EFFECTS OF DENERVATION AND BOTULINUM TOXIN ON MUSCLE SENSITIVITY TO ACETYLCHOLINE AND ACCEPTANCE OF FOREIGN INNERVATION IN THE FROG

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SUMMARY

1. The effects of denervation and local paralysis produced by botulinum toxin (type D) on the sensitivity of skeletal muscle to ACh and its ability to accept innervation by an implanted foreign nerve were investigated in the frog.

2. Denervated muscles developed supersensitivity to ACh within 2 weeks and became extensively innervated by an implanted foreign nerve after about 4 weeks.

3. Chronic electrical stimulation of denervated muscles (50 Hz for 1 sec every 60 sec) did not prevent the development of supersensitivity.

4. Muscles paralysed by botulinum toxin did not usually develop supersensitivity to ACh until after 2–3 months and the extra-junctional sensitivity of individual fibres was generally less than after denervation. Significant innervation of the paralysed muscles by an implanted foreign nerve did not occur until after 2–3 months.

5. The results suggest that in the frog nerves are able to control muscle sensitivity to ACh and to prevent innervation by foreign nerves by some mechanism other than muscle activity. Prolonged inactivity seems to result in some development of extra-junctional sensitivity and acceptance of foreign innervation.

INTRODUCTION

It is well known that skeletal muscle fibres of frogs and mammals are normally sensitive to acetylcholine (ACh) only at the motor end-plate and that they do not become innervated by an implanted foreign nerve. After denervation muscle fibres develop extra-junctional sensitivity to ACh and may become innervated by a foreign nerve.

The way by which nerves control the sensitivity of muscles to ACh and prevent their acceptance of foreign innervation is not certain. In mammals there is evidence that these influences are mediated mainly through the activity induced in the muscle through synaptic transmission. Muscular paralysis in rats and mice caused by blocking the conduction of nerve action potentials by local anaesthetics (Lømo & Rosenthal, 1972; Jansen, Lømo, Nicolaysen & Westgaard, 1973) or blocking neuromuscular transmission with botulinum toxin which prevents the release of ACh (Thesleff, 1960; Fex, Sonesson, Thesleff & Zelena, 1966; Tonge, 1974*a*, 1977) or the

post-synaptic blocking fractions of *Bungarus multicinctus* and *Naja siamensis* (Berg & Hall, 1975; Tonge, 1978) cause the development of extra-junctional sensitivity to ACh and the acceptance of foreign innervation. Both these changes may be prevented by direct electrical stimulation of denervated muscle (Lømo & Rosenthal, 1972; Jansen *et al.* 1973).

In amphibians the role of activity in controlling muscle sensitivity to ACh is less certain. Evidence that activity by itself is not sufficient to control sensitivity is given by the observation of Miledi (1960*a*) that partially denervated fibres of sartorius muscles in frogs develop extra-junctional sensitivity around the denervated end-plate even though action potentials may still be evoked in the muscle fibre by nerve stimulation via the intact end-plate. Miledi (1960*b*) also observed that during re-innervation of the sartorius muscle the extra-junctional ACh sensitivity of fibres began to decline prior to the restoration of neuromuscular transmission and suggested that sensitivity was controlled by some factor from nerve terminals whose release was independent of that of ACh.

Botulinum toxin when injected in sublethal doses causes prolonged local paralysis of skeletal muscle by inhibiting the release of ACh from motor nerve terminals (Burgen, Dickens & Zatman, 1949) without causing structural damage to either nerve of muscle (Thesleff, 1960; Duchen, 1971). In the present investigations the effects of denervation or paralysis caused by the injection of botulinum toxin on the sensitivity of muscle fibres to ACh and their ability to accept foreign innervation in the frog were compared in order to determine to what extent these were controlled through activity. A preliminary account of some of these findings has already been published (Tonge & Wernig, 1978).

