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ABSENCE OF ACTION POTENTIALS IN FROG SLOW MUSCLE FIBRES PARALYSED BY BOTULINUM TOXIN

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SUMMARY

1. As shown previously, slow muscle fibres of the frog develop the ability to produce action potentials in 2 weeks after denervation.

2. Normal transmission at the slow fibre neuromuscular junction gives a junction potential of 11 mV average (range 6–20 mV), caused by summation of potentials from several motor axons.

3. Botulinum toxin injected intramuscularly into the iliofibularis blocks the neuromuscular junction of slow fibres in 6 days at room temperature. Single nerve stimuli give junction potentials of 0·05 mV average (range 0–1·6 mV). Contraction of slow fibres in response to tetanic stimulation is eliminated.

4. No action potentials could be elicited in botulinum treated fibres, even 6½ weeks after injection of toxin. This finding is discussed in relation to the possible involvement of a 'trophic' factor regulating the action potential mechanism in frog slow muscle fibres.

INTRODUCTION

In contrast to twitch muscle fibres, the slow muscle fibres of the frog are normally incapable of generating an action potential (Kuffler & Vaughan Williams, 1953*a*; Burke & Ginsborg, 1956*a*; Peachey, 1961; Orkand, 1963; Stefani & Steinbach, 1969; Forrester & Schmidt, 1970). However, upon denervation slow fibres develop the ability to generate action potentials in response to depolarizing current pulses (Miledi, Stefani & Steinbach, 1971). Furthermore, slow muscle fibres which are experimentally innervated by axons previously going to twitch fibres are also capable of generating action potentials (Stefani & Schmidt, 1972*b*). When these experimentally innervated muscles later receive their original innervation, the ability to produce an action potential is lost

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(Miledi *et al.* 1971; Stefani & Schmidt, 1972*a*). Since this membrane property is regulated by the presence or absence of a particular nerve, it is of interest to ask whether the control is exerted by patterns of muscle activity or by some 'trophic' interaction between nerve and muscle.

Botulinum toxin acts presynaptically at the skeletal neuromuscular junction, blocking the release of transmitter evoked by nerve stimulation, and having no direct effect on the sensitivity of the post-synaptic muscle membrane (Burgen, Dickens & Zatman, 1949). Conduction of action potentials along nerve terminals of intoxicated preparations appears normal (Harris & Miledi, 1971). However, when transmission to twitch muscle fibres is blocked for prolonged periods the extrajunctional muscle membrane becomes highly sensitive to acetylcholine (ACh) (Thesleff, 1960; Lømo & Rosenthal, 1972; N. Spitzer, unpublished). The experiments reported here were made to see if the action potential mechanism would develop in slow fibres after blocking neuromuscular transmission (and thus muscle activity) with botulinum toxin.

METHODS

Experiments were made on the iliofibularis muscle of frogs (*R. temporaria*) of both sexes.

Operations. Surgical procedures were carried out on animals under ether anesthesia (Miledi, 1960). For the injection of toxin a small incision was made in the skin on the back of the right thigh and a solution injected with a microsyringe into the middle one-third of the iliofibularis muscle, identified by its prominent, white, distal tendon. Within 3–4 days animals began dragging their right legs. It was found that 200 MLD (mouse lethal dose) in 2 μl. was the maximum dose consistent with long term survival of the animals. Higher doses blocked transmission more completely, but the toxin also blocked the lymph heart; frogs were unable to maintain their water balance and became swollen and oedematous. Such systemic effects of the toxin led to death within a few weeks. Toxin (Type A, from Porton Down, 10^5 (MLD)/mg) was dissolved in 1 part 1 % Na_2HPO_4 (pH 6·5) and 9 parts normal Ringer, which had the following composition in m-mole/l.: NaCl, 115·6; KCl, 2·0; $CaCl_2$, 1·8; PO_4, 1·0. To denervate muscles an incision was made at the hip, the sciatic nerve sectioned and a portion removed. Animals were kept on moist paper towels in small tanks (5–6/tank) at room temperature, and intermittently fed with small chunks of liver. Control responses to indirect stimulation of slow muscle fibres at 1 Hz were obtained from preparations of normal iliofibularis muscle in which the twitch fibres on either side of the central tonus bundle had been cut at the start of the experiment to reduce movement artifacts. To evaluate synaptic transmission at higher frequencies the transverse tubular system of twitch, and perhaps also slow, fibres was disrupted by osmotic shock (Eisenberg, Howell & Vaughan, 1971). Muscles were incubated in 400 mM glycerol for 3 hr at room temperature and then returned to high Ca^{2+} Ringer (see below) and examined 1 hr later. This procedure abolished nearly all contraction to stimulation at 50 Hz. The absence of any effect on slow fibres previously reported (Stefani & Steinbach, 1968) may have been due to the briefer period of incubation (1 hr); perhaps equilibration of the glycerol solution in the T system takes longer in slow than in twitch fibres.

Intracellular recording. Both recording and current passing micropipettes were filled with 3 M potassium acetate and had resistances of 15–40 MΩ. The current pipette was connected by a chlorided silver wire and a 50–100 MΩ series resistance to a DC voltage supply in parallel with a square-pulse generator. Current was monitored across a 50 kΩ resistance from the bath to ground. Since slow fibres are easily damaged by the insertion of micro-electrodes, with considerable loss in resting potential (Stefani & Steinbach, 1969), the DC source was used to pass a steady hyperpolarizing current across the membrane to bring the resting potential (E_{RP}) to -90 mV (E_m). The electrical properties of slow fibre membranes and synaptic transmission were studied at this membrane potential.

