles (Fig. 1) although reinnervated muscles had returned to their normal values by 21 days (Table 2). However, when the values for 15 and 21 days were combined, the TTX-reinnervated muscles (e.d.l., $76{\cdot}1 \pm 9{\cdot}1$; $n = 11$ and soleus, $101{\cdot}4 \pm 10{\cdot}6$; $n = 11$) were significantly different (Student's t test, $P < 0{\cdot}05$) from the denervated muscles (e.d.l., $122{\cdot}0 \pm 9{\cdot}4$; $n = 12$ and soleus, $133{\cdot}8 \pm 9{\cdot}5$; $n = 12$).

TABLE 2. Influence of TTX-inactive nerves on the resting membrane potential and ACh receptor density of reinnervated muscles

		Resting membrane potential (mV)		ACh receptors (f-mole α-neurotoxin bound/mg protein	
Preparation	n	e.d.l.	Soleus	e.d.l.	Soleus
Control	10	$79{\cdot}7 \pm 0{\cdot}5$	$76{\cdot}4 \pm 0{\cdot}5$	$8{\cdot}9 \pm 0{\cdot}5$	$6{\cdot}9 \pm 0{\cdot}6$
Reinnervated					
10 days	3	$73{\cdot}1 \pm 1{\cdot}8$	$72{\cdot}8 \pm 1{\cdot}4$	—	—
15 days	4	$79{\cdot}3 \pm 0{\cdot}8$	$77{\cdot}1 \pm 0{\cdot}4$	$15{\cdot}7 \pm 0{\cdot}9$	$6{\cdot}4 \pm 0{\cdot}6$
21 days	4	$79{\cdot}8 \pm 0{\cdot}6$	$76{\cdot}8 \pm 0{\cdot}3$	$11{\cdot}4 \pm 0{\cdot}5$	$6{\cdot}6 \pm 0{\cdot}8$
TTX-reinnervated					
10 days	5	$69{\cdot}0 \pm 1{\cdot}6$	$69{\cdot}7 \pm 1{\cdot}2$	—	—
15 days	6	$70{\cdot}3 \pm 1{\cdot}8$	$68{\cdot}7 \pm 1{\cdot}5$	$72{\cdot}1 \pm 9{\cdot}1$	$92{\cdot}1 \pm 12{\cdot}0$
21 days	5	$72{\cdot}9 \pm 1{\cdot}7$	$68{\cdot}9 \pm 1{\cdot}6$	$80{\cdot}9 \pm 17{\cdot}8$	$112{\cdot}4 \pm 18{\cdot}6$
Denervated					
10 days	5	$63{\cdot}4 \pm 0{\cdot}9$	$63{\cdot}6 \pm 1{\cdot}1$	—	—
15 days	7	$64{\cdot}3 \pm 1{\cdot}6$	$62{\cdot}9 \pm 0{\cdot}5$	$119{\cdot}5 \pm 13{\cdot}9$	$127{\cdot}8 \pm 12{\cdot}2$
21 days	5	$63{\cdot}8 \pm 1{\cdot}4$	$64{\cdot}1 \pm 1{\cdot}3$	$115{\cdot}9 \pm 11{\cdot}9$	$144{\cdot}3 \pm 16{\cdot}0$

Each value is the mean ± S.E. of the number of muscles indicated by n and the resting membrane potential of each muscle was the mean of at least fifteen to twenty penetrations. Unoperated control muscles were taken from the contralateral limb.

Axonal transport. The pattern of movement of labelled material along the sciatic nerve was similar to that described by Griffin, Price, Drackman & Engel (1976). TTX capillaries implanted into the nerve had little effect on axonal transport (Fig. 4), although occasionally they caused a small localized accumulation of radioactivity. However, the extent of this accumulation was minor when compared to the amount that occurred proximal to a ligation (Fig. 4). When it did occur, the small accumulation of radioactivity in the vicinity of the TTX capillary was found along the whole length of the capillary and not just at the point of TTX release. The most likely explanation for this minor accumulation is the oedema and ingrowth of connective tissue associated with the capillary implants.