<div align="center">METHODS</div>

Adult *Rana pipiens* were used in all experiments because of their ability to tolerate high ambient temperatures (20–25 °C) and surgical operations. They were fed live crickets two to three times per week.

Botulinum toxin (type D) was used in preference to the more commonly used type A toxin because it is more effective in producing local paralysis in frogs. Injections of the toxin were made either subcutaneously over the cutaneous pectoris muscle or percutaneously into the extensor digitorum brevis *IV* (e.d.b.) of the foot whilst the frogs were lightly anaesthetized with tricaine methanesulphonate (Sigma). The toxin was in aqueous solution and the volume injected (0·03–0·05 ml.) contained 3–5 mouse lethal doses which was close to the maximum which the frogs could tolerate. After the injections most frogs remained healthy for several months and continued to feed for themselves. The only sign of an effect of the toxin in these animals was a slight flexion of the toes of the injected foot. Some frogs became weak and lost their appetite soon after the injections and usually died within 3–4 weeks.

Surgery. The cutaneous pectoris muscle was denervated by making a small incision through the skin close to its lateral border and removing a few mm of its nerve. The e.d.b. was denervated by cutting the peroneal branch of the sciatic nerve at the knee.

The nerve chosen for implantation into e.d.b. was the branch of the peroneus communis inferior which supplies the extensor muscle of toes II and III. The nerve was cut and its proximal stump inserted into e.d.b. close to its proximal tendon. In some frogs the e.d.b. was denervated during the same operation by tying a fine ligature of silk around its nerve.

Chronic stimulation. In some frogs the iliofibularis muscle was denervated by cutting the ishiadicus nerve and then stimulated electrically for up to 2 weeks with platinum foil electrodes implanted into the muscles on either side of the iliofibularis. The electrodes were connected to an external stimulator by fine insulated copper wires which were implanted under the frog's

skin from the back of its head to the thigh. The muscle was stimulated with 50 Hz a.c. for 1 sec every 60 sec and the voltage of the stimulus was adjusted to give a strong local contraction. The duration of effective stimulation as judged by observing the animals varied from 10 to 14 days. The frogs were kept without restraint in a Perspex container with a little water. In control animals the iliofibularis was denervated and the electrodes implanted but without stimulation.

Muscle sensitivity to ACh was assessed in two ways. The method used for e.d.b. and iliofibularis was to measure the isometric tension developed by the muscles in the presence of 3×10^{-5} M-ACh chloride and to compare this with the tension developed by the muscle during supramaximal stimulation at 50 Hz. The sensitivity of muscle fibres in cutaneous pectoris was determined by ionophoretic application of ACh. A glass micropipette filled with 3 M-ACh chloride was positioned using a double-headed micromanipulator (Narashigi) so that its point was within a few micrometres of the recording electrode. The relative positions of the points of the electrodes were such that the muscle fibre could be impaled with the recording electrode, leaving the ACh pipette close to (but not penetrating) the fibre. ACh was ejected from the pipette by positive pulses of 5–50 msec duration and regulated at values of 1–50 nA by a constant current supply. A negative backing voltage prevented leakage of ACh from the pipette. The voltage was adjusted so that the fibre gave a maximal response to a given pulse applied but without occurrence of any depolarization prior to the test pulse. The resistance of the micropipettes was usually 50–100 mΩ. The sensitivity of fibres was measured approximately half-way between the end-plate zone and the insertion of the muscle with the skin.

Assessment of the extent of innervation of e.d.b. by the foreign nerve was achieved by comparing the isometric tensions developed by the muscle during repetitive nerve stimulation or direct supramaximal stimulation at 50 Hz. In some muscles intracellular recordings were made with micro-electrodes to see if end-plate potentials (e.p.p.s.) could be recorded during nerve stimulation.

The Ringer solution had the following composition (mM) NaCl 111; KCl 3; CaCl$_2$ 1·8; glucose 10.

