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INCREASED EXTRAJUNCTIONAL ACETYLCHOLINE SENSITIVITY PRODUCED BY CHRONIC POST-SYNAPTIC NEUROMUSCULAR BLOCKADE

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SUMMARY

1. Anaesthetized rats were paralysed for periods of up to 3 days by chronic administration of D-tubocurarine (DTC), succinylcholine or α-bungarotoxin.

2. After 3 days of treatment with DTC, the phrenic nerve remained active. Neuromuscular transmission and spontaneous miniature end-plate potentials (m.e.p.p.s) were restored after removal of the DTC. Resting potentials and input resistances of muscle fibres that had been paralysed for 3 days were similar to those in denervated fibres.

3. Chronic neuromuscular blockade increased the binding of [^{125}I]-α-bungarotoxin by extrajunctional regions of muscle. The time course of the increase was similar to that seen after denervation. Binding to muscles from animals that were anaesthetized and respirated, but not paralysed, was not increased.

4. Three days of paralysis increased the sensitivity of the extrajunctional muscle membrane to acetylcholine (ACh) applied by iontophoresis.

5. Approximately the same proportion of muscle fibres from muscles paralysed for 3 days gave overshooting action potentials in the presence of tetrodotoxin 10^{-6} g/ml. as did fibres from muscles denervated for 3 days.

6. Chronic paralysis did not change the accumulation of acetylcholinesterase above a ligation in the sciatic nerve.

7. These results are consistent with the idea that extrajunctional ACh sensitivity is normally controlled by muscle activity.

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INTRODUCTION

The region of highest sensitivity to the transmitter ACh in vertebrate skeletal muscle is at the neuromuscular junction (Kuffler, 1943; Axelsson & Thesleff, 1959; Miledi, 1960*a*) where most of the receptors, as measured by binding of α-bungarotoxin, occur (Miledi & Potter, 1971; Barnard, Wieckowski & Chiu, 1971; Berg *et al.* 1972; Fambrough & Hartzell, 1972). ACh receptors also occur in muscle membrane that is not associated with the neuromuscular junction. The number of these extrajunctional receptors varies widely according to the state of innervation of the muscle, in contrast with the end-plate receptors, whose number appears to remain relatively constant. In newly innervated embryonic muscle fibres, the entire muscle surface is sensitive to ACh (Diamond & Miledi, 1962). After neuromuscular transmission has been established, sensitivity outside the end-plate to ACh declines until few, if any, extrajunctional receptors are found in the adult. The number of extrajunctional receptors in the adult can be increased, however, by denervation of the muscle (Axelsson & Thesleff, 1959; Miledi, 1960*a*) and again decreased by subsequent reinnervation (Miledi, 1960*b*; McArdle & Albuquerque, 1973).

The presence or absence of the nerve thus influences the number of extrajunctional receptors on the muscle surface. One mechanism by which the nerve exerts its influence is an indirect one, that is, by eliciting muscle activity. Direct electrical stimulation of denervated muscle, either *in vivo* (Lømo & Rosenthal, 1972; Drachman & Witzke, 1972; Jones & Vbrová, 1974), or in organ culture (Purvis & Sakmann, 1974) can prevent or reverse the development of high extrajunctional sensitivity to ACh. Similarly, chronically stimulated myotubes in cell culture have significantly lower ACh sensitivities than do myotubes cultured in tetrodotoxin (Cohen & Fischbach, 1973).

In addition to evoking muscle activity, the nerve may also influence the ACh sensitivity of the muscle in a direct way. For instance, when dually innervated fibres in frog sartorius muscle are partially denervated, ACh sensitivity increases around the denervated end-plates, although the fibres presumably remain active. This observation led Miledi (1960*a*) to propose that extrajunctional ACh sensitivity is regulated by a specific neural factor. Several groups have observed that colchicine applied to motor nerves results in the appearance of ACh sensitivity in innervated muscles although neuromuscular transmission remains intact (Albuquerque *et al.* 1972; Hoffman & Thesleff, 1972; Cangiano, 1973). These experiments have been interpreted as implying the existence of such a neural factor carried by axonal transport, although recent work (Lømo, 1974;

Cangiano & Fried, 1974) makes it likely that colchicine achieves its effect by acting directly upon the muscle.

To determine whether factors other than activity affect extrajunctional ACh sensitivity, we have produced chronic post-synaptic blockade of innervated muscles *in vivo* without disruption of impulses in the nerve. If sensitivity is related only to muscle activity, it should be increased after chronic block of the muscle. Alternatively, failure of the blockade to increase levels of extrajunctional receptor would suggest that the nerve exerts an influence on the muscle that is independent of muscle activity. We report here that chronic treatment with D-tubocurarine, succinylcholine or α-bungarotoxin dramatically increases the level of extrajunctional ACh receptors in rat diaphragm muscle.

METHODS

Chronic neuromuscular blockade. White, male, Sprague–Dawley rats (80–140 g) were anaesthetized with ether and cannulated with Intramedic PE 200 tubing inserted into the tracheae and fastened with nylon surgical thread. All rats were maintained throughout the period of the investigation (which lasted up to 86 hr) on a continuous anaesthetic mixture of N_2O and O_2 to give a total N_2O content of 80 %. The respirator (Harvard Apparatus Co.) was set at 80–100 strokes/min and 2 ml./stroke. The animals were covered lightly and were warmed with a Gorman-Rupp Model K-1-3 water-heating pad. Rectal temperatures and electrocardiograms were monitored periodically.