Muscles were pinned at each end to clear plastic (Sylgard) on the bottom of the bath and the connective tissue along the lateral margins pulled out to the sides and pinned down. This procedure exposed more slow fibres in the central tonus bundle to the surface and improved transillumination of the superficial fibres by thinning the body of the muscle. All surface fibres were examined during each traverse of the muscle from one margin to the other, at several points along its length. Single muscle fibres were visualized with a compound microscope at a magnification of 190 × during impalement; the two electrodes were separated by about 100 μm.

One to five slow muscle fibres were examined in a muscle.

After stimulating the nerve with square pulses of 0·1 msec duration, at frequencies up to 50 Hz, and observing the muscle under the microscope to determine the gross presence or absence of contraction, the normal Ringer was replaced with a solution containing an additional 10 m-mole/l. $CaCl_2$ (total 11·8 m-mole/l.). The higher Ca^{2+} ion concentration makes impalements both easier and more stable because it promotes sealing of the membrane around the electrodes. High Ca^{2+} also helps in the identification of slow fibres because of its marked effect in prolonging their membrane time constant (Stefani & Steinbach, 1969).

Prostigmine (10^{-6} g/ml.) was present in all bathing solutions. Most experiments were performed at room temperature (22° C), but a few were at 5° C.

RESULTS

Action potentials in denervated slow muscle fibres

Both denervated and normally innervated slow muscle fibres can be distinguished from twitch (fast) fibres by the electrical characteristics of their membranes (Miledi & Stefani, 1970; Miledi, Stefani & Steinbach, 1971). The membrane time constant of the former often exceeds 200 msec, while that of the latter is about 20 msec. This membrane property was used to identify slow muscle fibres.

In confirmation of previous results (Miledi *et al.* 1971) 2 weeks after transection of the sciatic nerve the slow fibres of the denervated muscle were able to produce action potentials. Resting potentials of 40–50 mV were obtained, and the membrane potential then held at -90 mV. Fig. 1 illustrates a typical response from a 14-day denervated slow fibre to depolarizing current pulses. The threshold for spike initiation occurred at -45 mV. Action potentials were not observed in slow fibres denervated for 8 or 10 days, but were easily elicited from all slow fibres examined in preparations denervated more than 2 weeks. See Table 1. In contrast,

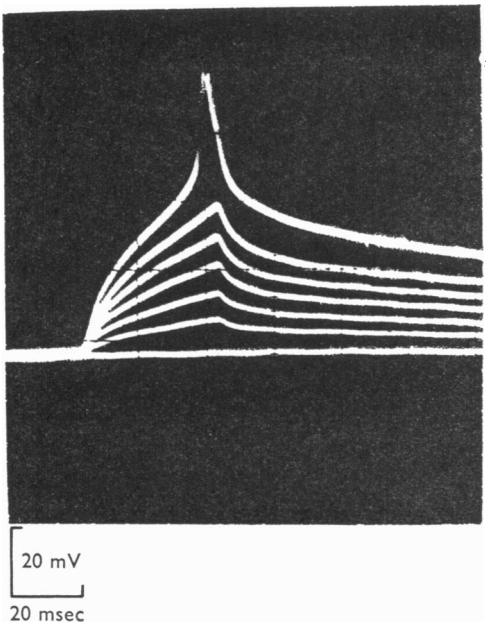

Fig. 1. Action potential from a slow muscle fibre elicited by depolarizing current pulses. Iliofibularis muscle denervated 14 days. Strongest pulse 4×10^{-8} A. RP (resting potential) -46 mV; E_m (membrane potential) -90 mV.

TABLE 1. Time course of appearance of action potentials in frog slow muscle fibres following denervation. Twitch fibres in the same muscles all had high resting potentials and gave action potentials on stimulation. RP (resting potential after impalement); E_m (membrane potential at which fibre examined); AP (presence or absence of action potential in response to depolarizing current pulse)

Days denervated	Slow fibres		
	RP (mV)	E_m (mV)	AP
8	−52	−90	−
	−53	−90	−
10	−46	−90	−
	−41	−90	−
	−48	−90	−
14	−46	−90	+
	−43	−90	+
19	−50	−90	+
	−52	−90	+
	−42	−90	+
26	−43	−90	+
	−46	−90	+

denervated twitch fibres examined in all of the same preparations had resting potentials greater than 80 mV on impalement and gave action potentials in response to depolarizing currents.

Normal transmission in slow fibres

It was important to assess to what degree neuromuscular transmission was impaired by botulinum intoxication. A quantitative estimate of normal synaptic transmission at slow fibre neuromuscular junctions, at a stimulation frequency of 1 Hz, was made on preparations in which most of the twitch fibres had been inactivated by transection (see Methods). In six fibres (four muscles) every stimulus to the nerve gave rise to a junction potential in the muscle, and the response in these fibres was always between 6 and 20 mV (mean 11 mV). Fig. 2 shows potentials from a normal fibre studied in this way; the mean junction potential amplitude in this fibre was 11·5 mV (range 9·1–18·2). These values are comparable to those reported previously for the iliofibularis muscle (Kuffler & Vaughan Williams, 1953*a*; Burke & Ginsborg, 1956*a*, *b*; Orkland, 1963), and for slow fibres in the rectus abdominis (Forrester & Schmidt, 1970), although it was expected that holding potentials of $E_m = -90$ mV would yield somewhat higher values (Burke & Ginsborg, 1956*b*). The actual level of transmission *in vivo* may be smaller than the values reported here, as the prostigmine and high Ca^{2+} solution used in these experiments would be expected to have increased the amplitude of the junction potential as well as prolonged its decay time.