RESULTS

Neuromuscular transmission after botulinum toxin was blocked within 3 days in the injected muscles which usually remained completely paralysed to nerve stimulation for at least 3 months. Some muscles were still paralysed when examined after 5 months. It was possible to record occasional subthreshold e.p.p.s of up to 3 mV with micro-electrodes during repetitive nerve stimulation (50 Hz) at end-plates in the paralysed muscles. In muscles which were recovering from the effects of the toxin (3–5 months) high frequencies of nerve stimulation (100–200 Hz) caused facilitation of e.p.p.s so that some fibres contracted.

ACh sensitivity. The tensions developed by e.d.b. muscles in the presence of ACh are shown in Figs. 1 and 2. The mean response (± S.D.) of thirteen muscles examined 2–7 weeks after denervation was 54·6 ± 17 % whereas the response of the nine contralateral muscles examined during this period was 20·2 ± 16·2 %, indicating that the denervated muscles had become supersensitive to ACh. The response to ACh of fourteen muscles examined 2–12 weeks after the injection of botulinum was 21·7 ± 14·9 % whereas the response of nine contralateral muscles (which were not paralysed) was 15·9 ± 12·4 %. The mean response of the muscles injected with botulinum toxin was slightly greater than that of the non-injected muscles, but the difference was not significant.

In six frogs the sensitivity of e.d.b. muscles to 3×10^{-5} M-carbamylcholine chloride was determined 16–17 days after denervation or injection of botulinum toxin. The mean tension ± S.D. developed by three denervated muscles in the presence of carbamylcholine was 19·1 ± 5·9 % whereas that of the contralateral muscles was 8·8 ±

5·1 %. The response of the three botulinum toxin injected muscles was $9·1 \pm 3·4$ % which was similar to that of the non-injected muscles ($10·7 \pm 5·4$ %). This result indicates that it is unlikely that the differences in sensitivity to ACh after denervation or botulinum could have been due to changes in cholinesterase activity.
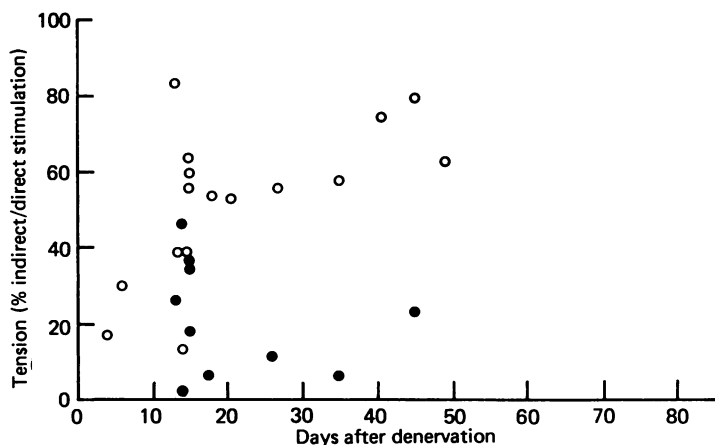

Fig. 1. The tensions developed by denervated ($\bigcirc$) and control ($\bullet$) e.d.b. muscles in the presence of 3×10^{-5} M-ACh. The tensions are expressed as % responses to direct stimulation at 50 Hz. Note that after 2 weeks the response of the denervated muscles is greater than that of the control, indicating the development of supersensitivity to ACh.
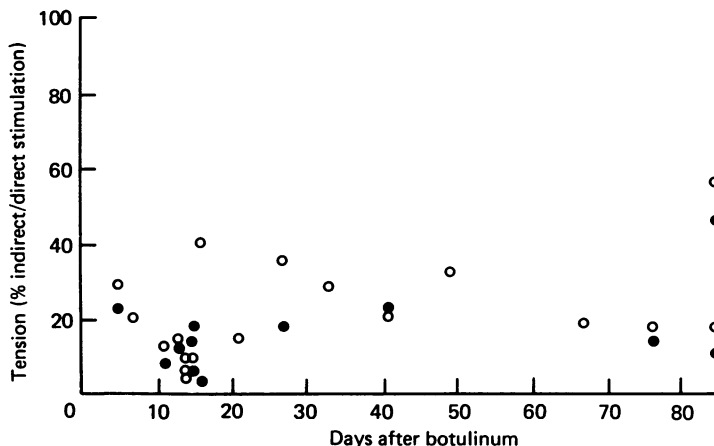

