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CHRONIC EFFECTS OF BOTULINUM TOXIN ON NEUROMUSCULAR TRANSMISSION AND SENSITIVITY TO ACETYLCHOLINE IN SLOW AND FAST SKELETAL MUSCLE OF THE MOUSE

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SUMMARY

1. A sublethal dose of botulinum toxin (type A) was injected into the muscles of one hind limb of the mouse causing local paralysis.

2. Neuromuscular transmission and muscle sensitivity to acetylcholine (ACh) were studied *in vitro* in soleus and extensor digitorium longus (EDL) from 6 hr to 4 months after the injection of toxin.

3. Both soleus and EDL failed to respond to nerve stimulation within 6 hr of the injection of toxin.

4. In muscle fibres in which neuromuscular transmission was blocked, subthreshold end-plate potentials (e.p.p.s) were recorded. The amplitude of the e.p.p.s increased during recovery from the effects of the toxin and both muscles contracted in response to nerve stimulation after 2–3 weeks.

5. For about 2 months muscles fatigued more rapidly than normal during repetitive nerve stimulation because of the low quantal content of e.p.p.s.

6. Supersensitivity to ACh developed in 3–5 days and persisted after the return of neuromuscular transmission. Muscle sensitivity to ACh became normal when the rate of fatigue during nerve stimulation was normal.

INTRODUCTION

The effect of botulinum toxin on cholinergic transmission is known to be due to its selective action on nerve terminals whereby the release of acetylcholine (ACh) is impaired (Burgen, Dickens & Zatman, 1949; Ambache, 1949). Most physiological studies which have included intracellular recording with micro-electrodes have been done on acutely paralysed muscles (Brooks, 1956; Harris & Miledi, 1971; Spitzer, 1972) since the toxin is lethal in minute quantities. The finding by Guyton &

MacDonald (1947) that the local injection of botulinum toxin directly into the muscles of one hind leg of an experimental animal caused prolonged paralysis in those muscles showed that the chronic effects of the toxin could be studied. Thesleff (1960) found that muscles paralysed by botulinum toxin became supersensitive to ACh as if they had been denervated. Duchen & Strich (1968) showed that, in the mouse, the muscles paralysed by a local injection of toxin eventually recovered and that the recovery of transmission seemed to be mediated by axonal sprouting and the formation of new motor end-plates. The morphological changes in the end-plates and the characteristics of the new nerve-muscle junctions have been described (Duchen, 1970, 1971) with both light and electron microscopy and there was evidence which suggested that recovery of transmission occurred in slow muscles earlier than in fast muscles.

The purpose of this paper is to report the electrophysiological findings in muscles both acutely paralysed by botulinum toxin and also during their recovery from paralysis when axonal sprouting and the formation of new neuromuscular junctions are taking place. Differences between slow and fast muscles were also examined.

METHODS

Male albino mice weighing about 30 g were used. Unpurified botulinum toxin (Type A) dissolved in 0·05–0·1 ml. sterile phosphate buffer containing 0·2 % gelatine was injected into the muscles of the right leg under light ether anaesthesia. The dose of toxin used (0·05–0·1 μg) was sufficient to cause paralysis of the hind limb for several weeks. About 10 % of the mice died within the first week. A gauge-27 needle was introduced through the skin over gastrocnemius and pushed through to the anterior compartment of the leg where half of the dose of toxin was injected. The needle was then withdrawn into the posterior compartment of the leg where the remaining half of the dose of toxin was injected. Mice were allowed to survive for periods of time varying from 1 day to 4 months after the injection of toxin.

Motor nerve sprouting caused by botulinum toxin in the mouse has been shown to occur earlier in slow muscle (soleus and deeper parts of gastrocnemius) than in fast muscle (outer parts of gastrocnemius) (Duchen, 1969, 1970). Soleus and extensor digitorum longus (EDL) were used for *in vitro* physiological studies since their composition is mainly of slow and fast fibres respectively. Gastrocnemius was not used because the fast fibres are limited to the outer parts only.

