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OBSERVATIONS ON THE ACTION OF TYPE A BOTULINUM TOXIN ON FROG NEUROMUSCULAR JUNCTIONS

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SUMMARY

1. Progressive block of neuromuscular transmission in frog sartorius and gastrocnemius preparations by haemagglutinin-free crystalline Type A botulinum toxin (BTX) was investigated by *in vitro* application and by injection of the toxin into living animals.

2. Neuromuscular block was characterized by (*a*) decline in amplitude of evoked twitch contractions, (*b*) decline in amplitudes of end-plate potentials (e.p.p.s) and (*c*) changes in statistical characteristics of spontaneous miniature end-plate potentials (m.e.p.p.s).

3. Progress of the block was enhanced by nerve stimulation.

4. A decrease in frequency to less than 0·1/sec and decreased average amplitudes of m.e.p.p.s preceded observable impairment of neuromuscular transmission. These changes occurred as early as 3 hr after injection of the toxin into dorsal lymph sacs.

5. The amplitude distributions of m.e.p.p.s changed from a normal distribution to one that showed an increased skewness toward smaller amplitudes as the block progressed. These changes were first detectable as early as 75 min following addition of the toxin to the bath.

6. At later stages of toxin action, e.p.p.s began to decrease in amplitude and eventually failed altogether. E.p.p.s showed a normal quantal variation at very early stages in the block in Mg^{2+}-treated preparations. At later stages of the block, it was not possible to test the quantal make-up of the e.p.p.

7. At all stages before complete failure it was possible to obtain normal

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or greater than normal degrees of synaptic facilitation with paired stimuli to the nerve. This aspect of the coupling of nerve terminal depolarization to transmitter release appears to be relatively unaffected by BTX.

8. Electrical depolarization of nerve terminals in partially blocked preparations evoked a maintained discharge of m.e.p.p.s with an amplitude distribution similar to that of the spontaneous m.e.p.p.s; hyperpolarization of the terminals evokes a distinctly larger class of m.e.p.p.s. In fully blocked preparations, depolarization of the terminals does not evoke transmitter release whereas hyperpolarization continues to yield the larger class of m.e.p.p.s.

9. It is proposed that the neuromuscular block caused by BTX is due to impairment of a process by which vesicles become charged with transmitter before release.

INTRODUCTION

The toxin produced by *Clostridium botulinum* (BTX), a protein with a molecular weight of 150,000, is known to block the release of acetylcholine (ACh) from the presynaptic nerve terminals in skeletal muscle (Ambache, 1949; Burgen, Dickens & Zatman, 1949; Brooks, 1954, 1956; Thesleff, 1960; Harris & Miledi, 1971) and other cholinergic synapses (Ambache, 1949, 1952; Kupfer, 1958; Shankland, Rose & Donninger, 1971). Since the minimal lethal dose (MLD) of BTX for the mouse is only about 10 pg, i.e. less than 10^8 molecules, it is unlikely that more than 10 molecules of toxin are needed to block a single cholinergic synapse.

BTX does not seem to interfere with ACh synthesis (Burgen *et al.* 1949) and ACh is stored in apparently normal amounts in fully blocked nerve–muscle preparations (Brooks, 1954; Thesleff, 1960). Thus, it appears that the toxin acts on a critical step in the chain of events that link the presynaptic action potential with transmitter release. An alternate possibility is that it may interfere with the processes by which ACh is stored ready to be liberated by the depolarization of the presynaptic membrane.

Previous electrophysiological studies on the effects of BTX have been conducted mainly on fully blocked preparations and relatively little was known about the changes that occur during the onset of the paralysis. For this reason we have attempted to follow the development of the block in single end-plates of the frog sartorius.

In addition, previous work reported in the literature concerning the influence of BTX on neuromuscular transmission was done either with crude, partially purified preparations or with toxin isolated and crystallized by the methods described by Lamanna, McElroy & Ecklund (1946)

and Duff, Wright, Klerer, Moore & Bibler (1957). These preparations contained impurities not found in the toxin preparations used in the present studies.

METHODS

Preparations. The sciatic-sartorius preparation of the frog (*Rana pipiens*) has been employed throughout this work for electrical recording. In most experiments the sartorius muscle was attached, inner surface up, to a layer of Sylgard (Dow-Corning) on the bottom of a Petri dish. To polarize the nerve terminals by applied currents the sartorius muscle was wound around a Perspex rod which could be rotated to allow proper positioning of the junctions with respect to the oil-Ringer interphase. End-plates were located by visual inspection but due to the extremely low frequency or even absence of miniature end-plate potentials (m.e.p.p.s.) the position of the junction was confirmed in some instances by the electrophoretic application of ACh pulses from a pipette.

A few sciatic-gastrocnemius preparations were also used to record the gradual decrease in twitch tension caused by BTX.