Fig. 2. The tensions developed by botulinum injected ($\bigcirc$) and control ($\bullet$) e.d.b. muscles in the presence of 3×10^{-5} M-ACh. The tensions are expressed as % responses to direct stimulation at 50 Hz. Note that the responses of the botulinum injected and the control muscles to ACh are similar.

The sensitivities of fibres in cutaneous pectoris muscles to ionophoretically applied ACh are shown in Tables 1 and 2. In eight muscles examined 15–29 days after denervation every fibre tested (except one) showed extra-junctional sensitivity > 0·1 mV/nC. Most fibres (fifty-nine out of seventy-six) had sensitivities > 1 mV/nC. Reinnervation occurred after about 3 weeks when nerve stimulation caused the contraction of some muscle fibres.

After the injection of botulinum toxin many fibres did not develop extra-junctional sensitivity to ACh. In five muscles examined 16–34 days after the injection of botulinum only nine out of fifty-two fibres were found to be sensitive. In the eight muscles examined 41–171 days after botulinum the proportion of fibres with extra-junctional sensitivity was greater (sixty-two out of ninety-seven tested) but of these only thirty-

TABLE 1. The sensitivity of cutaneous pectoris muscle fibres to ionophoretically applied ACh after denervation

Days after denervation	ACh sensitivity (mV/nC ± s.d.)	Numbers of fibres with sensitivities:			
		> 10 mV/nC	1–10 mV/nC	0·1–1 mV/nC	< 0·1 mV/nC
10	0·4 ± 0·4	0	0	7	4
15	0·7 ± 0·5	0	2	8	0
16	12·5 ± 7·9	7	4	0	0
20	40·8 ± 37·5	8	2	0	0
21	25·4 ± 25·6	4	1	0	0
22	4·1 ± 2·4	0	10	0	0
27	4·8 ± 5·7	1	7	2	0
28	1·6 ± 1·9	0	4	6	0
29	19·2 ± 17·8	6	3	0	1

TABLE 2. The sensitivity of cutaneous pectoris muscle fibres to ionophoretically applied ACh after injection of botulinum toxin

Days after injection	ACh sensitivity (mV/nC ± s.d.)	Numbers of fibres with sensitivities:			
		> 10 mV/nC	1–10 mV/nC	0·1–1 mV/nC	< 0·1 mV/nC
7	0·02 ± 0·06	0	0	1	9
10	0·05 ± 0·13	0	1	1	9
16	1·18 ± 3·12	1	1	2	6
21	0·08 ± 0·26	0	0	1	9
27	0·00 ± 0·00	0	0	0	10
28	0·09 ± 0·28	0	0	1	9
34	0·02 ± 0·03	0	0	3	9
41	3·90 ± 3·20	1	11	2	0
49	0·00 ± 0·00	0	0	0	12
98	9·10 ± 9·00	3	6	0	0
100	0·29 ± 0·53	0	2	1	7
149	0·77 ± 0·89	0	3	7	4
161	0·23 ± 0·25	0	0	9	3
170	0·61 ± 1·12	0	2	5	7
171	2·26 ± 2·24	0	8	2	2

six had sensitivities > 1 mV/nC. The rate of development of extra-junctional sensitivity in muscles paralysed by botulinum is slower than after denervation and the degree of sensitivity is usually less.