Mice were anaesthetized with sodium pentobarbitone (100 mg/kg) and soleus and EDL of the right leg dissected out together with short lengths of their nerves. The muscles were placed in a chamber containing mammalian Ringer solution which was continuously gassed with 95 % O_2/5 % CO_2. Muscle tensions were recorded isometrically using a Grass mechano-electrical transducer (FT. 03C). This device had been modified by the addition of a small spring which could be adjusted to counterbalance the tension of the muscle, and also a small damping device to prevent oscillation of the transducer. The muscles were stimulated indirectly using suction electrodes into which the nerves were drawn. For direct stimulation of the muscle two platinum wire electrodes were placed, one on each side of the muscle and a current passed between them and the contents of the bath.

Intracellular recordings of electrical potentials were made with conventional glass micro-electrodes filled with 3 M-KCl. The sensitivity of muscles to ACh was assessed by two methods, the first being iontophoretic application of 3 M-ACh Cl from a micro-pipette. ACh was ejected from the pipette by the application of positive pulses 0·05–0·5 sec in duration. The strength of the pulses was regulated at values between 1 nA and 1 μA by a constant current supply. Leakage of ACh from the pipette was prevented by the continuous application of a negative backing voltage. ACh sensitivity was estimated by the method of Miledi (1960) as the membrane potential change in mV produced per nC of current applied through the pipette. Because of the development of severe atrophy of muscle fibres in the experimental animals, it was in general not possible to map the sensitivity to iontophoretically applied ACh along the entire length of single fibres. For this reason a method similar to that described by Lømo & Rosenthal (1972) was used. The sensitivity to ACh was determined in five fibres about 0·5 mm proximal to the end-plate zone and then repeated at parallel 0·5 mm intervals passing distally through the end-plate region towards the tendon. This method was found to be satisfactory in soleus which has a compact band of end-plates almost at right angles to the long axis of the muscle. In EDL this method was not practicable because the end-plates are arranged in a complex diagonal fashion across the muscle. The second method of assessing muscle sensitivity to ACh was by recording the tension developed by the whole muscle in response to the addition of ACh to the bathing solution. The muscle was placed in a chamber containing 10 ml. Ringer solution to which 0·2 ml. ACh solution was then added to produce a final concentration of 2×10^{-5} M. The muscle response was recorded isometrically.

The mammalian Ringer solution used had the following composition (in m–mole/l.) NaCl 115; KCl 3·5; $CaCl_2$ 2; $MgSO_4$ 1; $NaHCO_3$ 25; KH_2PO_4 1; glucose 10. All experiments were done at 30° C.

RESULTS

Clinical. Within 24 hr of the injection of botulinum toxin the right leg became paralysed and remained so for about 1 month. More general signs of toxicity were evident during the first week when about 10 % of the mice died. The general condition of the mice improved after the first week but full recovery of the use of the right leg was seen only after 2–3 months.

Response to nerve stimulation. The tension developed by soleus and EDL in response to single nerve stimuli are shown in Figs. 1 and 2. Both muscles failed to respond to nerve stimulation within 6 hr of the injection of toxin and remained completely paralysed for 10 days. After 22 days every muscle studied responded to nerve stimulation. Considerable variation in rates of recovery between different muscles was found but EDL tended to remain paralysed for longer than soleus. Between days 11 and 21, six out of eleven EDL were paralysed compared with only three out of fourteen soleus. The tension developed by both muscles in response to direct or indirect stimulation after recovery was generally less than normal.