Chronic neuromuscular blockade was produced in three ways. One group of rats was given an initial I.P. injection of 3 mg DTC (Squibb) in 1 ml. isotonic saline followed by a continuous I.P. infusion (8–12 ml./day) of isotonic saline solution containing DTC, 1·07 mg/ml., glucose, 32 mg/ml. and penicillin G, $5{\cdot}6 \times 10^3$ u./ml. (Squibb). In several of the rats, plasma levels of DTC at the end of three days infusion were determined using a modification of the method of Elert & Cohen (1962) in which all volumes were scaled down tenfold. To obtain standard values, DTC was added to the plasma of normal rats, and a correction for the blank was made using plasma from rats injected I.P. (8 ml./day) with infusion solution that did not contain DTC. Plasma DTC values in the treated animals ranged from 1 to 3×10^{-5}g/ml.

A second group of rats was injected initially with 25 mg succinylcholine (Squibb) in 1 ml. isotonic saline and maintained on the same infusion fluid as above, except that succinylcholine, 19·5 mg/ml., replaced the DTC. A third group of rats was treated with α-bungarotoxin by infusing the animals with isotonic saline containing only glucose and penicillin, and giving four to five injections at approximately 12 hr intervals of α-bungarotoxin, purified as previously described (Berg *et al.* 1972). The first two injections contained 20–30 μg toxin and later injections 5–15 μg for a total of approximately 70 μg/100 g body weight, given over a 48 hr period. After the final injection, respiration and the infusion were continued for an additional 12–24 hr to allow loss of toxin from extrajunctional receptors (Berg & Hall, 1974) before the animals were sacrificed.

Another group of anaesthetized animals (sham-treated) was respirated and warmed, but the rats were mechanically restrained by taping them to a board with

masking tape to prevent movements that might dislodge the cannulae. These animals were not infused, but were given 3–4 ml. injections 3–4 times daily of an isotonic saline solution containing glucose and penicillin as described above. In addition, some of the sham-treated animals received a single injection of 0·1 mg atropine sulphate (Eli Lilly Co.) shortly before the tracheotomy was performed in an effort to reduce fluid accumulation in the respiratory tract.

Both control and treated animals lost 20–30 % of their original body weight during the 3 days of treatment.

Assay of the ACh receptor by binding of [^{125}I]-*α-bungarotoxin.* Diaphragms were removed from the rats and over a period of about 1 hr were rinsed in several changes of a Krebs solution of the following composition: 138 mM-NaCl, 4 mM-KCl, 2 mM-$CaCl_2$, 1 mM-KH_2PO_4, 1 mM-$MgSO_4$, 12 mM-$NaHCO_3$ and 11 mM glucose. The muscles were then incubated with [^{125}I]-α-bungarotoxin and washed extensively (Berg *et al.* 1972). End-plate-free regions of muscle (Hebb, Krnjević & Silver, 1964) were then dissected out, homogenized and the radioactivity of the homogenate determined. In some experiments the radioactivity bound by muscles from DTC-treated animals and from animals that had been denervated for three days was examined by zone sedimentation. The non-innervated portions of muscles to which radioactive toxin was bound were homogenized at a concentration of 50 mg muscle/ml. in a solution containing 1 % Lubrol PX, 1 M-NaCl, 20 mM Tris-HCl, pH 7·0, and 0·2 mM-EDTA (homogenate buffer). Lubrol PX was used rather than Triton X-100 as described previously because better solubilization was achieved. After the homogenates were shaken for 1 hr and centrifuged at 28,000 *g* for 30 min over 90 % of the radioactivity was recovered in the supernatant. Samples of the supernatants (0·2 ml.) plus marker enzymes were then layered on 4·8 ml. linear gradients of 5–20 % sucrose in homogenate buffer and centrifuged for 13 hr at 44,000 rev/min using an SW 50·1 rotor and Spinco Model L-2 centrifuge. All operations were carried out at 4° C. The marker enzymes were bacterial alkaline phosphatase (6·1 S) and catalase (11·3 S) and were assayed as described previously (Berg *et al.* 1972). Under the conditions used here, toxin-receptor complex sediments at approximately 7 *S* (A. Rao and P. Hogan, unpublished observations).

Intracellular recording. Muscles were dissected and pinned out at room temperature in a dish containing Krebs solution that was continuously bubbled with 95 % O_2, 5 % CO_2. In some experiments the dishes (7 ml.) were perfused (3 ml./min) with oxygenated Krebs fluid. Intracellular recordings were made using glass micro-electrodes filled with 3 M-KCl, whose resistances ranged from 15 to 40 MΩ. The signal was amplified by a preamplifier (Model M4-A, W-P Instruments, Inc.), displayed on an oscilloscope and photographed.

ACh sensitivity of muscle fibres was measured by iontophoresis using micropipettes that contained 3 M acetylcholine chloride and had resistances of 50–100 MΩ. A braking current (1–10 nA) was applied to prevent leakage from the tip. ACh sensitivity was measured in the nerve-free portion of the muscle (1–2 mm from the central tendon) in all experiments except those in which miniature end-plate potentials (m.e.p.p.s) and the input resistance were also measured in the same fibre. For each fibre the ACh pipette was positioned to give the maximum response, and the strength and duration of the current pulse adjusted to give a depolarization of 2–4 mV. The ACh sensitivity was then expressed as mV/nC (Miledi, 1960*a*).