The normal frequency of impulse traffic along the nerves to the tonus bundle of the iliofibularis is not known, but stimulation at 50 Hz was chosen to test maximal transmission. Kuffler & Vaughan Williams (1953*b*) demonstrated that maximum tension is generated by the slow fibres at that frequency, higher frequencies serving only to increase the rate of rise of tension. These tests were carried out after treating the muscles with glycerol (see Methods). Slow fibres in glycerol treated muscles in which excitation and contraction were uncoupled had resting potentials in the same range as untreated fibres; Stefani & Steinbach (1968) have shown that this treatment does not significantly impair neuromuscular transmission at 1 Hz. The response of a fibre to nerve stimulation at different frequencies is shown in Fig. 3. Stimulation at 1 Hz gave a junction potential of 11·6 mV mean amplitude (range 10·0–14·2). A train of stimuli at 50 Hz produced a sustained depolarization of 40–50 mV (four fibres in three muscles) and could be obtained repeatedly from the same fibre. Two of the four fibres gave a small contraction in response to this tetanic stimulation; no contraction was detectable in the other two.

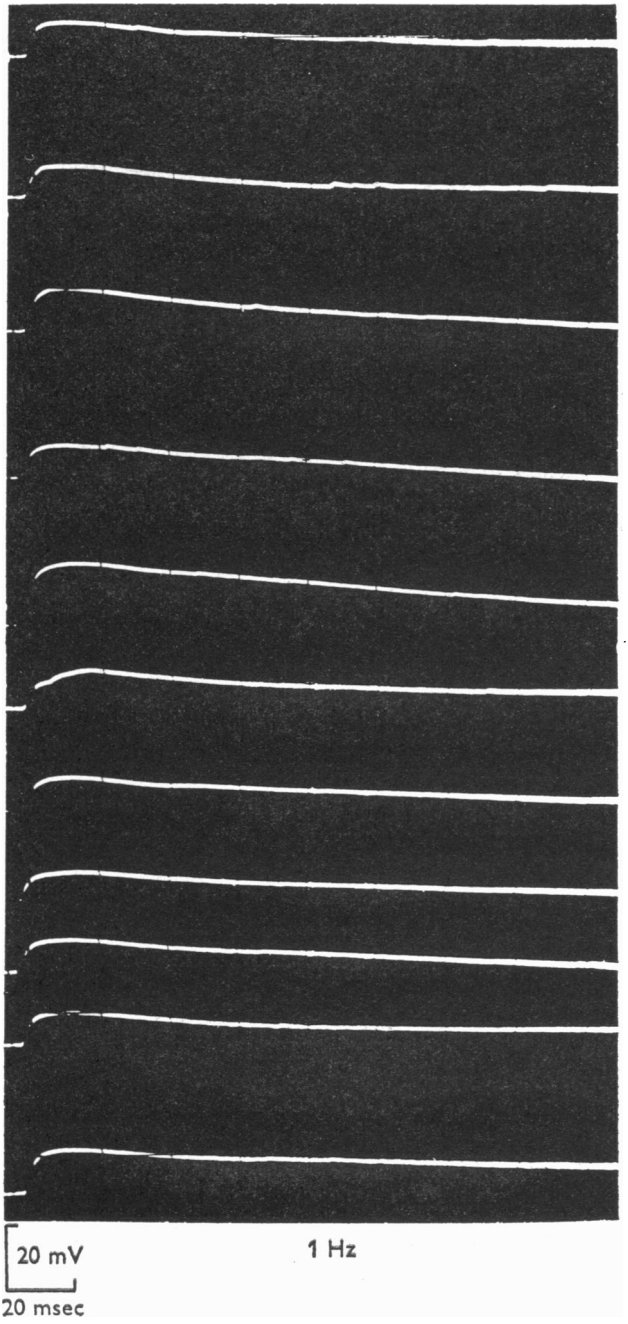

Fig. 2. Transmission in normal slow muscle fibre; adjacent twitch fibres cut. Responses to stimulation at 1 Hz. RP -43; E_m -90 mV.

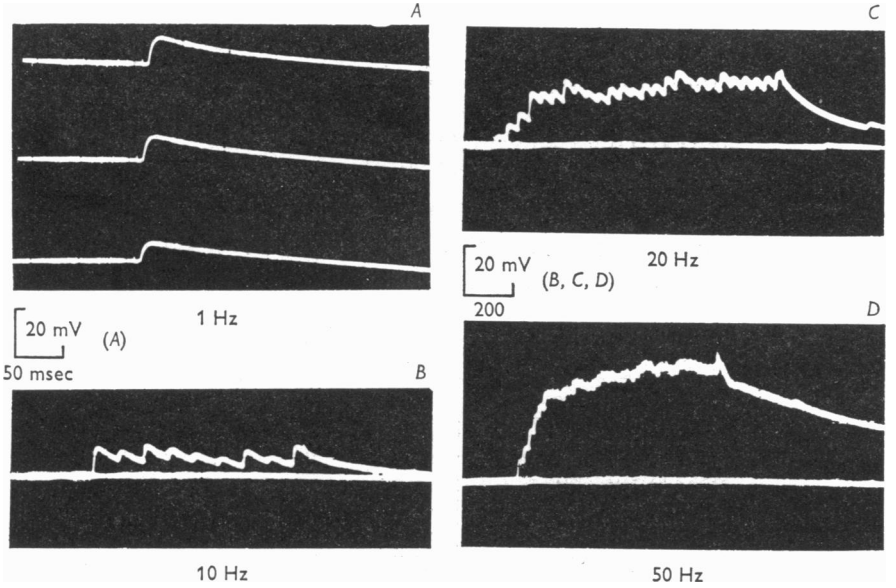

Fig. 3. Transmission in a normal slow muscle fibre following glycerol treatment. Stimulation frequency *A*, 1 Hz; *B*, 10 Hz; *C*, 20 Hz; *D*, 50 Hz. RP −48, E_m −90 mV.