Histology. Segments of the sciatic nerve (proximal, adjacent and distal to the capillary), peroneal, tibial and sural nerves were evaluated histologically at 7, 10 and 15 days after implantation of TTX capillaries. Transverse sections of osmium-fixed nerve showed a normal myelinated fibre density compared with control nerve

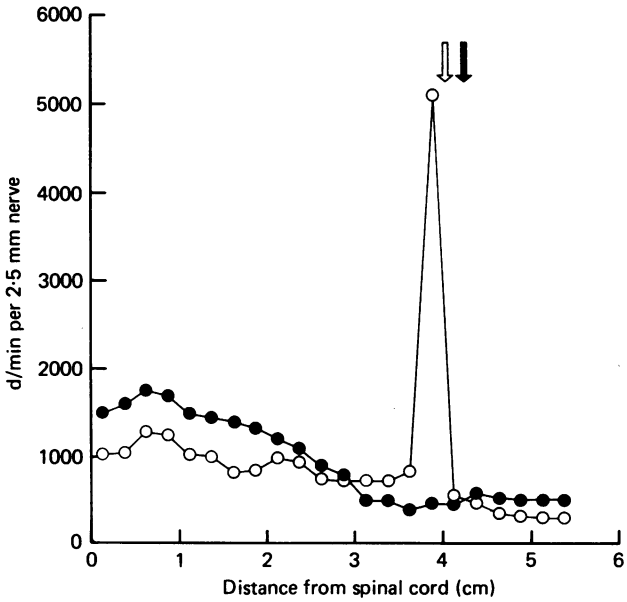

Fig. 4. Effect of TTX capillaries on axonal transport. A typical example is shown in which a TTX capillary was implanted for 7 days in the sciatic nerve (closed circles) and the animal was killed 18 hr after the injection of [^{3}H]proline as described in the text. The contralateral sciatic nerve (open circles) did not contain a capillary but was ligated just prior to the time of injection. The position of the tip of the TTX capillary is indicated by the closed arrow and the ligature by the open arrow. Each value indicates the disintegrations per min (d/min) of a 2·5 mm segment of nerve.

with no evidence of axonal degeneration. Teased-fibre analysis distal to the capillary, likewise, revealed no abnormality.

Presynaptic effect of inactivity. While measuring the resting membrane potential of the TTX-paralysed muscles it became obvious that in these muscles the m.e.p.p. frequencies were higher than those seen in unoperated controls. Since the distribution of m.e.p.p. frequencies of both e.d.l. and soleus are wide and skewed (McArdle & Albuquerque, 1973) the geometric means ($\pm$ S.E.), rather than the arithmetic means, were determined after 10 and 21 days paralysis. The m.e.p.p. frequency of 6–8 fibres was measured in each muscle (n = no. of muscles). The mean frequencies for the TTX-paralysed e.d.l. muscles were $6{\cdot}2 \pm 0{\cdot}5$ sec^{-1} ($n = 5$) and $5{\cdot}7 \pm 0{\cdot}5$ sec^{-1} ($n = 7$) at 10 and 21 days respectively, while in the control muscles it was $3{\cdot}5 \pm 0{\cdot}5$ sec^{-1} ($n = 7$). The mean frequencies in the TTX-paralysed soleus muscles were $2{\cdot}8 \pm 0{\cdot}5$ sec^{-1} ($n = 5$) and $2{\cdot}7 \pm 0{\cdot}5$ sec^{-1} ($n = 7$) at 10 and 21 days respectively, while in control soleus muscles it was $1{\cdot}1 \pm 0{\cdot}5$ sec^{-1} ($n = 7$). In each case the difference between the TTX-paralysed and control muscles was significant (Student's t test, $P < 0{\cdot}05$).

DISCUSSION

The effects of denervation on the intrinsic properties of skeletal muscle have been variously attributed to (1) inactivity, (2) neurotrophic factors and (3) nerve degenera-

tion products. In the present experiments a comparison has been made between the effects of denervation and inactivity at a time when the nerve degeneration process is probably complete (Lømo & Westgaard, 1976; Miledi & Slater, 1968) and nerve degeneration products are unlikely to be a contributing factor. These studies were also performed on reinnervated muscles to ensure that any reaction due to nerve degeneration products was initially present in both denervated and TTX-paralysed muscles.

The techniques used in the past to investigate the role of activity and trophic substances have been subject to the criticism that there may be incomplete loss of activity (e.g. tenotomy, cordotomy), partial denervation (e.g. pressure cuffs, Silastic cuffs) or inhibition of axonal transport (e.g. application of local anaesthetics). Our experiments with small TTX-filled capillaries inserted into the sciatic nerve indicate that muscular inactivity of the hind limb of rats can be maintained for quite long periods. As to the effectiveness of the TTX block, we could only be guided by the absence of the toe-spreading reflex and direct stimulation of the nerve. It seems unlikely that recovery from the block would have gone undetected, and if it had, the effects would not have been great as full recovery from the block took 4–6 days. One of the main reasons for using TTX to block impulses was because it has been convincingly demonstrated that TTX does not interfere with fast axonal transport (Ochs & Hollingsworth, 1971; Lavoie *et al.* 1976; Pestronk *et al.* 1976). The TTX capillaries, likewise, did not block axonal transport, nor did they cause any signs of axonal degeneration. Therefore, it is unlikely that interruption of axonal transport contributed to the influence of nerves made inactive with TTX capillaries.