To relate m.e.p.p. size and input resistance the average m.e.p.p. amplitude of a fibre was measured and corrected to a resting potential of −80 mV (Katz & Thesleff, 1957). Only those fibres were used in which the m.e.p.p.s had a rise time of less than 1·5 msec. The input resistance of fibres was measured by passing current with a second micro-electrode placed within 50 μm of the recording micro-electrode. The

potential change produced by several positive and negative current pulses of 100 msec duration was measured and the calculated resistances averaged. The input resistance was independent of voltage over the range in which the experiments were done. Fibres were acceptable only if the final resting potential was at least -45 mV.

To determine tetrodotoxin sensitivity we measured the ability of muscle fibres to produce conducted action potentials in the presence of tetrodotoxin 10^{-6} g/ml. Fibres were impaled with two micro-electrodes less than 50 μm apart, one for passing current and the other for recording membrane potential. The membrane potential was initially adjusted to -90 mV by a steady d.c. potential upon which depolarizing pulses of 10 msec duration and varying amplitude were superimposed (Redfern & Thesleff, 1971*a*).

Several of the oscilloscope records were retouched for clarity.

Recording from the phrenic nerve. Normal and DTC-treated rats were anaesthetized with ether and the left thoracic cavity opened and filled with mineral oil at 37° C. The phrenic nerve was dissected free and lifted on to a recording electrode consisting of two bent platinum wires that were insulated except for a small groove. The electrical activity of the nerve was displayed on an oscilloscope and photographed. No attempt was made to resolve single units.

Axonal transport of aceylcholinesterase. Sciatic nerves were ligated with nylon surgical thread under ether anaesthesia. The distribution of acetylcholinesterase proximal to the ligation was determined by removing the nerve, sectioning it into 3 mm pieces with a razor blade, and homogenizing each section in 0·1 ml. 0·05 M Tris-HCl, pH 7·4, 0·2 mM-EDTA. Samples of the homogenate were then assayed by a radiochemical method using [^{3}H]ACh as described previously (Hall, 1973). Assays were performed in the presence of 2×10^{-5} M *iso*-OMPA (tetraisopropylpyrophosphoramide, Koch-Light, Ltd) to inhibit non-specific cholinesterase activity. One unit of enzyme activity is defined as 1 μmole ACh hydrolysed per hour under standard assay conditions.

RESULTS

Chronic neuromuscular blockade in rats was produced for up to 86 hr by continuous I.P. infusion of DTC. During the period of infusion no spontaneous or reflex movements of the animals were observed. In several animals that had been maintained on DTC for 3 days, the phrenic nerve and diaphragm were exposed under ether anaesthesia and the nerve stimulated. No visible response of the muscle was detected.

In other experiments extracellular recordings were made under ether anaesthesia from the phrenic nerve to test whether the motor neurones continued to discharge impulses after 3 days of DTC treatment. Rhythmic bursts of activity were observed occurring at the same rate as the respirator strokes (Fig. 1). Occasionally, an extra burst, out of rhythm with the respirator, was seen. When the respirator was stopped, the rhythmic bursts of activity either continued at the same rate or, after a brief pause, continued at a different rate. The discharge pattern continued after the phrenic nerve was cut between the electrodes and the muscle, indicating that the afferent axons were not involved. Thus extended DTC treatment did not inhibit normal activity in the phrenic nerve.

Neuromuscular transmission. The neuromuscular blockade produced by DTC was reversible. Reflex and spontaneous movements of animals treated with DTC for up to 3 days usually returned within a period of several hours after the DTC infusion was discontinued. When animals in which spontaneous movement had returned were removed from the respirator, they resumed normal breathing movements and were capable of maintaining respiration.

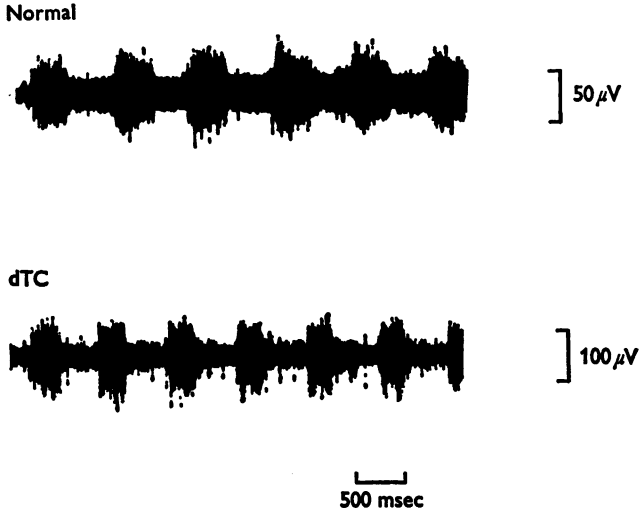

Fig. 1. Extracellular recordings from the phrenic nerves of a normal animal and of an animal after 3 days treatment with DTC.