Block of transmission with toxin: onset and extent of block

It was of interest to examine slow muscle fibres whose synapses had been blocked with botulinum toxin for periods equal to and greater than 14 days, i.e. longer than the time of denervation required for the development of the action potential mechanism. Accordingly the onset and degree of neuromuscular block were evaluated at various times after application of botulinum.

Three to 4 days after the intramuscular injection of 200 MLD of toxin the iliofibularis gave no twitch response, or slow contracture, to electrical stimulation at frequencies from 1 to 10 Hz. Tetanic stimulation at 20 and 50 Hz gave rise to intermittent twitching of a few fast fibres at the margins of the muscle and a small, slow contracture of the tonus bundle in the midline.

At 5–6 days after the application of botulinum the iliofibularis failed to contract after stimulation at 50 Hz, as observed under the compound microscope at 100× magnification. Subsequent intracellular recording from individual slow fibres provided more detailed information about the level of block. Fig. 4*A* shows responses from a slow fibre 6 days after injection of toxin, stimulated at 1 Hz. There are many failures of transmission (116 in 151 trials) and when a response occurs it is much smaller

than control junction potentials observed in normal slow fibres. If transmitter release at slow fibre neuromuscular junctions obeys Poisson statistics, the quantal content (m) of release will be given by m = ln (no. of trials/no. of failures) = 0·26 (del Castillo & Katz, 1954). Stimulation at 20 and 50 Hz (4 *B*, *C*) which would produce a large depolarization and a contracture in a normally innervated fibre, here yielded a maximal depolarization of only 3–4 mV. This tetanic stimulation was repeated many times without dislodging the electrodes from the cell; no muscle contraction was detectable.

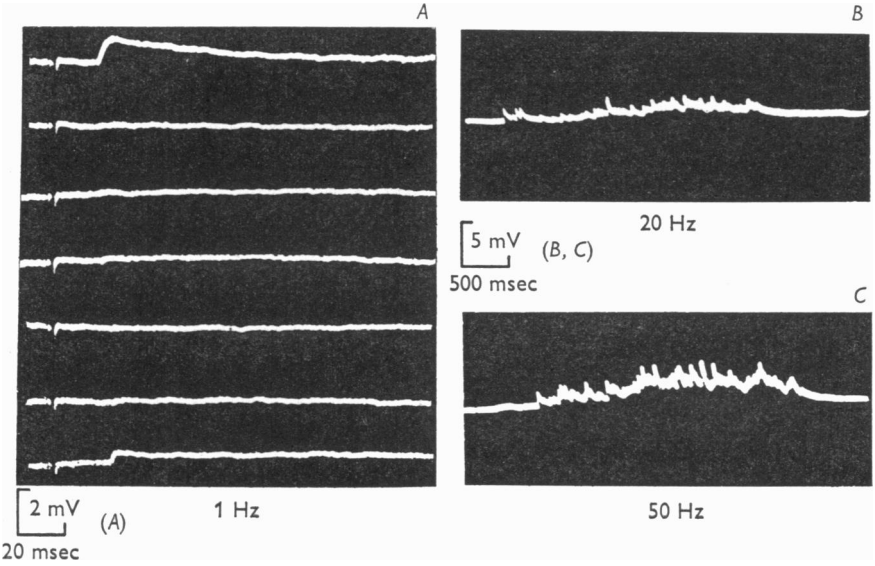

Fig. 4. Transmission in a slow fibre from a muscle injected with 200 MLD botulinum toxin 6 days previously. Compare with Fig. 2. *A*, consecutive traces, stimulation 1 Hz, 151 trials, thirty-five responses. m = 0·26. *B*, stimulation 20 Hz. *C*, stimulation 50 Hz. RP −42, E_m −90 mV.

The neuromuscular block obtainable with this dose of toxin appeared to be fully developed by 5–6 days; preparations examined at later times showed the same degree of block (see Table 2). It was important to ascertain the degree of impairment of transmission in every fibre examined, since a slow fibre was occasionally encountered in which there were no failures of synaptic transmission although the amplitude of the junction potential was still much less than in normally innervated fibres. These fibres were not studied further.

TABLE 2. Evaluation of block of neuromuscular transmission to slow fibres by botulinum toxin. Short term. The mean amplitude of the junction potential at stimulation of 1 Hz is reduced by a factor of 200 in 5–6 days. Mean = mean response/trial. m = ln trials/failures. Other abbreviations as in Table 1

Days since injection	Slow fibres			Nerve stimulation at 1 Hz				
	RP (mV)	E_m (mV)	AP	Trials	Responses	Range (mV)	Mean (mV)	m
5	−46	−90	–	104	15	0–0·8	0·07	0·15
6	−42	−90	–	50	4	0–1·0	0·05	0·08
6	−37	−90	–	96	10	0–0·8	0·06	0·11
6	−37	−90	–	30	0	—	0	0·02*
6	−42	−90	–	151	35	0–1·2	0·10	0·26
8	−50	−90	–	124	1	0–0·7	0·005	0·01
8	−31	−90	–	140	3	0–0·5	0·01	0·02
							0·04 mV Mean	0·09 Mean

* Assumes 0·5 response.