Both denervation and TTX inactivity produced a fall in resting membrane potential but their effects were not equivalent (Fig. 1). At 21 days in both the e.d.l. and soleus, the fall that can be attributed to TTX inactivity was 61 % and 49 % respectively of that which follows denervation. However, as the resting membrane potential of both the TTX-paralysed e.d.l. and soleus muscles stabilized at about -69 mV and those of the denervated muscles were about -63 mV, it appears that the trophic influence of the TTX-inactive nerves is similar in both muscles. The different resting membrane potentials of the control muscles probably arises as a result of the different patterns of activity the muscles are subject to in normal use. Cangiano, Lutzemberger & Nicotra (1977) who used Silastic cuffs to induce a pressure block of nerve impulses to the rat e.d.l. have reported a difference in resting membrane potential between blocked and denervated muscles. However, these authors favoured the interpretation that some factor released by the degenerating nerve was responsible for the difference. In our experiments this is unlikely to be the case, because TTX-inactive nerves had a similar effect (Table 2) on the resting membrane potential of normal and of reinnervated muscles which had been subject to the influence of the products of nerve degeneration.

The greatest disparity between the TTX-paralysed and denervated muscles was found in their ACh receptor densities (Fig. 2). These results are consistent with those reported by Gilliatt, Westgaard and Williams (1977, 1978), who have examined the extrajunctional sensitivity of inactive muscle fibres from baboons after nerve pressure block of up to 63 days, and indicate that a trophic factor is involved in the regulation of extrajunctional ACh receptors. While the effect of inactivity on the

two muscle types did not differ greatly, the difference between inactivity and denervation was most marked on the soleus. At 21 days the ACh receptor density of the inactive soleus was only 21 % of the denervated level while that of the inactive e.d.l. was 34 % of the denervated level. As seen in Fig. 2 this difference is largely the result of the soleus having a greater response to denervation than the e.d.l. The reason for this is uncertain at present but it could result from the soleus being more dependent on the supply of trophic factors. Another possibility is that the spontaneous release of ACh or other substances contained in nerve terminal vesicles might influence extrajunctional ACh receptor density, and that this could account for the difference between the two muscles. This is unlikely to be the explanation because the effect should be greater in the muscle with the higher m.e.p.p. frequency. Our results indicate that trophic regulation of extrajunctional ACh receptor density is greater in the soleus, which has a lower m.e.p.p. frequency in both normal and inactive muscles.

A trophic influence of reinnervating nerves has been demonstrated previously in frog (Miledi, 1960) and avian muscle (Bennett, Pettigrew & Taylor, 1973). When examined 15 weeks and 2 weeks after nerve section the respective frog and avian muscles contained fibres which had non-transmitting junctions but reduced ACh sensitivity. This was not the case during the reinnervation of rat skeletal muscle (McArdle & Albuquerque, 1973), but it did occur during the prolonged recovery from botulinus poisoning (Bray & Harris, 1975). The TTX reinnervation experiments reported here indicate that inactive reinnervating nerves can have a trophic influence on the resting membrane potential and ACh receptor density of mammalian muscle.

In agreement with others (Lomø & Rosenthal, 1972; Cangiano *et al.* 1977) activity was found to be the major factor controlling muscle weight or protein content. In contrast, Gilliatt *et al.* (1978) found that the neural influence appeared to be more effective in preventing spontaneous fibrillation. In our experiments no attempt was made to quantify fibrillatory activity, but the occurrence of visible fibrillations in most of the TTX-inactive muscles suggests that activity is an important factor in the control of spontaneous fibrillation in the rat.

From the foregoing discussion, it appears that a neural influence, independent of nerve impulses, is involved in the long-term maintenance of the resting membrane potential and ACh receptor density of skeletal muscles. This could be attributed to either (as yet unidentified) neurotrophic factors or, simply, to nerve-muscle contact. It should be stressed that the observations made indicate only the minimum contribution of the trophic influence because (1) such an influence might be synergistic, rather than additive, with activity in regulating muscle properties; (2) nerve impulses might increase the amount of trophic factors released at the nerve terminal; (3) inactive muscles might be in a pathological state and unresponsive to the trophic influence as emphasized by Deshpande, Albuquerque & Guth (1976). The observation that m.e.p.p. frequency is increased after 10 and 21 days inactivity indicates that nerve action potentials, as well as being important in the long-term maintenance of skeletal muscle properties, are involved in the long-term regulation of the spontaneous release of transmitter from nerve terminals.

We wish to thank Dr M. Pollock for performing the histological examination of the nerves and B. Child for technical assistance. The research was supported by a grant from the Medical Research Council of New Zealand.

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