The electrophysiological properties of muscles from animals that had been paralysed for 3 days with DTC were examined *in vitro* by intracellular recording. Before the diaphragms were removed, DTC infusion was stopped and the animals allowed to recover spontaneous respiratory movements under continued anaesthesia. The muscles were extensively washed *in vitro* with oxygenated Krebs solution before recording.

Resting potentials obtained from fibres in muscles treated with DTC were significantly lower than those from normal muscles, as were the resting potentials in diaphragms that had been denervated for 3 days (Table 1). The average input resistance of fibres from DTC-treated and denervated muscles was also significantly higher than that in normal muscles (Table 1). Essentially all (thirty-five of thirty-seven) fibres tested in three muscles that had been subjected to DTC and allowed to recover gave propagated action potentials in response to nerve stimulation.

M.e.p.p.s were also detected in muscles from DTC-treated animals. Their average frequency and average amplitude were similar to those

in normal muscles (Table 1). Since DTC treatment increases the input resistance of the muscle fibres, however (Table 1), the average amplitude of the m.e.p.p.s should also be increased over those found in normal muscles. To investigate this point, m.e.p.p. amplitude and input resistance were measured in individual fibres. In many of the fibres in muscles from animals treated with DTC, the relation between input resistance and m.e.p.p. amplitude was the same as that observed for control fibres (Fig. 2).

TABLE 1. Electrical properties of muscles from normal, denervated and DTC-treated animals. Values refer to the mean ± S.E. of mean. The first number in parentheses refers to the number of muscles used, and the second the number of fibres studied. M.e.p.p. amplitudes have been corrected to a fibre resting potential of −80 mV

Muscle	Resting potential	M.e.p.p. freq. (sec^{-1})	M.e.p.p. ampl. (mV)	Input resistance (MΩ)
Normal	78·0 ± 0·8 (3, 39)	1·06 ± 0·10 (3, 21)	1·29 ± 0·10 (3, 16)	0·61 ± 0·04 (5, 17)
Denervated (3 days)	68·9 ± 1·0 (2, 49)*	—	—	1·06 ± 0·17 (3, 14)*
DTC-treated (3 days)	71·4 ± 0·9 (3, 43)*†	1·01 ± 0·11 (2, 21)	1·06 ± 0·09 (3, 15)	1·04 ± 0·09 (4, 16)*†

* Significantly different from normal, $P < 0·01$.
† Not significantly different from denervated, $P > 0·05$.

In other fibres from the treated animals, however, the relative m.e.p.p. amplitudes appeared to be reduced. It seemed unlikely that this effect was due to residual DTC in the preparation, since in all experiments the animals were allowed to recover, and in several experiments the muscles were perfused for 1–2 hr *in vitro* before recordings were made. Other possible causes for the reduction were not explored.

Binding of [^{125}I]-*α-bungarotoxin.* The effect of chronic nerve–muscle block on the number of extrajunctional ACh receptors in diaphragm was first investigated by examining the amount of [^{125}I]-α-bungarotoxin bound to non-end-plate regions of diaphragms from rats paralysed for 3 days. The diaphragms were removed, washed extensively, and incubated with radioactive toxin. Following chronic treatment with either DTC, succinylcholine or α-bungarotoxin the amount of radioactive toxin bound per unit weight in extrajunctional regions of muscle was over tenfold higher than that found with normal diaphragms and was approximately the same as the amount of toxin bound by extrajunctional regions of diaphragms that had been denervated for 3 days (Table 2). Extrajunctional regions of diaphragms from control anaesthetized rats that were respirated, mechanically restrained, and given saline instead of curare

(sham-treated animals) bound the same amount of toxin as did tissue from normal rats.

For each of the three treatments used to produce paralysis, the radioactivity bound to non-innervated portions of muscle after incubation with toxin was examined by extraction of the tissue with detergent and sucrose-gradient zone sedimentation of the resultant extract. Most of the

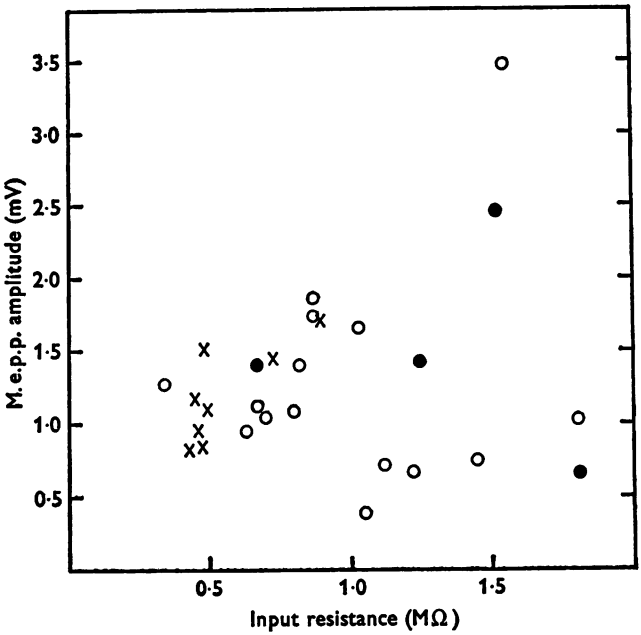

Fig. 2. M.e.p.p. amplitude as a function of input resistance. ×, normal fibres; ○, fibres that had been blocked by DTC for 3 days. The ACh sensitivities of the fibres represented by filled circles were measured 0·5 mm or greater from the end-plate; the sensitivities were 11, 75, 0·6 and 4·5 mV/nC in order of increasing input resistance.