Muscle weight. The mean wet weight of soleus in eight normal mice was $8·6 \pm 1·3$ mg and of EDL $11·7 \pm 2·1$ mg. After the injection of toxin the weights of both muscles fell during the first 3–4 weeks to about a third of

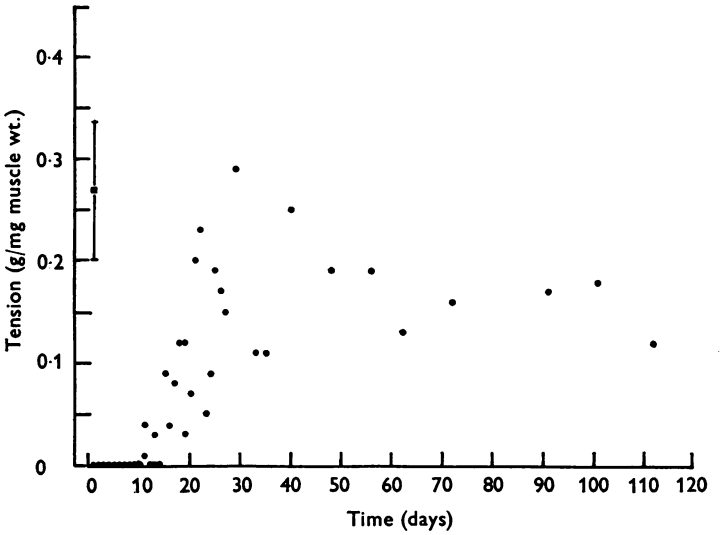

Fig. 1. Isometric twitch tension (g/mg muscle wt.) developed by soleus in response to nerve stimulation. The mean tension developed by muscles from eight normal mice (■) was 0·27 ± 0·07 g (S.D. indicated by vertical lines). After the injection of botulinum toxin muscles from experimental animals (●) were paralysed for 11–15 days before recovery occurred.

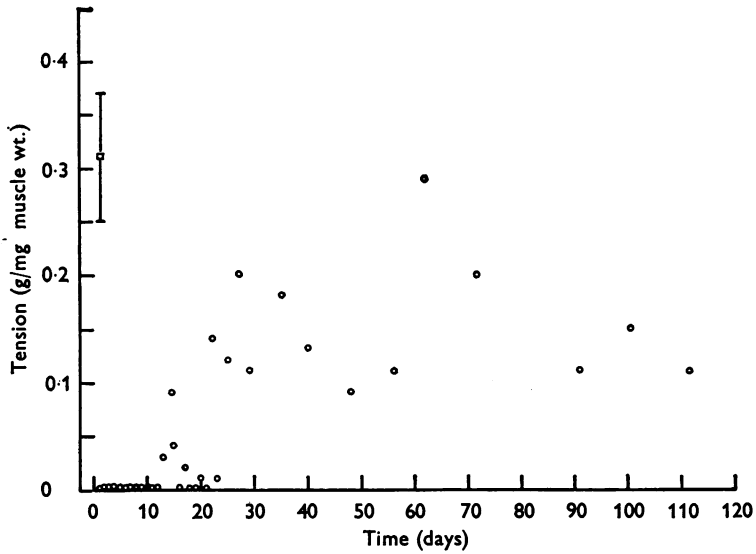

Fig. 2. Isometric twitch tension (g/mg muscle wt.) developed by EDL in response to nerve stimulation. The mean tension developed by muscles from seven normal mice (□) was 0·31 ± 0·07 g (S.D. indicated by vertical lines). After the injection of botulinum toxin muscles from experimental animals (○) were paralysed for 12–21 days before recovery occurred.

normal before slowly recovering again to normal after approximately 3 months.

Miniature end-plate potentials. Botulinum toxin has been shown to inhibit both the spontaneous release of ACh and that elicited by nerve stimulation (Brooks, 1956; Thesleff, 1960; Harris & Miledi, 1971; Spitzer, 1972).