Ringer solution. The saline solution employed had the following ionic composition (mM): Na, 113; K, 2; Ca, 3·6; Cl, 122·2. The pH was adjusted to 7·25 with phosphate buffer. High Mg solutions were prepared by mixing normal Ringer solution with 'Mg-Ringer' in which all NaCl had been replaced by isotonic $MgCl_2$ (84 mM). In some instances neostigmine bromide at a concentration of 3×10^{-6} (w/v) was added to the Ringer solution.

Botulinum toxin. The toxin employed was obtained from a type A crystalline preparation having $4{\cdot}3 \times 10^7$ MLD (for a 20 g mouse) per mg of protein. In spite of such a high activity, the neurotoxin made up only 10–20 % of the crystals in the preparation; the rest was represented by another biologically active substance, a powerful haemagglutinin, which invariably co-purified with the toxin but is irrelevant to it. The two substances were separated by the method described by DasGupta, Boroff & Rothstein (1966) and Boroff, DasGupta & Fleck (1968). The toxin fraction obtained, which was free of the haemagglutinin and appeared pure and homogeneous by all criteria used, was stabilized in 20 % sucrose. The final solution contained 5×10^6 mouse MLD/ml. as assayed at regular intervals by the method described by Boroff & Fleck (1966). In most cases, this solution was injected into the dorsal lymph sac of the frog and the animal was sacrificed at different stages of development of paralysis. In a few instances BTX was added to the muscle bath dissolved in Ringer solution to contain from 5×10^4 to 5×10^5 MLD/ml. Ringer. The former procedure was far more convenient in most cases, due to the long time course of development of toxin effects.

Electrical recording. The electrical activity of muscle fibres was recorded intracellularly with glass micro-electrodes filled with a 3 M solution of either KCl or K acetate. Since the m.e.p.p.s. recorded from muscle exposed to BTX often have a low average amplitude, it was essential to minimize noise by employing low resistance micro-electrodes. Most of our recordings were done with pipettes with a tip resistance of between 5 and 10 MΩ and selected for low noise.

Nerve stimulation. The nerve was mounted on a pair of platinum electrodes and stimulated by brief electric pulses via a stimulus isolation unit.

Electrical polarization of the motor nerve terminals. In some experiments the potential difference across the membrane of the motor nerve terminals was altered by externally applied currents, as described by del Castillo & Katz (1954*c*). The sartorius nerve was passed through a paraffin oil–Ringer interphase close to the point where it branches upon entering the muscle. An electric current was applied

between two Ag-$AgCl_2$ electrodes; one in contact with the nerve in the paraffin oil layer and the other in the bath. A fraction of this current penetrates into the nerve fibres at the oil–Ringer interphase giving rise to electrotonic potentials which reach the motor terminals at nearby junctions. The polarizing current was monitored by a 10 kΩ resistor placed between the bath and ground.

Mechanical recording. Nerve–muscle preparations were mounted in a vertical chamber filled with frog Ringer which was aerated continuously. The nerve was raised into a layer of paraffin oil for stimulation. The muscle was fixed to the bottom of the chamber and the opposite end was attached to a Grass Polygraph. The solutions were removed by aspiration into a flask containing 0·1 N-NaOH to inactivate the BTX.

Temperature. All the experiments were done at temperatures of between 21 and 23° C.

RESULTS

Blocking action of BTX in vitro

In a few experiments purified BTX solution diluted 1:10 to 1:1000 (v/v) in Ringer solution was substituted for pure Ringer in a chamber containing a sciatic-gastrocnemius preparation. Twitches recorded in response to nerve stimulation at 1/sec for 1 min test periods at occasional intervals over a period of several hours began to decline in amplitude 2–3 hr after addition of the toxin. Virtually all of the decline in twitch amplitude occurred during the periods of stimulation and there was little recovery following rest periods of 30 min or more. This loss of twitch amplitude was permanent and not due to normal fatigue, as control preparations recovered rapidly following the stimulation periods even after the BTX-treated preparation no longer responded at all. Therefore, in agreement with the results of Hughes & Whaler (1962), development of the neuromuscular block is greatly accelerated by nerve stimulation.

Overt effects of BTX on the intact frog

The dose usually employed was 0·1 ml. (about 10^5 mouse MLD) of purified BTX injected into the dorsal lymph sac. The effects of this dose were apparent 2–3 hr after the injection. The first sign was a change in the habitual resting posture of the frog. Instead of keeping its head high and the forelegs extended, under the influence of the toxin the frog tends to adopt a sagging posture. At the same time the animals appeared to be relatively irresponsive to their environment, although they were still able to jump when stimulated. Subsequently, the level of motor activity decreased further. Tremor was observed in some frogs before they became fully paralysed.

Since respiration in the frog is largely cutaneous, the heart continues to beat in the fully paralysed animals. At this stage the frog shows considerable retention of fluid, due presumably to paralysis of the lymph

hearts which are activated by cholinergic synapses (del Castillo & Sánchez, 1961).