Slow muscle fibres after blocking transmission for prolonged periods

As mentioned earlier, the membrane of denervated muscle fibres acquires the action potential mechanism within 2 weeks. In contrast, no action potentials were seen in innervated slow fibres after botulinum intoxication, even more than 6 weeks after toxin injection. For instance,

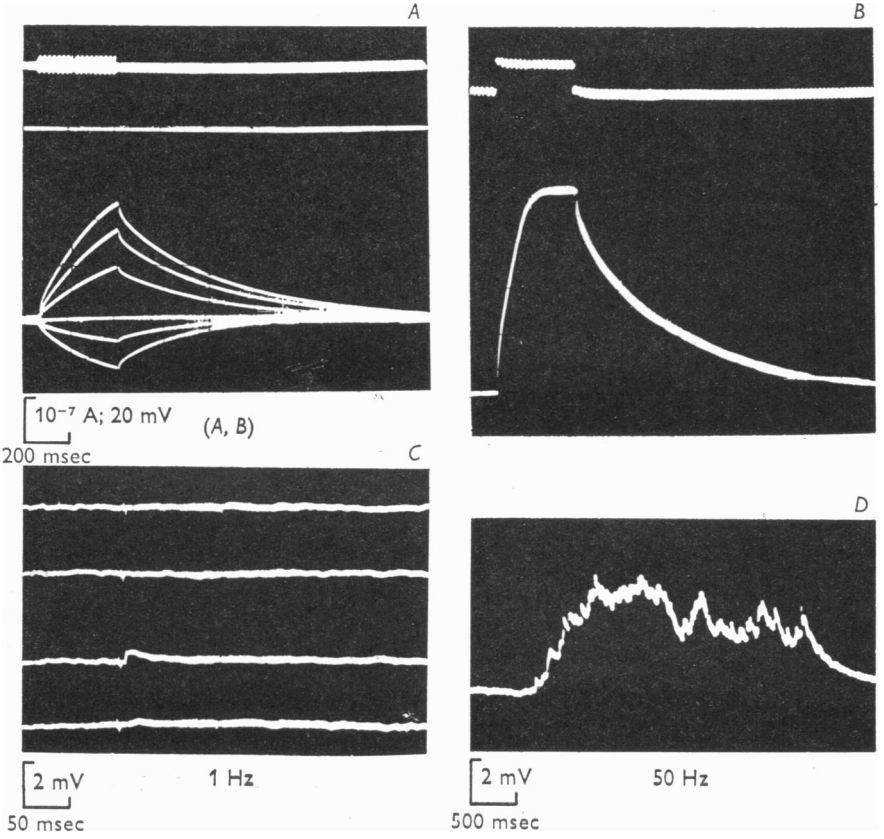

Fig. 5. Records from a slow fibre exposed to toxin 19 days previously. *A*, *B*, responses of muscle fibre to current pulses. Compare with Fig. 1. *C*, stimulation of nerve at 1 Hz, consecutive traces, 293 trials, nineteen responses. $m = 0{\cdot}07$. *D*, nerve stimulation, 50 Hz. RP -64, $E_m - 90$ mV.

Fig. 5 shows results obtained from a preparation examined 19 days after the injection of toxin. The degree of block was evaluated by stimulation at frequencies of 1 and 50 Hz, and is comparable to that seen at 5–6 days after exposure to toxin. Large depolarizing pulses, bringing E_m from -90 to 0 mV were ineffective in eliciting an action potential. Similar

TABLE 3. Long term evaluation of block of neuromuscular transmission to slow fibres by botulinum toxin. Block is comparable to that seen at 5–6 days. Action potentials were absent in slow fibres that had been blocked for periods up to 5½ weeks. Abbreviations as in Table 2. † Degree of block examined only by nerve stimulation at 50 Hz; peak response 3·6 mV.

Days since injection	Slow fibres			Nerve stimulation at 1 Hz				
	RP (mV)	E_m (mV)	AP	Trials	Responses	Range (mV)	Mean (mV)	m
19	−64	−90	–	293	19	0–1·6	0·08	0·07
	−50	−90	–	18	0	—	0	0·03*
	−42	−90	–	80	0	—	0	0·007*
26	−50	−90	–	92	2	0–0·7	0·01	0·02
	−48	−90	–	107	28	0–1·1	0·15	0·30
29	−54	−90	–	209	58	0–1·5	0·18	0·32
	−55	−90	–	110	6	0–1·1	0·04	0·05
45	−43	−90	–	176	21	0–1·0	0·06	0·13
	−28	−90	–	†				
	−44	−90	–	82	0	—	0	0·007*
							0·05 mV Mean	0·10 Mean

* Assumes 0·5 response.

results were obtained from other fibres with longer times after botulinum intoxication; there was no evidence of regenerative electrical activity (cf. Table 3).