TABLE 1. The effect of botulinum toxin on the frequency and amplitude of spontaneous m.e.p.p.s in soleus and EDL

Days after botulinum toxin	Soleus		EDL	
	m.e.p.p.s/sec	amplitude in mV	m.e.p.p.s/sec	amplitude in mV
Controls	3·2 ±0·9	0·35±0·11 (56, 5)	4·46±2·86	0·34±0·16 (53, 5)
1	0·02±0·02	0·31±0·21 (14, 2)	0·12±0·19	0·17±0·06 (14, 2)
2	0·04±0·04	0·35±0·21 (6, 1)	0·07±0·16	0·31±0·19 (6, 1)
3	0·0 —	— — (8, 1)	0·02±0·03	0·18±0·03 (5, 1)
14	0·14±0·09	0·43±0·16 (6, 1)	0·40±0·21	0·27±0·11 (8, 1)
15	0·32±0·21	0·70±0·24 (10, 1)	0·39±0·15	0·34±0·09 (10, 1)
27	0·03±0·05	0·44±0·36 (10, 1)	0·14±0·15	0·22±0·06 (10, 1)
40	0·21±0·11	0·44±0·19 (9, 1)	0·45±0·34	0·43±0·25 (10, 1)
48	0·16±0·11	0·56±0·45 (10, 1)	0·39±0·38	0·28±0·14 (10, 1)
72	0·35±0·27	0·41±0·28 (10, 1)	0·59±0·41	0·21±0·10 (10, 1)
91	0·50±0·34	0·24±0·11 (10, 1)	0·82±0·65	0·26±0·12 (10, 1)
101	1·08±0·5	0·16±0·05 (10, 1)	2·5 ±1·8	0·15±0·04 (10, 1)
112	0·85±0·68	0·19±0·11 (10, 1)	1·8 ±2·2	0·26±0·09 (10, 1)

Frequency and amplitude values are expressed as means ± S.D. The first figure shown in the brackets refers to the number of fibres tested and the second to the number of muscles studied. The means were calculated from spontaneous m.e.p.p.s recorded for 30 sec.

The mean frequency and amplitude of m.e.p.p.s in soleus of normal and experimental animals is shown in Table 1. It was found that in both muscles there was a great reduction in the frequency of m.e.p.p.s for many weeks after the injection of toxin and this had not returned to normal even after 4 months.

The frequency of m.e.p.p.s in muscles paralysed by botulinum toxin has been found to be temporarily increased by repetitive nerve stimulation (Brooks, 1956; Harris & Miledi, 1971; Spitzer, 1972). In the present investigations, however, no increase in the frequency of m.e.p.p.s was observed during repetitive nerve stimulation.

The amplitude of spontaneous m.e.p.p.s showed great variation. At most end-plates the distribution of amplitudes was skew instead of normal which is in agreement with the observations of Harris & Miledi (1971) and Spitzer (1972). The skew distribution was due mainly to the presence of small potentials of less than 0·2 mV and, to a lesser extent, larger potentials

of up to 2 mV. The larger amplitudes may have been due to the increased input resistance associated with denervated muscle fibres (Albuquerque & Thesleff, 1968). The amplitude of m.e.p.p.s has been shown to be correlated with the input resistance of muscle fibres (Katz & Thesleff, 1957).

End-plate potentials. During the first few days after the injection of botulinum toxin, nerve stimulation at 50–100/sec evoked occasional end-plate potentials (e.p.p.s) of low amplitude at practically every end-plate

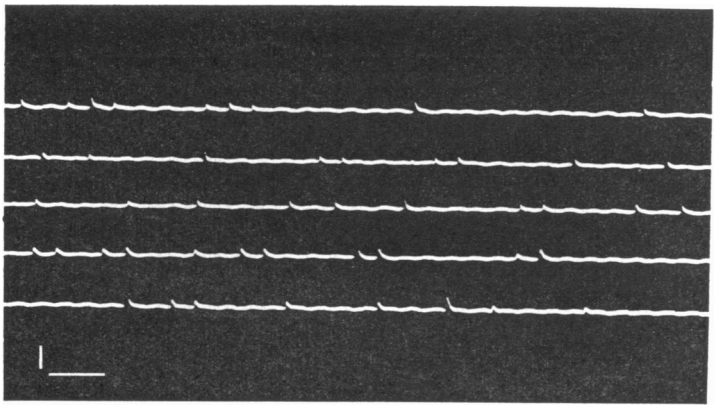

Fig. 3. Intracellular recording from a muscle fibre of soleus 24 hr after the injection of botulinum toxin showing e.p.p.s evoked by repetitive nerve stimulation (50/sec). Scale: vertical 1 mV, horizontal 50 msec.