TABLE 2. Binding of [^{125}I]-α-bungarotoxin to non-innervated regions of muscle. Hemidiaphragms were removed from animals after 3 days of the indicated treatment and [^{125}I]-toxin binding measured as described in Methods. Values refer to the mean ± S.E. of mean and the numbers in parentheses indicate the number of muscles used

Muscle	Toxin bound (f-mole/mg tissue)
Normal	0·4 ± 0·2 (23)
Denervated	6·2 ± 0·8 (3)
Paralysed	
DTC	4·8 ± 2·5 (8)
Succinylcholine	6·4 ± 0·6 (3)
α-Bungarotoxin	5·2, 10·3 (2)
Sham-treated	0·2 ± 0·1 (4)

radioactivity migrated with a sedimentation constant characteristic of the toxin–receptor complex. Sedimentation analyses of the detergent extracts of DTC-treated and denervated muscles are shown in Fig. 3.

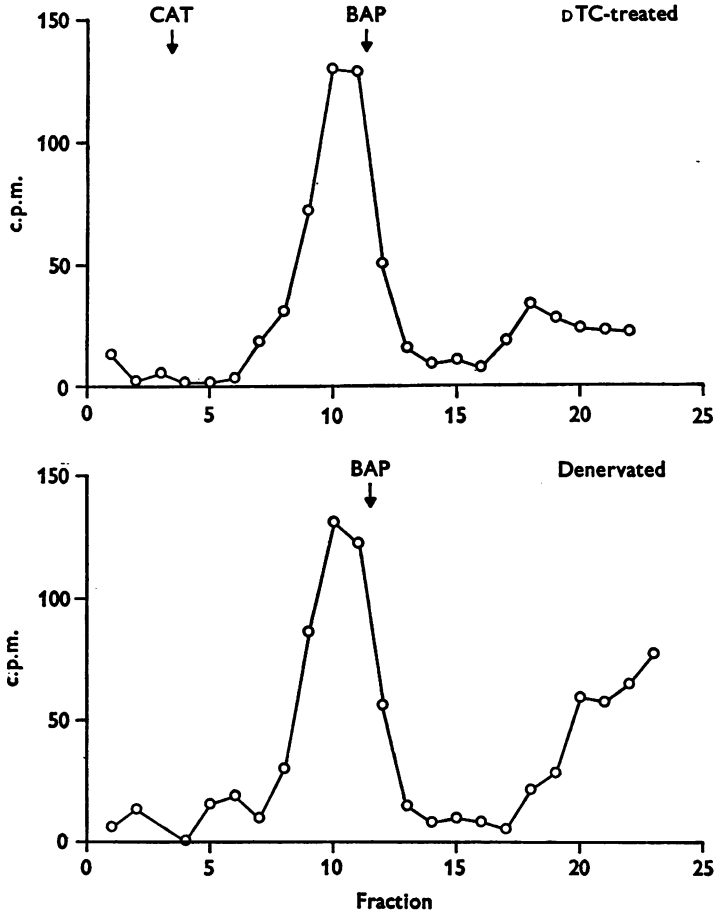

Fig. 3. Zone sedimentation of the radioactivity bound to non-innervated regions of muscles denervated for 3 days and of muscles treated with DTC for 3 days. Muscles were incubated with [^{125}I]-toxin, extensively washed, and extracts prepared in Lubrol PX as described in Methods. Sedimentation is from right to left in the Figure and was performed as described in Methods. Cat and BAP refer to the marker enzymes, catalase (11.3 *S*) and bacterial alkaline phosphatase (6.1 *S*).

Normal and denervated diaphragms from curarized animals. Although we observed no significant difference in the number of extrajunctional receptors in paralysed and denervated muscles, we examined this point more closely in the following way. The left phrenic nerves of several animals

were transected immediately before DTC paralysis. After 3 days of neuromuscular block, the binding of toxin to extrajunctional regions of right and left hemidiaphragms was compared. No consistent difference between the two sides was found (Table 3). The ratio of bound toxin for right/left hemidiaphragm was $1{\cdot}01 \pm 0{\cdot}14$ (mean $\pm$ S.E. of mean) compared to a ratio of $0{\cdot}94 \pm 0{\cdot}07$ obtained for four animals maintained on curare without denervation. Thus the presence of the intact nerves in the innervated hemidiaphragm did not significantly reduce the number of extrajunctional receptors that accumulate in response to the chronic treatment with curare.

TABLE 3. Binding of [^{125}I]-α-bungarotoxin to non-innervated regions of innervated and denervated hemidiaphragms from rats treated for 3 days with DTC. The left hemidiaphragms of four rats were denervated immediately before treating with DTC. Left and right hemidiaphragms were removed after the treatment and the [^{125}I] toxin bound to non-innervated regions of muscle determined as described in Methods

	Toxin bound (f-mole/mg tissue)	
Animal	Innervated (right)	Denervated (left)
1	9·5	7·2
2	6·0	5·2
3	9·6	13·7
4	6·4	7·3

Time of appearance of toxin-binding sites. Further experiments were done to determine whether the toxin-binding sites in non-innervated regions of muscle following neuromuscular blockade by DTC appeared at a rate similar to that following denervation (Fig. 4). In both cases the first detectable binding was observed after 24 hr, and an approximately parallel increase in binding sites occurred after that time.