By sacrificing the frogs at different times after the injection of the toxin, nerve–muscle preparations were obtained at various stages of block. For convenience, *fully blocked* preparations are those taken from completely paralysed frogs 12 or more hours after BTX injection. No end-plate potentials (e.p.p.s) were recorded in response to nerve stimulation in these preparations. *Partially blocked* preparations are those taken from the frog from 2 to 5 hr after injection of BTX. These muscles still twitched following nerve stimulation; the twitch tension decreased gradually with time in the absence of stimulation and more rapidly under the influence of high frequency stimuli. When the contractions had become weak, intracellular recording without large movement artifacts was possible.

It should be emphasized that the effects of BTX are not reversible. The changes elicited by exposure of the muscle to the toxin continue *in vitro* even after repeated washing with Ringer solution. In this respect our observations confirmed those of Hughes & Whaler (1962) and will not be described in detail here.

Effects of BTX on spontaneous miniature end-plate potentials

The earliest electrophysiological changes observed in muscles taken from BTX-injected frogs are those in m.e.p.p.s. Their frequency, usually 1 or more per second in normal muscles, decreased gradually in most preparations under the influence of the toxin to less than 0·1/sec.

In addition, the m.e.p.p.s recorded from BTX-treated preparations were characterized by a low mean amplitude and, more typically, by a skewed distribution of amplitudes due to the occurrence of a large number of m.e.p.p.s lower than 200 μV. Such a distribution is illustrated by histograms *A*, *B* and *C* of Fig. 1, which should be compared with histograms 1-*A* and 2-*A* of Fig. 3 obtained from untreated, control junctions.

These observations are in agreement with the findings of Harris & Miledi (1971) in frog muscles fully blocked *in vitro* by type D BTX. It should be emphasized, however, that the changes described above were observed as early as three hours after injection of BTX, when the muscles still contracted vigorously in response to nerve stimulation.

The first columns of histograms *A*, *B* and *C* of Fig. 1 probably underestimate the real number of small m.e.p.p.s, since even when recording with low-noise micro-electrodes it was difficult to resolve m.e.p.p.s almost obscured in the base line noise. An example of this difficulty is illustrated in Fig. 2 which shows the potential across the end-plate membrane of a junction partially blocked by BTX. A m.e.p.p. can be seen near the right end of the upper trace. In addition, a number of positive-going deflexions

marked by vertical bars can be seen. These may well be m.e.p.p.s but the signal-to-noise ratio is so small that deflexions of this type were never included in the histograms.

To determine the time course of these changes, m.e.p.p.s were recorded from four easily identifiable junctions in a sartorius muscle in normal Ringer. Purified BTX was then added to the bath in a concentration of 1:70 (v/v) and the m.e.p.p.s were recorded again from the four fibres at approximately 1·25 hr intervals.

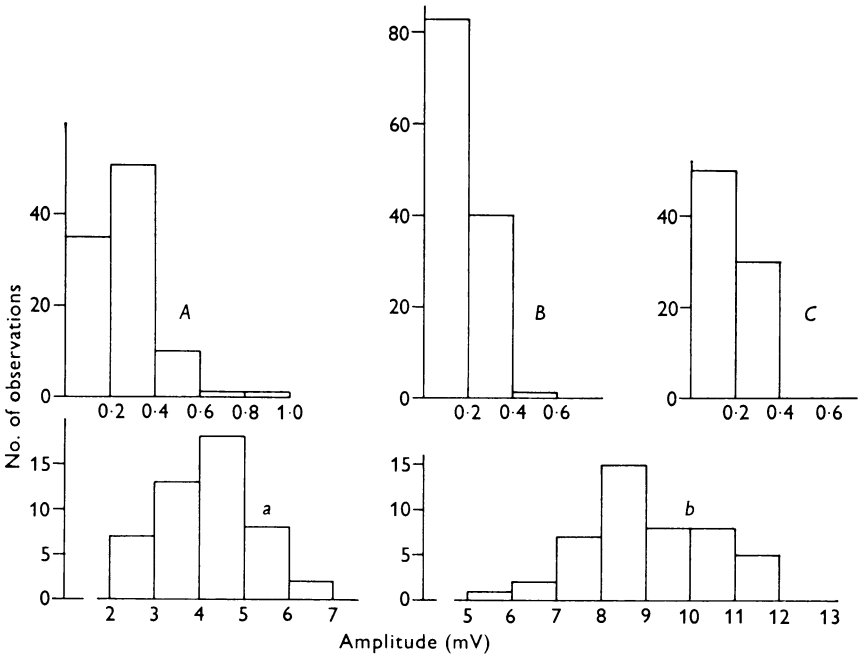

Fig. 1. *A*, *B* and *C* are amplitude histograms of the m.e.p.p.s recorded from three different fibres of a sciatic-sartorius preparation partially blocked by BTX. The m.e.p.p.s were recorded approximately 3 hr after injection of the toxin, at a time when the muscle still responded with a strong twitch to nerve stimulation. Notice the skewed distribution of the amplitudes in contrast to the approximately gaussian distribution observed in normal muscles (see the histograms 1*A* and 2*A* of Fig. 3). Histograms *a* and *b* show the distribution of amplitudes of e.p.p.s recorded from the fibres *A* and *B* after the muscles had stopped twitching, 8–10 hr after injection of the toxin. Amplitudes of e.p.p.s recorded from fibre *C* fluctuated between 8 and 16 mV.