tested in both soleus and EDL (Fig. 3). Since in frog (Harris & Miledi, 1971) and rat (Spitzer, 1972) m.e.p.p.s (but not e.p.p.s) were observed in muscles paralysed with botulinum toxin, the effect of a larger dose of toxin was investigated in order to see if this would abolish e.p.p.s. The dose of toxin injected was increased by a factor of 10 to $LD_{10} \times 10$ and muscles studied within 24 hr before the mice died. It was found that e.p.p.s could still be elicited in most muscle fibres by nerve stimulation.

The amplitude of e.p.p.s and also the regularity with which they were evoked by nerve stimulation began to increase during the first week after the injection of toxin and after 2–3 weeks they became large enough to initiate action potentials in some muscle fibres. It was found that, in general, action potentials could be recorded from fibres in the centre of the muscles sooner than from the superficial fibres.

Quantal content of end-plate potentials. Although by 22 days soleus and EDL were no longer paralysed, neuromuscular transmission was abnormal. During repetitive nerve stimulation (100/sec) the muscles developed fatigue much more rapidly than normal (Fig. 4) but still responded to direct stimulation. Abnormally rapid fatigue persisted for up to 2 months in

both soleus and EDL. Since raising the magnesium content of the Ringer solution has a similar effect on the rate of development of fatigue (unpublished observations) and it is known that this inhibits the release of ACh (del Castillo & Katz, 1954*a*) it seemed likely that the release of ACh from nerve terminals recovering from botulinum toxin was impaired. In order to investigate this possibility the mean quantal content of e.p.p.s was estimated from their coefficient of variation (del Castillo & Katz,

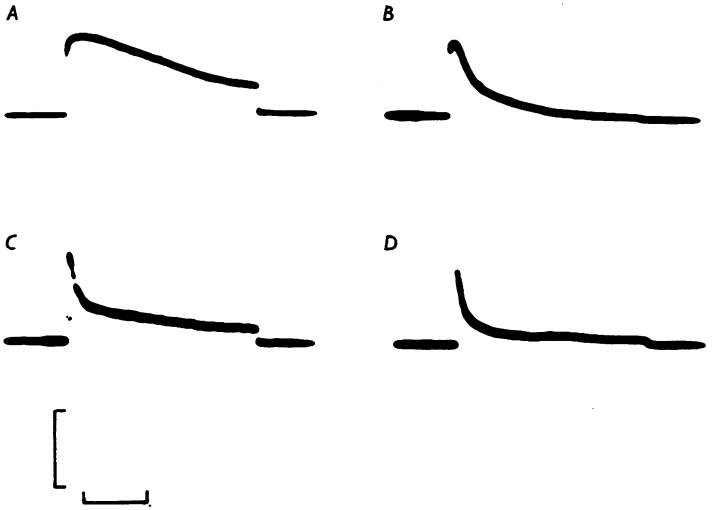

Fig. 4. Records of isometric tension developed by soleus and EDL of normal and experimental animals during repetitive nerve stimulation (100/sec). In normal mice the tension developed by both muscles is well maintained for several seconds before fatigue occurs first in EDL (*B*) and then in soleus (*A*). During recovery from botulinum toxin injected 40 days previously both soleus (*C*) and EDL (*D*) show marked fatigue within the first second of repetitive nerve stimulation. Scale: vertical = 10 g (*A* and *B*) and 5 g (*C* and *D*), horizontal = 10 sec.