ACh sensitivity measured by iontophoresis. In addition to assaying ACh receptors by the binding of α-bungarotoxin, we determined the sensitivity of non-innervated portions of paralysed muscles to iontophoretically applied ACh. Measurements were made 1–2 mm from the central tendon, an area which in normal muscles gave no detectable response to ACh (less than 0·2 mV/nC). In diaphragms from five animals treated with DTC for three days, a total of thirty-five out of fifty-two fibres responded to ACh with sensitivities ranging up to 125 mV/nC. An example of a response to ACh recorded near the central tendon is given in Fig. 5. In comparable experiments on two animals that had been denervated for three days, thirty-one out of thirty-five fibres were found to be sensitive. The frequency distribution of sensitivities in the two cases was similar (Fig. 6).

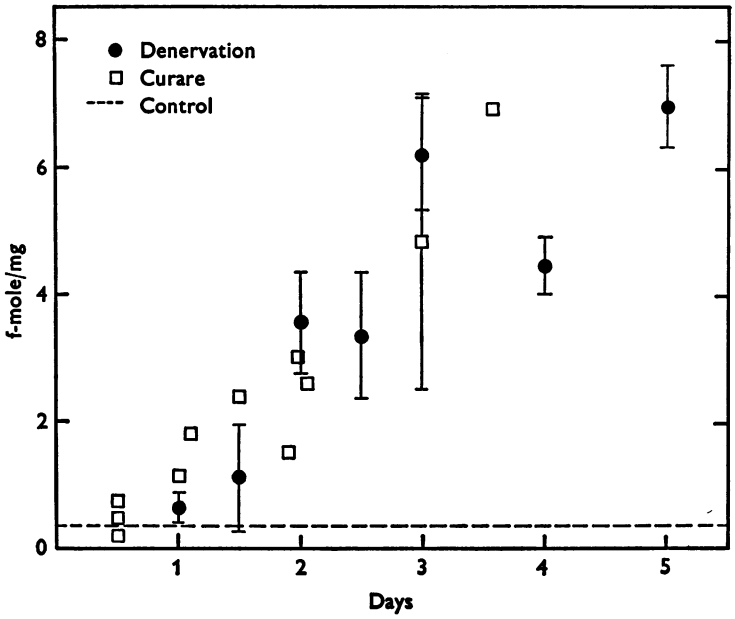

Fig. 4. Appearance of toxin-binding sites in non-innervated regions of muscles after denervation or during treatment with DTC. Animals were denervated or received DTC treatment as described in Methods. Muscles were removed and assayed for toxin-binding in non-innervated regions at the indicated times. Points with error bars represent the mean ± S.D. for four or more muscles. Otherwise, points represent individual determinations.

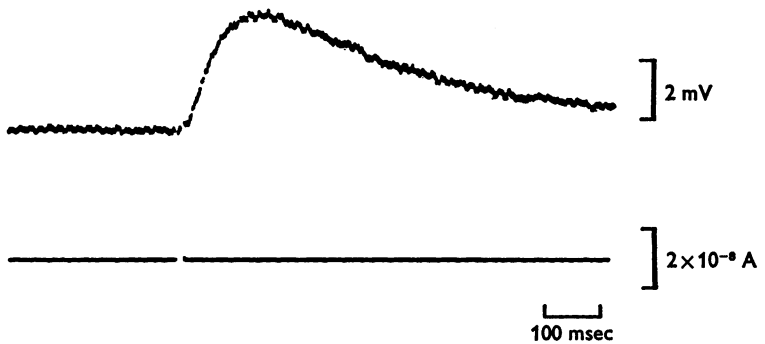

Fig. 5. ACh potential recorded near the central tendon of a muscle after 3 days of DTC treatment. The upper trace is the membrane potential recorded intracellularly and the lower trace represents current passed through the micropipette containing ACh. The sensitivity of the fibre was 21 mV/nC.

The finding that many fibres were not sensitive to ACh near the tendon after DTC treatment raised the possibility that post-synaptic block was not the important factor and that only those fibres with reduced m.e.p.p.s became sensitive. Accordingly m.e.p.p. amplitude, input resistance and extrajunctional ACh sensitivity were measured in individual fibres. Fibres with both normal m.e.p.p.s and those with reduced m.e.p.p.s were found to be sensitive. Values for four fibres whose ACh sensitivity was determined at least 0·5 mm away from the end-plate are given in Fig. 2.

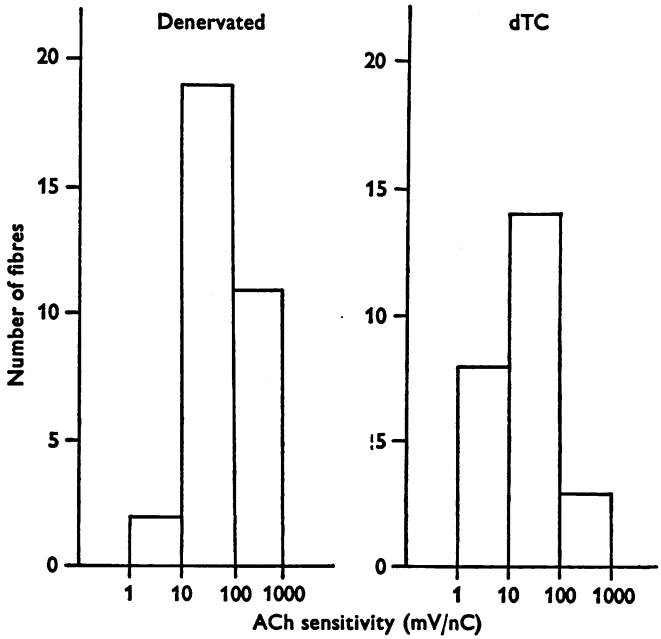

Fig. 6. Extrajunctional ACh sensitivities of fibres from muscles denervated for 3 days and from muscles treated for 3 days with DTC. All values were obtained 1–2 mm from the central tendon.