The histograms 1-*A* and 2-*A* of Fig. 3 show amplitude distributions of the m.e.p.p.s recorded from two fibres before the addition of toxin. As early as 75 min after introducing the toxin into the bath, the histograms became slightly skewed towards the smaller classes (1*B* in Fig. 3). During

successive sampling periods (*C* and *D* of Fig. 3) there was a distinct and progressive skewing to the left of the histograms and a drop in frequency of the m.e.p.p.s from the initial 2–3/sec, to about 0·5/sec.

It is worth noting that in both these and similar experiments, a small number of large m.e.p.p.s (up to 1·5 mV in amplitude) appeared between 150 and 225 min after the addition of the toxin. No inflexions could be seen in the rising phases of these potentials and, at the low frequencies encountered, it is unlikely that they represent events due to the simultaneous discharge of more than one quantum.

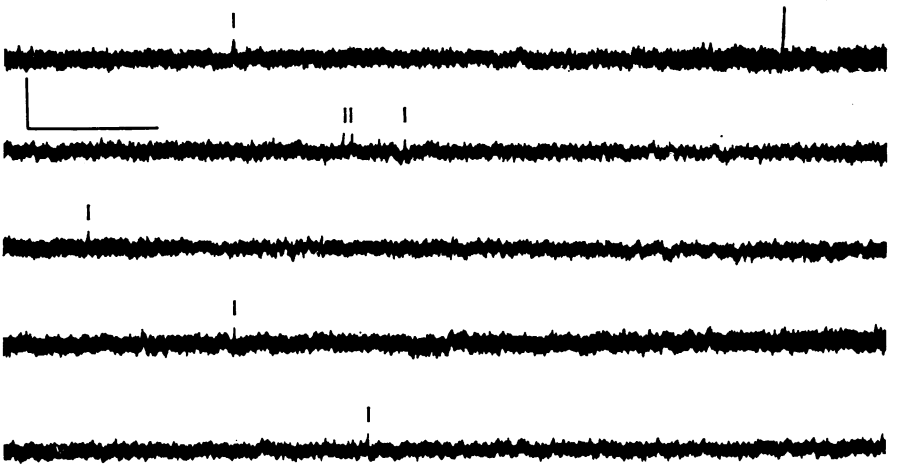

Fig. 2. Records showing the potential across the end-plate membrane of a preparation partially blocked by BTX. A m.e.p.p. is seen near the right end of the uppermost trace. The vertical bars mark deflexions that probably represent very small m.e.p.p.s barely larger than the noise level. These types of deflexions were not included in the histograms. Calibrations: vertical, 200 μV; horizontal 2 sec.

225 minutes after addition of the toxin, two of the four fibres still had resting potentials greater than 90 mV but the m.e.p.p.s recorded were in the lower ranges of the distribution of the previous samples.

The results obtained in the other two fibres studied were similar to those shown in Fig. 3, but the resting potential of one was too low at the time of the fourth impalement, and in the other the nerve terminals were accidentally damaged by the recording micro-electrode during the last insertion.

At the end of this experiment, when the preparation had been exposed to the toxin for about 4 hr, nerve stimulation still evoked a twitch. No attempt was made to record action potentials in the fibres from the samples described above, but all fibres appeared to twitch vigorously upon visual

observation through the microscope. Thus, the muscle was blocked only slightly, if at all, at this moment although the spontaneous m.e.p.p.s were already markedly altered. The longer persistence of neuromuscular transmission in this preparation, compared to that of the experiments with sciatic-gastrocnemius preparations can be attributed primarily to the total absence of nerve stimulation during the period of exposure to BTX.