1954*b*). Intracellular micro-electrodes were used to record e.p.p.s at neuromuscular junctions of normal and experimental animals. Tubocurarine ($0{\cdot}4$–$1{\cdot}1 \times 10^{-6}$ g/ml.) was added to the Ringer solution to paralyse the muscles when necessary. The nerve was stimulated at 50/sec for 2–3 sec. The first few e.p.p.s were generally larger than subsequent potentials of a series evoked by this frequency of stimulation in both normal and recovering muscles. The first 10 e.p.p.s were therefore disregarded and the mean quantal content of the next seventy-five calculated from their coefficient of variation. This procedure for the estimation of mean quantal content was chosen because, at the frequency of nerve stimulation used,

the rapid development of fatigue characteristic of recovery from botulinum toxin could be clearly seen in non-curarized preparations. No correction was made for non-linear summation of potentials (Martin, 1955) since the amplitude of e.p.p.s was less than 2 mV. A considerable variation in the mean quantal content of e.p.p.s was found in individual fibres within the same muscle (see Table 2). There was a prolonged reduction in the mean quantal content of e.p.p.s after the injection of botulinum toxin with recovery to normal occurring only after about 4 months. During the first 6 weeks it was possible in many fibres to calculate the mean quantal content of e.p.p.s from the proportion of nerve stimuli failing to evoke an e.p.p. (del Castillo & Katz, 1954*b*). Values obtained by this method were generally very close to those calculated from the coefficient of variation of the e.p.p.s. In individual muscles, when the mean quantal content of e.p.p.s was estimated to be above approximately 20, the rate of fatigue of that muscle during repetitive nerve stimulation at 50 or 100/sec was normal.

TABLE 2. The mean quantal content of e.p.p.s in soleus and EDL of control and experimental mice after botulinum toxin

Days after the injection of toxin	Soleus	EDL
Controls	53·4 ± 19·4 (49, 5)	65·5 ± 26·3 (48, 5)
1	0·15 ± 0·09 (14, 2)	0·19 ± 0·18 (14, 2)
2	0·11 ± 0·03 (6, 1)	0·16 ± 0·13 (6, 1)
3	0·46 ± 0·35 (8, 1)	0·40 ± 0·44 (5, 1)
27	2·09 ± 1·34 (10, 1)	4·88 ± 4·42 (9, 1)
40	1·73 ± 1·03 (10, 1)	15·52 ± 10·0 (6, 1)
48	4·16 ± 2·43 (10, 1)	19·9 ± 17·9 (10, 1)
72	14·7 ± 8·9 (10, 1)	25·4 ± 22·3 (10, 1)
91	20·2 ± 16·8 (9, 1)	33·4 ± 25·8 (10, 1)
101	30·8 ± 15·5 (10, 1)	19·4 ± 12·7 (10, 1)
112	43·7 ± 26·0 (10, 1)	53·2 ± 28·8 (10, 1)

Mean quantal content of e.p.p.s calculated from their coefficient of variation. The values given are means ± S.D. for the number of muscle fibres shown by the first figure in brackets. The second figure in brackets refers to the number of muscles studied. With the exception of days 1–3, curare was used to paralyse the muscles.

Sensitivity to ACh. It is well known that muscles paralysed by botulinum toxin become supersensitive to ACH as do surgically denervated muscles (Thesleff, 1960). In the present investigations, both soleus and EDL became supersensitive to ACh after 4 days (Fig. 5). The response to ACh was greatest after 2–3 weeks and then slowly declined to normal after 6–10 weeks. It appeared that the muscles were supersensitive to ACh after the return of neuromuscular transmission and remained super-

sensitive until neuromuscular transmission became normal. Every muscle examined which showed abnormally rapid fatigue during nerve stimulation was also supersensitive to ACh. Whilst these results suggested a correlation between abnormal neuromuscular transmission and persistence of supersensitivity to ACh, there remained the possibility that the increased muscle response to ACh could have been due to delayed return of transmission in some fibres and that only in those fibres was there supersensitivity. This possibility was investigated by applying ACh iontophoretically to muscle fibres. In normal soleus, muscle fibres responded to ACh only within a band (1·5–2·0 mm in width) corresponding to the location of motor end-plates (Fig. 6). After botulinum toxin, the muscle fibres