The longest survival following the standard injection of botulinum toxin was 45 days; results from a slow fibre in this preparation are illustrated in Fig. 6. Stimulation at 10 and 20 Hz yielded only very small depolarizations. Assuming that it took 6 days for neuromuscular block to

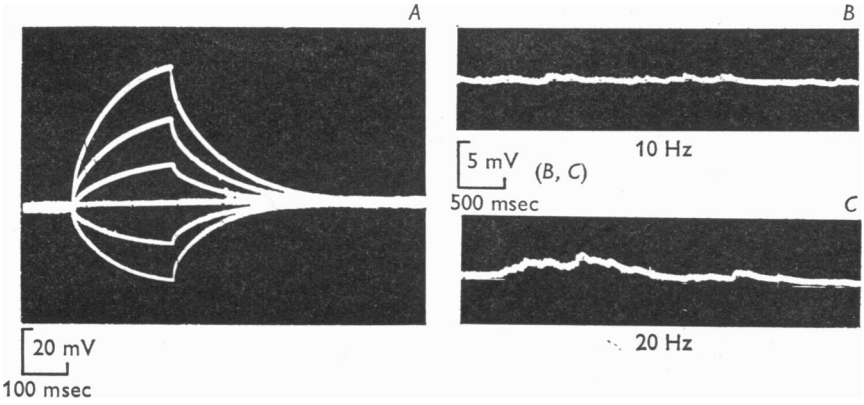

Fig. 6. Slow muscle fibre treated with botulinum toxin 45 days previously. *A*, responses to current pulses; input resistance about 2·6 MΩ. Fibre depolarized to −14 mV during course of examination; no action potential was elicited. *B*, stimulation of nerve at 10 Hz. *C*, stimulation at 20 Hz: Maximal response. Nerve stimulation at 1 Hz gave 0 responses in eighty-two trials. RP −44, E_m −90 mV.

reach this level, this experiment indicates that even after 5½ weeks of such depression of synaptic activity, large depolarizing current pulses did not elicit an action potential. Depolarizing currents applied to twitch fibres in these blocked preparations gave rise to normal action potentials and contractions; these observations demonstrate that the toxin does not have a direct effect on the action potential mechanism already present in muscle.

It may be thought that the toxin, even at the low dose and fairly well localized route of application used, might have some general, systemic effect which prevents the development of the action potential mechanism in slow fibres from the blocked muscle. To test this hypothesis, and provide a further control of the examination procedure, a series of animals were prepared in which the right iliofibularis muscle was injected with toxin while the left iliofibularis was denervated. Both muscles were subsequently examined in the same bath. Records from two slow fibres from such an

animal, 26 days following the operations, are shown in Fig. 7. As expected, the slow fibre from the denervated side was capable of generating action potentials. On the other hand, the slow fibre from the side paralysed by botulinum was not able to generate action potentials even though neuromuscular transmission was blocked as illustrated in Fig. 7 *B*, *C* ($m = 0{\cdot}30$).

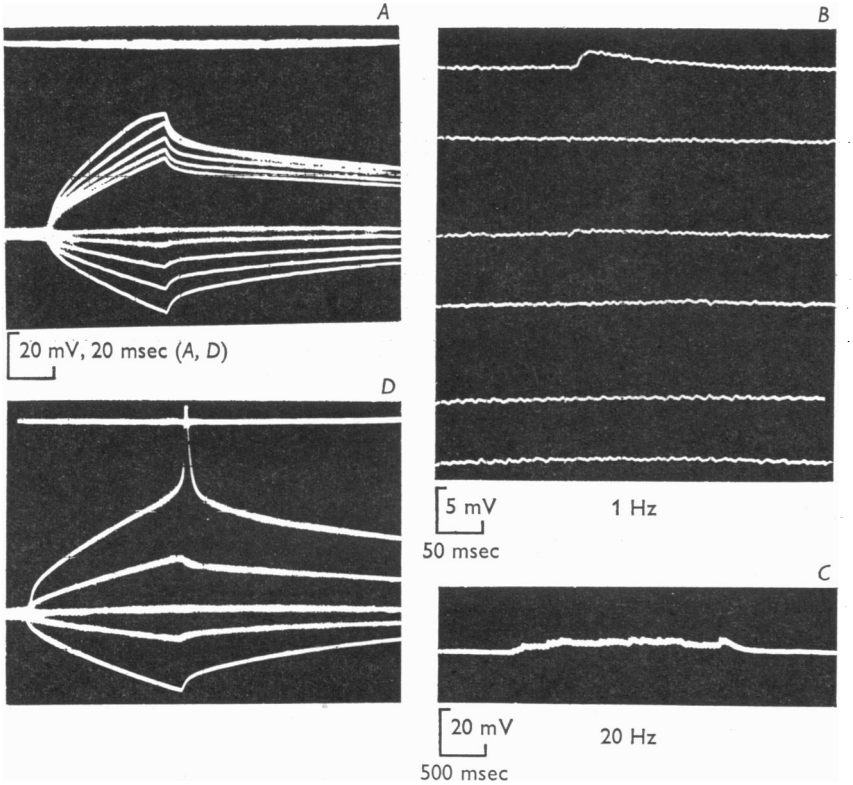

Fig. 7. *A–C*, responses from slow fibre in muscle injected with toxin 26 days earlier. RP -48, E_m -90. *A*, responses to depolarizing and hyperpolarizing current pulses. *B*, stimulation of nerve to injected muscle at 1 Hz; 107 trials, twenty-eight responses. $m = 0{\cdot}30$. *C*, stimulation of nerve to injected muscle at 20 Hz. *D*, responses to current pulses in a slow fibre in the muscle from the opposite leg, denervated when toxin was injected. RP -46, E_m -90. Note action potential arising from injected current pulses.

DISCUSSION

Complete blockade of transmitter release by nerve impulses at twitch fibre neuromuscular junctions has been demonstrated *in vitro* with higher doses of botulinum toxin than used in this study; spontaneous release

continued to occur at a low rate, however (Harris & Miledi, 1971; Spitzer, 1972). The toxin has no detectable direct effect on the sensitivity of the muscle to transmitter or on the conduction of the action potential in the nerve terminals, indicating that it affects the release process itself (Burgen *et al.* 1949; Harris & Miledi, 1971).