Sensitivity to tetrodotoxin. In addition to increasing the density of extrajunctional ACh receptors, denervation of a muscle decreases the sensitivity of conducted action potentials to tetrodotoxin (Redfern & Thesleff, 1971*b*). The effect of chronic neuromuscular blockade on the ability of fibres to conduct action potentials in tetrodotoxin 10^{-6} g/ml. was tested by recording from fibres 1–2 mm from the central tendon, using the method described by Redfern & Thesleff (1971*a*). In two normal muscles, tetrodotoxin 10^{-6} g/ml. abolished the action potential to suprathreshold stimuli in all fibres examined, while half of the fibres (six of twelve) tested in a muscle denervated for 3 days gave overshooting action potentials in the

presence of tetrodotoxin. When two muscles from animals maintained on DTC for 3 days were examined, approximately half of the fibres (eleven of twenty-one) also gave overshooting action potentials. Examples of fibres from denervated and DTC-treated muscles whose action potentials were not abolished by tetrodotoxin are given in Fig. 7. Thus chronic paralysis of innervated muscles can cause them to resemble denervated muscles not only with respect to extrajunctional ACh receptor density but also in their sensitivity to tetrodotoxin.

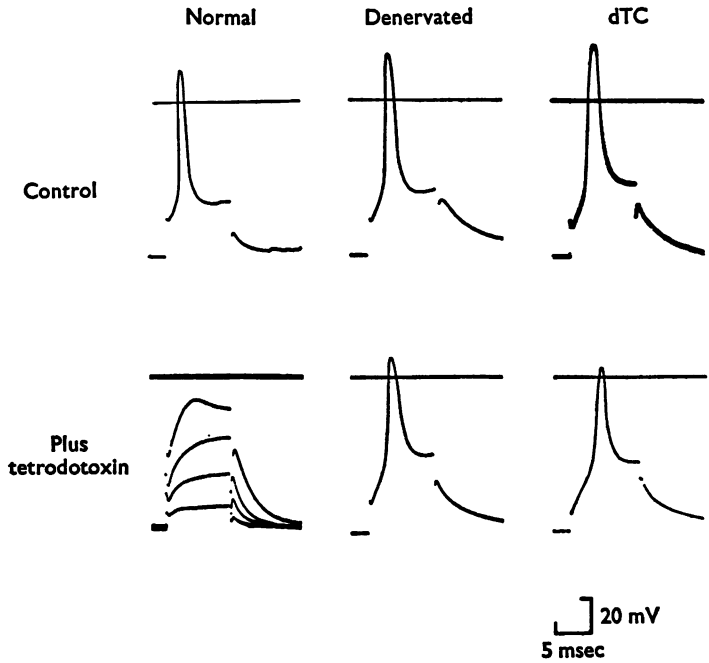

Fig. 7. Effect of tetrodotoxin on action potentials in normal, denervated (3 days) and DTC-treated (3 days) fibres. Fibres were depolarized by passing current through a micro-electrode within 50 μm of the recording electrode. The concentration of tetrodotoxin was 10^{-6} g/ml. Examples are given of denervated and DTC-treated fibres that gave overshooting action potentials in the presence of tetrodotoxin. Such fibres represented about half the total number of fibres examined. No normal fibres were observed to give potentials in tetrodotoxin 10^{-6} g/ml.

Axonal transport of acetylcholinesterase. Agents that block axonal transport also cause an increase in extrajunctional ACh sensitivity in innervated muscles (Albuquerque *et al.* 1972; Hoffman & Thesleff, 1972; Cangiano, 1973). We examined the effect of chronic DTC treatment on the axonal transport of acetylcholinesterase by measuring the accumulation of enzyme activity proximal to a nerve ligation. Sciatic nerves of two

groups of rats were ligated and in one group DTC treatment was begun immediately after the ligation. Twenty-one hours later, nerves were removed from both groups of animals, sectioned into 3 mm pieces, homogenized and assayed for acetylcholinesterase activity. The nerve segments immediately proximal to the ligations in each of three animals treated with DTC contained $2{\cdot}38 \pm 0{\cdot}17$ times more activity (mean $\pm$ S.E. of mean, six nerves) than did other segments of the nerves. Segments proximal to ligation in four control animals contained $2{\cdot}4 \pm 0{\cdot}12$ times more activity than other segments. The mean activities in nerve segments away from the ligation were 0·165 and 0·144 units per segment for curare-treated and control animals, respectively. No net accumulation was observed in animals assayed immediately after ligation. Thus axonal transport as reflected by migration of acetylcholinesterase appears to be unimpaired by the amounts of DTC used in these experiments.