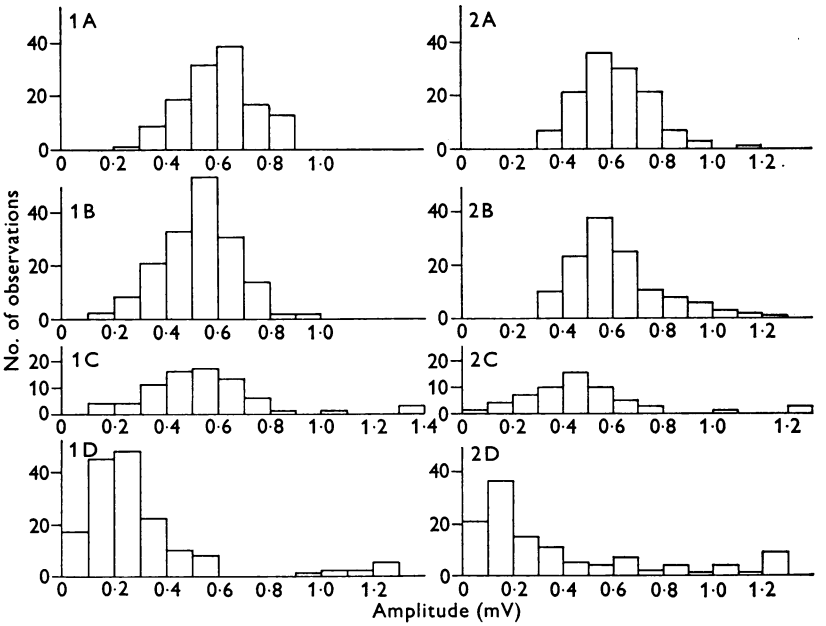

Fig. 3. Effect of BTX on the distribution of the amplitudes of m.e.p.p.s recorded from two different junctions of a sartorius muscle. Histograms 1*A* and 2*A* show the m.e.p.p.s recorded before the addition of the toxin to the bath. Purified BTX (1:70 v/v) was then added to the bath and the m.e.p.p.s were recorded again from the same fibres at approximately 1·25 hr intervals. Sampling periods ranged from 2–3 min at early stages to 7–8 min at later stages when the m.e.p.p. frequency had decreased markedly. See text for details.

Our observations on neuromuscular junctions of fully blocked preparations agree with those of Harris & Miledi (1971). In some junctions no m.e.p.p.s at all could be seen during the periods of observation, and electrophoretic application of ACh was needed to confirm that we were actually recording from the end-plate region of the muscle fibre. In other synapses m.e.p.p.s were seen only occasionally and no attempt was made to obtain statistical information from such infrequent events.

End-plate potentials in partially blocked preparations

After the twitch elicited by indirect stimulation was abolished under the influence of BTX, or reduced to a feeble contraction, recording microelectrodes could be introduced into the junctional regions of the muscle fibres while the nerve was stimulated. It was then seen that even when the muscle had become fully paralysed, the nerve impulse was still effective in liberating sufficient transmitter to give rise to large e.p.p.s, the properties of which are discussed below.

(*a*) *Amplitude.* The size of the e.p.p.s recorded from partially blocked preparations depended on both the time elapsed since the application of the toxin and the number of stimuli applied. In some instances e.p.p.s with an amplitude of over 25 mV were observed soon after the muscle stopped twitching. As the neuromuscular block gradually progressed, the amplitude of the e.p.p.s decreased until they merged into the noise. E.p.p.s of about 50 μV have been observed.

The e.p.p.s in BTX-blocked preparations show large fluctuations in size. For instance, in fibre *B* of the experiment illustrated in Fig. 1, e.p.p.s varied between 5 and 12 mV. In other fibres e.p.p.s with an amplitude of several mV were accompanied by a considerable number of 'failures'. To assure that such failures were not due to intermittent nerve block, perhaps at the stimulating electrodes, paired stimuli were applied to the nerve a few milliseconds apart. The amplitude of the e.p.p.s increased markedly due to facilitation, and no failures were observed. It is, therefore, clear that the genuine synaptic failures can occur at a time when the average size of the e.p.p.s is still relatively large.

(*b*) *Influence of the number of stimuli and rate of stimulation.* As mentioned above, the average amplitude of the e.p.p.s recorded during the sampling periods decreases gradually as the action of BTX progresses. In addition, reduction in the amplitude of the e.p.p.s is markedly accelerated by nerve activity. Stimulating the nerve at the rate of 10/sec for brief periods greatly increases the rate of paralysis of the preparation, and the point where intracellular recording becomes possible is soon reached. Stimulation of the partially blocked preparation illustrated in Fig. 4 resulted in nearly full block 7 min after the first recording, although the first few stimuli resulted in an initial 30% increase in e.p.p. amplitude that may have been due to post-activity potentiation.

(*c*) *Relationship between e.p.p.s and m.e.p.p.s.* As the block produced by BTX increases, the m.e.p.p.s become smaller and finally merge into the base line noise. It appears likely, therefore, that the size of the ACh packets continued to decrease beyond the limits of resolution of our recording system.

We observed, consistently that the decrease in the size of the m.e.p.p.s under the influence of BTX preceded the reduction of the amplitude of the e.p.p.s below the threshold level of the muscle fibres. It was interesting, therefore, to find out whether the spontaneously released transmitter packets were of the same average size as the units released by the nerve impulse.