Our results suggest that botulinum toxin blocks neuromuscular transmission to slow muscle fibres in the same way that it does in the twitch fibre system, by preventing the release of acetylcholine from motor nerve terminals. We were unable to apply such high doses to the iliofibularis muscle *in vivo*, and accordingly neuromuscular transmission was not fully blocked. Nonetheless, transmission was greatly depressed. Single nerve impulses produced small junction potentials, if any. Considering the ratio of mean junction potential amplitude of intoxicated preparations (0·05 mV; see Tables 2 and 3) to that of normal muscles (11 mV), then the toxin reduced transmitter release more than two hundred fold. Even the maximal summated potential evoked by tetanic stimulation was reduced to 1/10 or less of its control value.

One may envisage that a membrane's ability to generate action potentials depends on the presence of a macromolecule – perhaps a protein – which conveys to the membrane its voltage-dependent Na-permeability. The production of such a molecule and its insertion into the membrane might then be under neural control in a way resembling that of ACh-receptor protein in the muscle (Miledi, 1960). Whatever the basis for the action potential mechanism turns out to be, its induction in denervated slow muscle fibres could be explained in a number of ways.

For instance, it could be that normally innervated slow muscle fibres are incapable of generating action potentials because the mechanism is somehow suppressed by their contractile activity. A second possibility is that the control is exerted by depolarization of the muscle fibre membrane resulting from transmitter action associated with normal nerve impulse traffic. It has been shown (Lømo & Rosenthal, 1972) that muscular activity is an important factor controlling the extent of ACh sensitivity of mammalian twitch muscle.

A role for contraction *per se* in the normal suppression of the development of the action potential mechanism in slow muscle fibres seems remote. The maximum junction potential depolarization we observed after botulinum poisoning was only 4–5 mV; no contraction was detectable even when stimulating the nerve at frequencies of 50 Hz. Moreover, no contraction would be expected to occur from such small depolarization since in slow fibres the threshold for contraction lies at membrane potential of about −50 mV (Nasledov, 1969; Lännergren, 1967).

It would seem that the depolarizing, or any other action of ACh is not

the main factor controlling development of the action potential mechanism, because transmitter release was considerably reduced by botulinum toxin. In spite of this reduction, for periods up to 5½ weeks, the slow fibres remained incapable of generating action potentials. Furthermore, miniature end-plate potentials due to ACh released from Schwann cells are observed in denervated slow muscle fibres (Miledi & Stefani, 1970) which do become capable of generating action potentials. It is possible, but we feel unlikely, that the residual synaptic activity of intoxicated muscles is sufficient to prevent the development of the action potential mechanism.

Another argument against the hypothesis that contractile activity or transmitter action is suppressing the action potential mechanism in slow fibres should be noted. If these fibres are denervated long enough to develop the capacity to produce action potentials and then experimentally reinnervated with axons normally innervating twitch fibres, they continue to possess the ability to generate and propagate action potentials (Miledi & Orkand, 1966; Miledi *et al.* 1971; Stefani & Schmidt, 1972*b*). They retain this ability even though the regenerated end-plates transmit impulses and evoke contractions.

In view of these considerations, it is more likely that the action potential mechanism in slow fibres is under a neural 'trophic' control, which can be exerted in the absence of functional neuromuscular transmission as, for instance, in the muscles paralysed by botulinum toxin, or during regeneration of neuromuscular synapses before transmission is restored (Stefani & Schmidt, 1972*a*). There is good evidence that the ability of slow muscle fibres to generate maintained contractures is also neurally determined. Denervated slow fibres retain this capacity and lose it when innervated by a nerve which normally innervates twitch fibres (Miledi & Orkand, 1966). When these slow fibres are later reinnervated by axons originally running to slow fibres, the maintained contracture response returns; it is interesting that the return of the contracture response occurs before neuromuscular transmission is restored (Elul, Miledi & Stefani, 1968, 1970; Schmidt, 1971). The existence of a 'trophic' influence in a different system may also be indicated by the finding that apparently normally active twitch muscles develop generalized sensitivity to ACh when their nerves are treated with colchicine, which presumably blocks axoplasmic transport without directly affecting electrical activity or muscle metabolism (Albuquerque, Warnick, Tasse & Sansone, 1973).

It has been suggested (cf. Miledi *et al.* 1971) that the slow motor axons release a substance which represses the production, by the muscle, of a protein required for the 'Na channels' involved in action potential generation. The lack of this neural repressor, caused either by denervation or by experimental innervation with fast axons, would induce the synthesis

of the molecules required to produce action potentials. These ideas are consistent with Schmidt & Tong's (1973) recent finding that Actinomycin D prevents the induction of the action potential mechanism in denervated slow muscle fibres.

It is interesting to note that if the action potential mechanism and the distribution of ACh receptors are both under neurotrophic control, then the two systems are somewhat different. The ACh sensitivity of slow muscle fibres exposed to botulinum toxin was not measured in these experiments; however, 1/10 the dose of toxin, when injected into rat soleus muscle, blocked transmission and led to increased extrajunctional sensitivity (N. Spitzer, in preparation). In spite of the fact that botulinum toxin leads to increased ACh sensitivity of mammalian muscle (Thesleff, 1960), it does not induce the action potential mechanism in frog slow muscle fibres. Furthermore, the time course of development of the action potential mechanism and appearance of extrajunctional ACh receptors are dissimilar. The former occurs relatively abruptly during the second week following denervation; the latter occurs slowly and continuously from the first week, reaching a maximum at about 8 weeks. While membrane properties in different systems may be regulated to different degrees by both trophic factors and cell activity, it appears that the Na channels of the slow muscle fibre membrane are normally repressed by a 'trophic' neural influence, the release and action of which are not blocked by botulinum toxin.