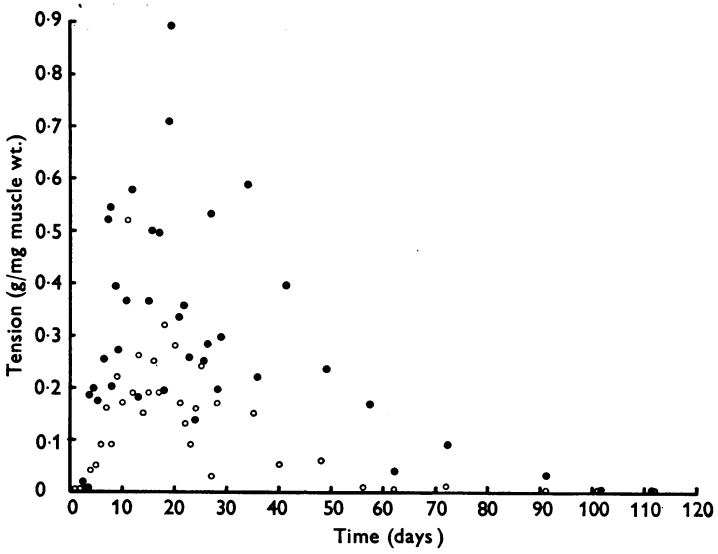

Fig. 5. Isometric tension (g/mg muscle weight), developed by soleus (●) and EDL (○) in response to 2×10^{-5} M-ACh. Within a few days of the injection of botulinum toxin, both muscles contracted strongly indicating supersensitivity to ACh. The muscle response to ACh was maximal at 2–4 weeks and then slowly declined to normal after about 2 months.

became uniformly supersensitive to ACh and remained in this state until after the return of neuromuscular transmission which in the superficial fibres occurred after 4–5 weeks. During the next few weeks the response of muscle fibres to ACh outside the end-plate region declined, especially towards the tendon. Later at 8–10 weeks whilst the whole muscle contracted slightly in the presence of ACh, it was no longer possible to determine with certainty whether the band of muscle sensitive to ACh was wider than usual or not. Since muscle fibres were insensitive to ACh 2·0–2·5 mm

away from the end-plate region, it seemed likely that the muscle response may have been due to a slight extension of the area of muscle fibre membrane sensitive to ACh adjacent to the motor end-plates.