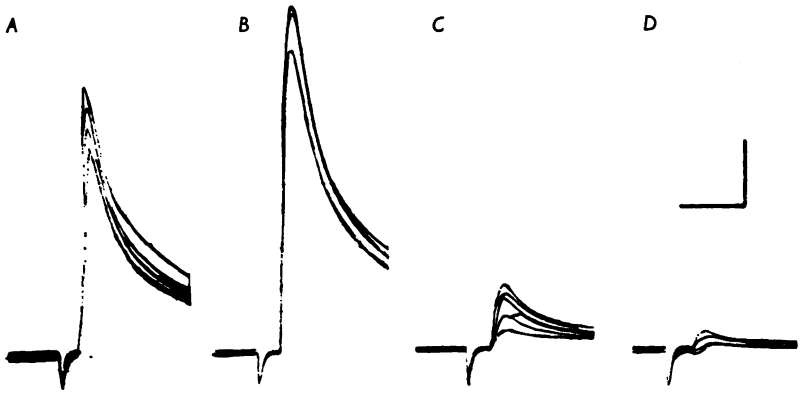

Fig. 4. Tracings of e.p.p.s recorded from a junction in a sartorius muscle partially blocked by BTX illustrating the effect of synaptic activity on the development of the neuromuscular block. *A*, are the e.p.p.s recorded just after the preparation had been set up in the chamber; only a few test stimuli had been applied to the nerve during dissection. Between *A* and *B* the nerve was stimulated for 2 min at 1/sec. Between *B* and *C* and *C* and *D* the nerve was stimulated during 2 min at a frequency of 10/sec. See text for comments. Calibrations, vertical 2 mV, horizontal, 5 msec.

Attempts to obtain information on this point by following the progressive action of the toxin to a point where e.p.p.s of low quantal content could be recorded were unsuccessful. As already mentioned, some junctions failed suddenly at a stage when the e.p.p.s were still relatively large. In other junctions when the size of the e.p.p.s was sufficiently small, the m.e.p.p.s had become too small and infrequent to be measured reliably. In addition, preparations poisoned by BTX are not in a steady state and, in the stages that precede complete block, the progressive reduction in the amplitude of the e.p.p.s is greatly accelerated by the nerve stimulation needed to elicit them.

To overcome these difficulties we placed partially blocked preparations in high Mg^{2+} Ringer so that the average quantal content of the e.p.p.s was decreased to a level where their statistical composition could be analysed. We were not able to achieve the right conditions for this experiment in the advanced stages of BTX blocking, because at this time the Mg^{2+} concentration is very critical and stimulation rapidly changes the preparation.

We succeeded in performing this experiment only at the very early stages of action of the toxin.

The results obtained in one preparation immersed in Ringer solution containing 17 mM-Mg^{2+} are illustrated in the histograms of Fig. 5. Although the average amplitude of the m.e.p.p.s was 238 μV, the histogram is only slightly skewed. Also, when the high-Mg^{2+} Ringer was replaced by normal Ringer at the end of the experiment and the nerve was stimulated, twitching of the muscle was seen. Both observations suggest that the action of the toxin was only beginning when the experiment was performed.

To relate the spontaneous m.e.p.p.s to the evoked responses, the average number of packets of transmitter released by each nerve impulse (m) was calculated from the records (i.e. average size of e.p.p.s/average size of m.e.p.p.s). According to eqn. [2] of del Castillo & Katz (1954*a*), if the e.p.p.s consist of units of the same average size as the spontaneous m.e.p.p.s m should be equal to the $\log_e$ of the number of stimuli/number of failures.

TABLE 1. Measurement of electrical potential after nerve stimulation

A	*B*	*C*	*D*	*M*	
E.p.p.s (mean, μV)	M.e.p.p.s (mean, μV)	No. of impulses	No. of failures	*A*/*B*	$\log_e$ *C*/*D*
284	Total 238 (n = 133)	117	33	1·19	1·26
	Before stimulation 252 (n = 93)	—	—	1·16	—
	After stimulation 204 (n = 40)	—	—	1·39	—

Note. A total number of 133 m.e.p.p.s were recorded, ninety-three before the period of stimulation and forty after stimulation. The average amplitude of the m.e.p.p.s decreased from 252 to 204 μV after stimulation. The value of $\log_e$ *C*/*D* is almost equal to the mean of these two values of *A*/*B*, i.e. 1·27.

In this experiment, m.e.p.p.s were measured before and after a period of nerve stimulation at 1/sec to evoke the e.p.p.s. As shown in Table 1, the two independent measures of the number of packets per impulse are in good agreement, although the relationship changed slightly after stimulation, due to a reduction in quantal size.

It can be concluded that at least at this stage in the action of the toxin, the spontaneous m.e.p.p.s recorded were of the same amplitude as the transmitter packets released by the nerve impulse. That is, both spontaneous and evoked units had been affected by the toxin to the same extent.

(*d*) *Facilitation.* Even in the advanced stages of its action, BTX does not prevent neuromuscular facilitation. Indeed, when the muscle twitch

in response to stimulation at 1/sec is fully abolished by the toxin, an increase in the frequency of stimulation usually restores the contractions, if only for a brief period before the responses are abolished again.