DISCUSSION

A number of experimental techniques have been used previously to reduce the activity of innervated muscles. These include spinal cord section (Johns & Thesleff, 1961), fixation of the limb (Fischbach & Robbins, 1971) and either a local anaesthetic (Robert & Oester, 1970; Lømo & Rosenthal, 1972), botulinum toxin (Thesleff, 1960) or β-bungarotoxin (Hoffman & Thesleff, 1972) applied to the motor nerve. All of these treatments, however, produce their effects presynaptically by reducing impulse frequency in the nerve or its ability to release transmitter. Hence one cannot distinguish effects of nerve activity or transmitter release from those of muscle activity. In particular the experiments do not exclude the possibility that a neural factor, released by nerve impulses, influences extrajunctional ACh sensitivity. For the present series of experiments, agents were chosen whose primary site of action is post-synaptic. DTC, succinylcholine, and α-bungarotoxin each cause neuromuscular block by interacting with the ACh receptors in the muscle membrane to prevent the post-synaptic conductance change produced by the transmitter: DTC and α-bungarotoxin are antagonists that compete with acetylcholine for binding to the receptor, and succinylcholine is a depolarizing agent that desensitizes the receptor. In addition, conditions of the experiments were such that impulse activity in the phrenic nerve continued despite the paralysis.

Each of the three drugs, when chronically applied to produce neuromuscular blockade, caused the appearance of extrajunctional acetylcholine receptors. When measured by iontophoresis, both the number of fibres sensitive to ACh and the average sensitivity appeared to be some-

what less in DTC-treated muscles than in denervated muscles. The wide variation in ACh sensitivity between different fibres (Hartzell & Fambrough, 1972) and along the length of each fibre after three days of denervation, however, makes the precision of the iontophoretic measurements low and the significance of the difference between treated and denervated muscles difficult to assess. The toxin-binding studies, which are more reliable since the toxin bound by a muscle represents an average of all the fibres, revealed no significant difference between denervated and innervated hemidiaphragms in DTC-treated animals. Nevertheless, a small difference would have gone undetected.

The simplest explanation for the increase in extrajunctional ACh sensitivity caused by DTC, succinylcholine and α-bungarotoxin is that they prevent muscle activity. Drug treatment could, however, have had secondary effects that would provide other explanations for the increased sensitivity. Several tests for such effects were carried out. Extracellular recording from the phrenic nerve revealed that the nerve fibres remained active after 3 days of DTC treatment. Although these relatively crude recordings would not detect small changes in nerve activity, it is clear that treatment did not block C.N.S. centres responsible for phrenic nerve activity, nor did it block conduction in the nerve trunk. In addition, the extended exposure to DTC did not appear to have profound irreversible effects on neuromuscular transmission, since essentially full recovery of function was observed after removal of the drug. One observation of interest is that many of the fibres in the DTC-treated preparations appeared to have spontaneous miniature end-plate potentials that were reduced in amplitude when the appropriate correction was made for the input resistance of the fibres. Since fibres with both normal and reduced m.e.p.p. amplitudes had extrajunctional ACh sensitivity, the reduction appeared to be unrelated to the change in sensitivity.

It is possible that the drugs used in these experiments reduced the amount of ACh released in response to a nerve impulse. Both DTC and succinylcholine, as well as cobra neurotoxin, affect the repetitive backfiring of motor nerves produced by stimulating either after a tetanus or in the presence of an anticholinesterase (Standaert, 1964; Chang & Lee, 1966; Riker & Okamoto, 1969), and thus presumably have a site of action on the presynaptic nerve terminal. The normal function of this site is unknown. Although both electrophysiological measurements and assay of collected transmitter have been used to test the effect of DTC on ACh release, the results have been conflicting (Krnjević & Mitchell, 1961; Beani, Bianchi & Ledda, 1964; Beránek & Vyskočil, 1967; Bowen & Merry, 1969; Fletcher & Forrester, 1970; Auerbach & Betz, 1971; Galindo, 1971; Gergis, Dretchen, Sokoll & Long, 1971; Hubbard & Wilson, 1973).

We have not examined release of ACh at the plasma levels of curare ($1–3 \times 10^{-5}$ g/ml.) that occurred during these experiments.

Another possible site of secondary action by the post-synaptic blocking drugs is on the transport by motor axons of material that might be required in the terminals to exert neural control. DTC had no detectable effect on the axonal transport of acetylcholinesterase, a protein that accumulates proximal to a ligation of the nerve at a rate characteristic of fast axonal transport (Ranish & Ochs, 1972).

The increase in extrajunctional ACh sensitivity and the development of tetrodotoxin insensitivity caused by DTC, succinylcholine and α-bungarotoxin in these experiments is therefore consistent with the idea that these properties are directly controlled by the electrical and/or contractile activity of the muscle. The experiments do not exclude a mechanism of neural control independent of activity, because of the possibility of secondary sites of action of the drugs. In addition, these experiments do not exclude a direct effect of the drugs on the muscle independent of activity. The results do indicate that the mechanism that ordinarily suppresses extrajunctional acetylcholine sensitivity in innervated muscle can be overcome *in vivo* by drugs that interact with a cholinergic site whose specificity resembles that of the ACh receptor in muscle.

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