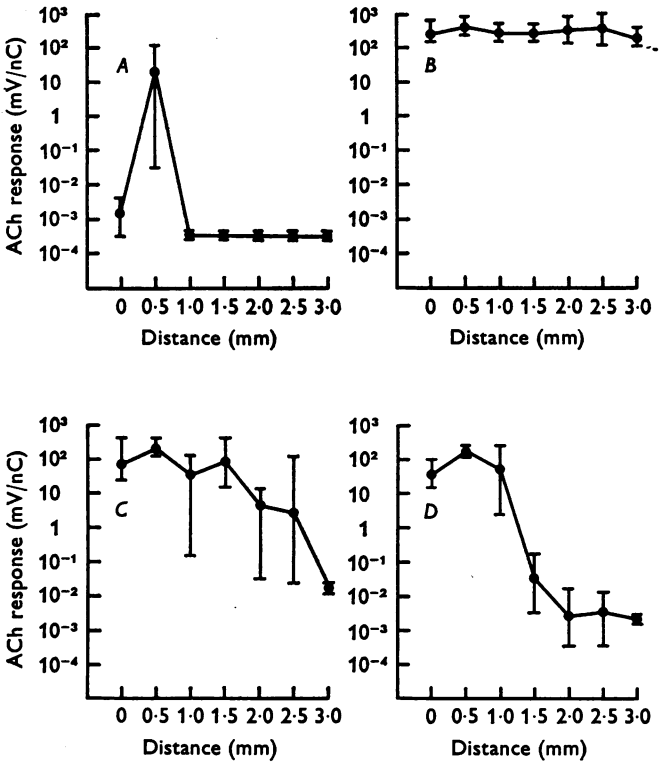

Fig. 6. The response of soleus to the iontophoretic application of ACh. The mean sensitivity to ACh (mV/nC) of 5 muscle fibres (range indicated by vertical lines) determined at 0·5 mm intervals between a position (○) 0·5 mm proximal to the end-plate zone and the distal tendon (3·0). In the soleus of a normal mouse (A) the muscle fibres respond to ACh only at the end-plate zone. After the injection of botulinum toxin (27 days previously) the muscle fibres respond to ACh outside the end-plate region (B). Later after 39 days (C) and 42 days (D) the sensitivity of the muscle fibres to ACh becomes progressively reduced, especially towards the tendon.

DISCUSSION

In the present investigations the injection of a sublethal dose of botulinum toxin caused paralysis of soleus and EDL within 6 hr which lasted for 2–3 weeks. Considerably larger doses of toxin in guinea-pigs, rats and rabbits have been shown to block neuromuscular transmission both *in vivo* and *in vitro* within 1–3 hr (Guyton & MacDonald, 1947; Burgen *et al.*

1949; Brooks, 1956; Spitzer, 1972). Recovery of muscle response to nerve stimulation after botulinum toxin in the guinea-pig was found to take several months (Guyton & MacDonald, 1947). In the cat, the release of ACh from motor nerve terminals was impaired for at least 1 month (Thesleff, 1960).

The acute effects of botulinum toxin in the mouse were similar to its effects on other species in that the spontaneous release of ACh was almost abolished. In contrast to the findings of other workers, however (Brooks, 1956; Thesleff, 1960; Harris & Miledi, 1971; Spitzer, 1972), occasional subthreshold e.p.p.s could be evoked at end-plates by nerve stimulation.

The amount of ACh released in response to nerve stimulation as estimated by the mean quantal content of e.p.p.s began to increase during the first week after the injection of toxin and continued to do so until full recovery occurred after 3–4 months. When the amplitude of e.p.p.s had first recovered sufficiently to cause action potentials in muscle fibres, their mean quantal content was still much lower than normal. At this stage of recovery from the effects of toxin, during repetitive nerve stimulation regression of e.p.p. amplitude occurred due to the progressive decline in the mean quantal content of e.p.p.s (Elmquist & Quastel, 1965) causing the muscles to fatigue more rapidly than normal. During later stages of recovery from the effects of the toxin, when the mean quantal content of e.p.p.s was greater than approximately one third of normal, the rate of development of fatigue was normal.

After neuromuscular transmission had been blocked by botulinum toxin both soleus and EDL became supersensitive to ACh and remained in this state until after nerve stimulation was capable of evoking action potentials in muscle fibres. The extrajunctional sensitivity to ACh in muscle fibres then slowly declined to normal. The persistence of extrajunctional sensitivity in muscle fibres during recovery from the effects of botulinum toxin was found to be correlated with the persistence of abnormally rapid fatigue during repetitive nerve stimulation. In the rat, extrajunctional sensitivity to ACh has been shown to be suppressed by direct electrical stimulation (Lømo & Rosenthal, 1972). It seems likely that during recovery from botulinum toxin extrajunctional sensitivity to ACh persists in muscle fibres until full recovery of neuromuscular transmission occurs when they are able to receive sufficient stimulation to reduce their sensitivity to ACh to normal.

In previous morphological studies (Duchen & Strich, 1968; Duchen, 1970) it was found that during recovery from botulinum toxin, new neuromuscular junctions were formed by axonal sprouts. These junctions were at first simple and superficial and the maturation into fully formed end-

plates with post-synaptic differentiation took place slowly over several weeks (Duchen, 1971). It seemed likely that the recovery of neuromuscular transmission was associated with the growth of motor nerves. From the present electrophysiological investigations it seems likely that e.p.p.s and probably action potentials can be evoked by nerve stimulation while axonal growth is still in progress and before the maturation of new motor end-plates is complete. It is possible that ACh may be released from the recovering original nerve terminal as well as from nerve sprouts.

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