A convenient way of showing facilitation in preparations partially blocked by curare or by high [Mg^{2+}] and low [Ca^{2+}] is to stimulate the nerve with pairs of pulses separated by an interval of a few milliseconds. If the resulting e.p.p.s are recorded extracellularly from the whole muscle

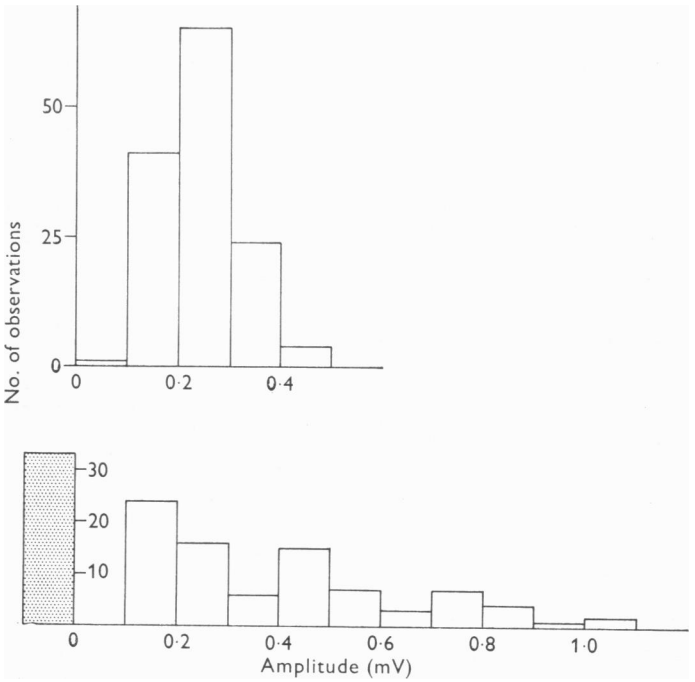

Fig. 5. Histograms showing the distribution of the amplitudes of m.e.p.p.s and e.p.p.s recorded from a junction exposed to BTX and immersed in Ringer solution containing 17 mM of Mg^{2+} ions. Upper histogram shows the m.e.p.p. amplitudes. Lower histogram represents the distribution of the amplitudes of the e.p.p.s. Dotted vertical bar shows the number of failures. See text and Table 1.

or intracellularly the second e.p.p. is found to be significantly larger than the first (Schaefer & Haass, 1939; Eccles, Katz & Kuffler, 1941; del Castillo & Katz, 1954*b*).

Analogous results were obtained from junctions almost fully blocked by BTX. In one experiment, illustrated in Fig. 6, 18 out of 33 first pulses of each pair failed to elicit an e.p.p., but all except two of the second pulses elicited an e.p.p. The mean amplitude of the response to the first pulse was 0·26 mV, whereas the mean response to the second was 1·42 mV

(including all failures in both instances). The facilitation ratio was 1:5·46, a higher value than that found in similar experiments in normal muscle (see Tables 1 and 2 in del Castillo & Katz, 1954*a*).

The effectiveness of two consecutive stimuli applied to the nerve of

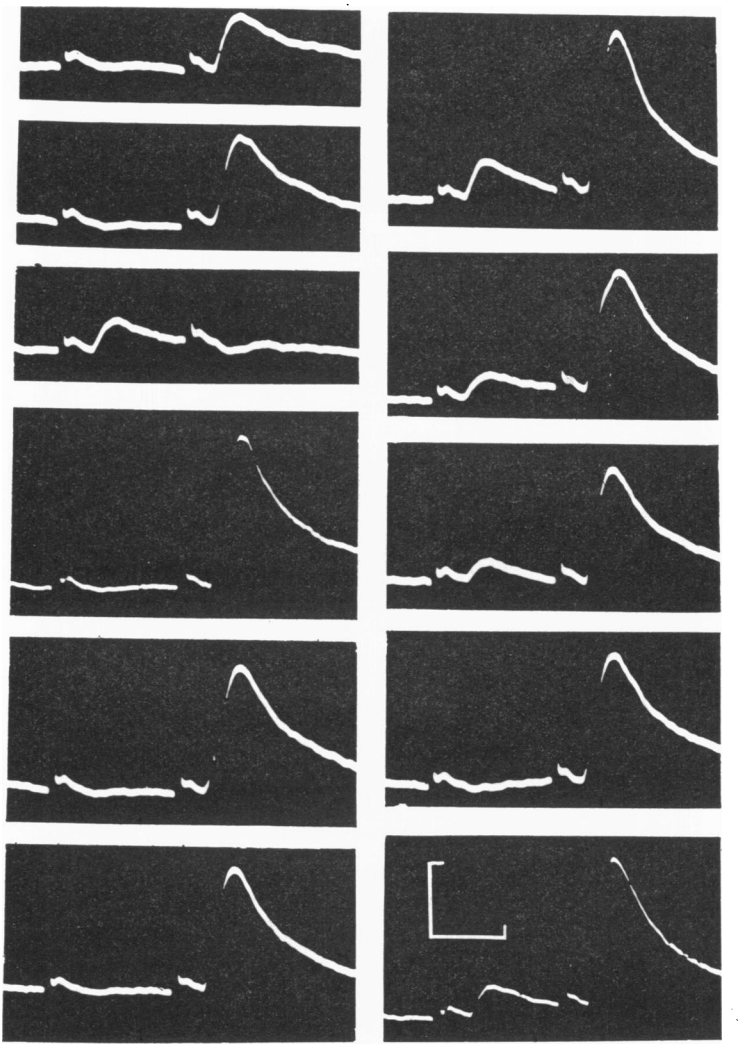

Fig. 6. Neuromuscular facilitation in a nerve–muscle preparation almost fully blocked by BTX. Pairs of stimuli separated by an interval of approximately 8 msec were applied to the nerve at a frequency of 1/sec. Notice the larger amplitudes of the second e.p.p.s, as well as the higher incidence of failures following the first stimulus. See text for details. Calibrations for all records; vertical 1 mV; horizontal, 5 msec.