Effects of nerve degeneration on ACh sensitivity. It has been suggested that degeneration of nerve may itself contribute to the development of extra-junctional sensitivity to ACh in denervated muscles (Jones & Vrbova, 1974). In order to investigate the possibility that nerve degeneration could account for the early development of supersensitivity after denervation, a branch of the tibialis nerve which innervates the flexor muscles of the IV toe was implanted into e.d.b. and subsequently caused

to degenerate by cutting the tibial branch of the sciatic nerve at the knee 30 days later. The sensitivity to ACh of e.d.b. muscles was measured at 16 days. The mean tension ($\pm$ S.D.) developed by four operated muscles in the presence of ACh was $12 \pm 1\cdot4\%$ of the response to direct stimulation whereas the response of three unoperated contralateral muscles was $16\cdot3 \pm 1\cdot5\%$. This result indicates that nerve degeneration by itself does not cause the development of supersensitivity to ACh.

TABLE 3. The tensions developed by iliofibularis muscles in the presence of 3×10^{-5} M-ACh after denervation with or without chronic stimulation (50 Hz, 1 sec/60 sec). The tensions are expressed as % responses to direct stimulation at 50 Hz

Denervated muscles			Unoperated contralateral muscles
Days after denervation	Days of stimulation	ACh response	ACh response
17	—	23	4
16	—	13	2
17	—	12	4
14	—	8	7
16	10	15	3
17	14	14	2
14	11	12·5	2·5
14	14	24·1	8·7

The effects of denervation and chronic stimulation of iliofibularis on ACh sensitivity are shown in Table 3. The mean tension ($\pm$ S.D.) developed by eight normally innervated muscles in the presence of ACh was $4\cdot1 \pm 2\cdot5\%$ of their response to direct stimulation (50 Hz). The response to ACh of four muscles examined 2–3 weeks after denervation was $14 \pm 6\cdot4\%$ which was similar to the response of four denervated muscles $16\cdot4 \pm 5\cdot2\%$ which had received chronic electrical stimulation (50 Hz for 1 sec every 60 sec). Chronic stimulation under the conditions used in this experiment did not affect the development of supersensitivity to ACh in denervated muscles of the frog, although similar frequencies of stimulation suppress extra-junctional sensitivity in denervated skeletal muscle of the rat (Lømo & Rosenthal, 1972; Lømo & Westgaard, 1975).

Innervation of muscle by an implanted foreign nerve. The tensions developed by e.d.b. during stimulation of the implanted nerve after ligaturing the original nerve or injecting botulinum toxin are shown in Fig. 3. The mean tension ($\pm$ S.D.) developed by seven muscles examined 24–71 days after the implantation of the foreign nerve but without further treatment was $2\cdot1 \pm 3\cdot3\%$ of the response to direct stimulation. The innervation of fibres in these muscles by the foreign nerve may have been the result of local damage caused by the implantation of the extra nerve (Miledi, 1963). The mean response of fourteen muscles to stimulation of the implanted nerve 19–62 days after the injection of botulinum toxin was $3\cdot2 \pm 3\cdot8\%$ indicating that during the first 2 months of paralysis by botulinum most muscle fibres had not become innervated by the foreign nerve. However, in four muscles examined 90–98 days after the injection of toxin the mean tension developed by muscles during stimulation of the implanted nerve was $34\cdot2 \pm 14\cdot2\%$, indicating that a large proportion of fibres had become innervated by the implanted nerve.