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REFERENCES

ALBUQUERQUE, E. X., WARNICK, J. E., TASSE, J. R. & SANSONE, F. M. (1973). Effects of vinblastine and colchicine on neural regulation of the fast and slow skeletal muscles of the rat. *Expl Neurol.* **37**, 607–634.

BURGEN, A. S. V., DICKENS, J. & ZATMAN, L. J. (1949). The action of botulinum toxin on the neuro-muscular junction. *J. Physiol.* **109**, 10–24.

BURKE, W. & GINSBORG, B. L. (1956*a*). The electrical properties of the slow muscle fibre membrane. *J. Physiol.* **132**, 586–598.

BURKE, W. & GINSBORG, B. L. (1956*b*). The action of the neuromuscular transmitter on the slow fibre membrane. *J. Physiol.* **132**, 599–610.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *J. Physiol.* **124**, 560–573.

EISENBERG, R. S., HOWELL, J. N. & VAUGHAN, P. C. (1971). The maintenance of resting potentials in glycerol-treated muscle fibres. *J. Physiol.* **215**, 95–102.

ELUL, R., MILEDI, R. & STEFANI, E. (1968). Neurotrophic control of contracture in slow muscle fibres. *Nature, Lond.* **217**, 1274–1275.

ELUL, R., MILEDI, R. & STEFANI, E. (1970). Neural control of contracture in slow muscle fibres of the frog. *Acta physiol. latinoam.* **20**, 194–226.

FORRESTER, J. & SCHMIDT, H. (1970). An electrophysiological investigation of the slow fibre system in the frog rectus abdominis muscle. *J. Physiol.* **207**, 477–491.

HARRIS, A. J. & MILEDI, R. (1971). The effect of type D botulinum toxin on frog neuromuscular junctions. *J. Physiol.* **217**, 497–515.

KUFFLER, S. W. & VAUGHAN WILLIAMS, E. M. (1953*a*). Small-nerve junctional potentials. The distribution of small motor nerves to frog skeletal muscle and the membrane characteristics of the fibres they innervate. *J. Physiol.* **121**, 289–317.

KUFFLER, S. W. & VAUGHAN WILLIAMS, E. M. (1953*b*). Properties of the 'slow' skeletal muscle fibres of the frog. *J. Physiol.* **121**, 318–340.

LÄNNERGREN, J. (1967). Contractures of single slow muscle fibres of *Xenopus laevis* elicited by potassium, acetylcholine or choline. *Acta physiol. scand.* **69**, 362–372.

LØMO, T. & ROSENTHAL, J. (1972). Control of ACh sensitivity by muscle activity in the rat. *J. Physiol.* **221**, 493–513.

MILEDI, R. (1960). The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. *J. Physiol.* **151**, 1–23.

MILEDI, R. & ORKAND, P. (1966). Effect of a 'fast' nerve on 'slow' muscle fibres in the frog. *Nature, Lond.* **209**, 717–718.

MILEDI, R. & STEFANI, E. (1970). Miniature potentials in denervated slow muscle fibres of the frog. *J. Physiol.* **209**, 179–186.

MILEDI, R., STEFANI, E. & STEINBACH, A. B. (1971). Induction of the action potential mechanism in slow muscle fibres of the frog. *J. Physiol.* **217**, 737–754.

NASLEDOV, G. A. (1969). Interrelationship between membrane potential level and contraction in tonic muscle fibres. *Sechenov. J. Physiol.* **55**, 569–575.

ORKAND, R. K. (1963). A further study of electrical responses in slow and twitch muscle fibres of the frog. *J. Physiol.* **167**, 181–191.

PEACHEY, L. D. (1961). Structure and function of slow striated muscle. In *Biophysics of Physiological and Pharmacological Actions*, ed. SHANES, A. M., pp. 391–411. Washington: Am. Ass. Adv. Sci.

SCHMIDT, H. (1971). Slow muscle fibres in the frog rectus abdominis muscle after denervation. *Proc. int. Eur. Congr. Biophys.* **5**, 375–379.

SCHMIDT, H. & TONG, E. Y. (1973). Inhibition by actinomycin D of the denervation-induced action potential in frog slow muscle fibres. *Proc. R. Soc.* B **184**, 91–95.

SPITZER, N. (1972). Miniature end-plate potentials at mammalian neuromuscular junctions poisoned by botulinum toxin. *Nature, New Biol.* **237**, 26–27.

STEFANI, E. & SCHMIDT, H. (1972*a*). A 'trophic' nerve influence independent of neuromuscular transmission: inhibition of action potential in denervated frog slow fibres. *Pflügers Arch. ges. Physiol.* **335**, R 64.

STEFANI, E. & SCHMIDT, H. (1972*b*). Early stage of re-innervation of frog slow muscle fibres. *Pflügers Arch. ges. Physiol.* **336**, 271–275.

STEFANI, E. & STEINBACH, A. (1968). Persistence of excitation-contraction coupling in 'slow' muscle fibres after a treatment that destroys transverse tubules in 'twitch' fibres. *Nature, Lond.* **218**, 681–682.

STEFANI, E. & STEINBACH, A. B. (1969). Resting potential and electrical properties of frog slow muscle fibres. Effect of different external solutions. *J. Physiol.* **203**, 383–401.

THESLEFF, S. (1960). Supersensitivity of skeletal muscle produced by botulinum toxin. *J. Physiol.* **151**, 598–607.