After denervation of e.d.b. by ligaturing its original nerve the rate of innervation of the muscle by the implanted nerve was faster than after the injection of botulinum toxin. The mean response to stimulation of the implanted nerve of seven muscles examined 25–70 days after denervation was $44 \cdot 7 \pm 25\%$ of the response to direct stimulation. In a further six muscles examined 25–55 days after ligaturing the original
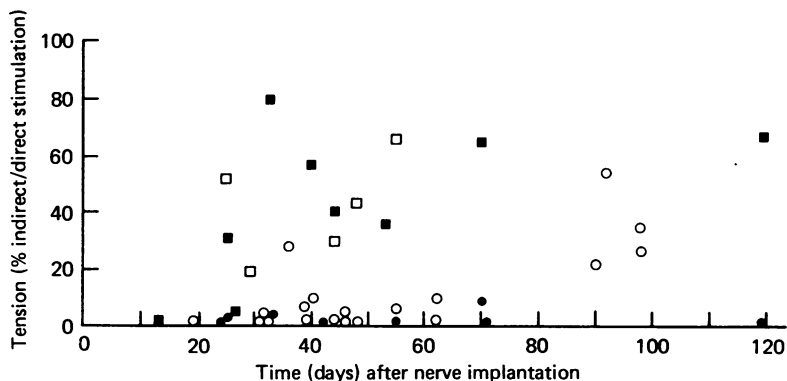

Fig. 3. The tensions developed by e.d.b. during repetitive stimulation (50/sec) of the implanted nerve as % response to direct stimulation after nerve implantation alone (●), injection of botulinum toxin (○), ligaturing the original nerve (■), or injection of botulinum toxin and ligature of the original nerve (□).

nerve and injecting botulinum toxin the mean response to stimulation of the implanted nerve was $42 \pm 18 \cdot 4\%$ of the response to direct stimulation. This indicated that the injection of botulinum itself did not prevent the formation of synapses by the implanted nerve.

DISCUSSION

The mechanisms by which nerves trophically influence muscle have been studied mainly in rats and mice and the importance of activity in controlling ACh sensitivity and in preventing innervation by foreign nerves is well established (Lømo & Rosenthal, 1972; Jansen *et al.* 1973; also reviews by Purves, 1976 and Fambrough, 1979). In the mouse, the soleus muscle becomes supersensitive within 3 days of denervation or injection of botulinum toxin and the degree of supersensitivity is similar in both cases (Tonge, 1974*a, b*). Similar findings were reported by Lømo & Rosenthal (1972) in muscles of the rat, which were denervated or paralysed by blocking the conduction of nerve action potentials with local anaesthetic. Pestronk, Drachman & Griffin (1976) however, found that denervation produced a greater increase in ACh receptors (as measured by the binding of ^{125}I-α-bungarotoxin) in skeletal muscle of the rat than did botulinum toxin.

In other species of mammals greater differences have been observed in ACh sensitivity of denervated muscles and those paralysed by other methods. Robert & Oester (1970) failed to observe any change in the sensitivity of muscles in the rabbit to close arterial injection of ACh after the blockade of the conduction of action potentials with local anaesthetic. Gilliat, Westgaard & Williams (1978) found that the rate of development of extra-junctional sensitivity to ACh was slower and its intensity less

in muscles which were paralysed by pressure-induced demyelination of the nerve than in denervated muscles in the baboon.

The results of the present investigation indicate that, in the frog, denervation of skeletal muscle produces effects on muscle sensitivity to ACh and its ability to accept foreign innervation different to those of paralysis by botulinum toxin. Denervated muscle fibres developed extra-junctional sensitivity to ACh within 2 weeks, confirming the findings of Nasledov & Thesleff (1974) and Dreyer & Peper (1974), whereas in muscles paralysed by botulinum toxin most fibres did not develop extra-junctional sensitivity until after 6 weeks. Denervated muscles readily became innervated within 4–5 weeks by the implanted foreign nerve, which was similar to the findings of Grinnell, Letinsky & Rheuben (1979) whereas a comparable degree of foreign innervation of muscles paralysed by botulinum toxin did not occur until after about 3 months. These findings suggest that the nerve may be able to control sensitivity to ACh and prevent innervation by other nerves by some other mechanism which does not involve muscle activity. The neural control of certain physiological properties of slow muscle fibres and the sensitivity to ACh of parasympathetic ganglion cells of the frog heart also seem to be mediated independently of synaptic transmission (Miledi & Spitzer, 1974; Schmidt & Stefani, 1977; Dennis & Sargeant, 1979).

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