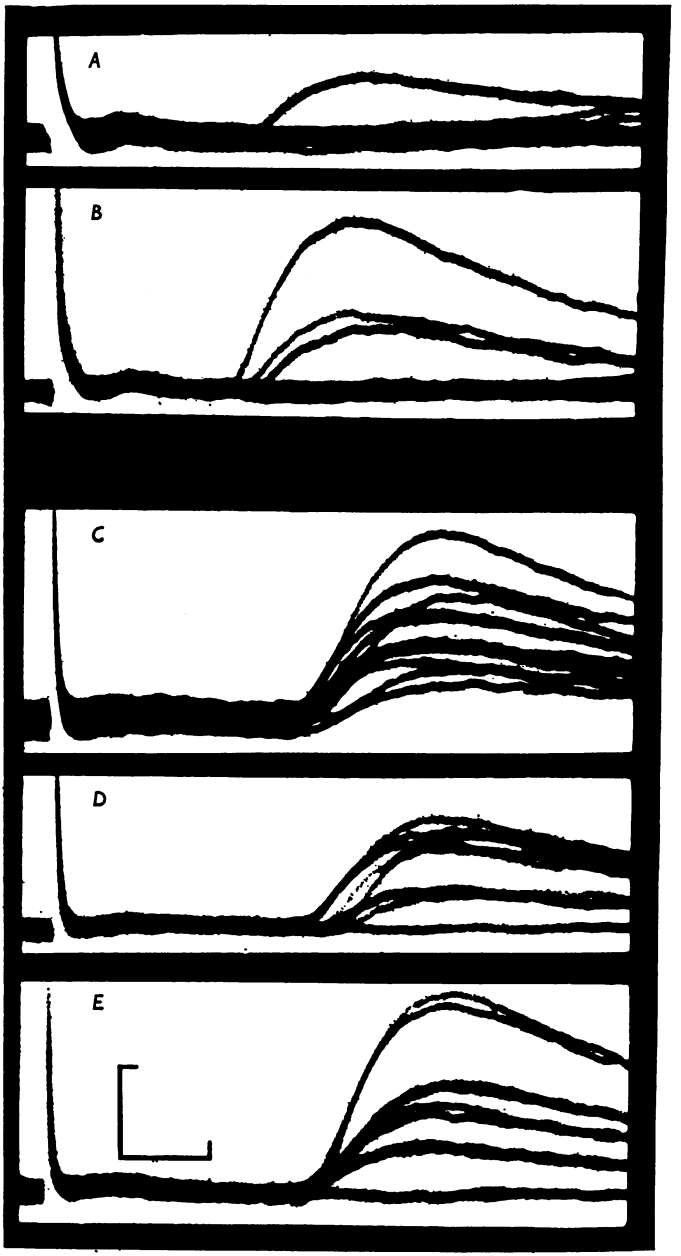

Fig. 7. For legend see facing page.

a sartorius muscle paralysed by BTX was first reported by Stover, Fingerman & Forester (1953). A muscle action potential was observed when the interval between the two stimuli was 4–18 msec. This was interpreted, however, as due to summation of e.p.p.s. A similar facilitation of BTX-blocked cat m. gracilis was seen with paired stimulation (Brooks, 1954).

Effects of increasing the external Ca^{2+} concentrations

Thesleff (1960) reported that doubling the Ca^{2+} concentration in the bath temporarily restores activity in junctions partially blocked by BTX. Simpson & Tapp (1967) also observed an antagonism between calcium and BTX, since increasing the concentration of this cation in the bath delayed the onset of paralysis in phrenic-hemidiaphragm preparations. Harris & Miledi (1971) did not obtain recovery in fully blocked preparations upon increasing the calcium concentration, but observed a brief enhancement of release upon tetanic nerve stimulation.

Our observations in partially and fully blocked frog sartorius muscle support the above reports. Contraction of the muscle to indirect stimulation at 1/sec was restored temporarily when the Ca^{2+} concentration in the Ringer was increased. This was seen also in junctions where most of the nerve impulses resulted in failures. An example is shown in Fig. 7. An increase in the calcium concentration from 1·8 to 21 mM resulted in a drastic reduction in the number of failures. However, this effect was very brief and a complete block of the junction occurred soon after these records were taken that was not reversible by subsequent washing and retreatment with Ca^{2+}.

Effects of electrical polarization of the motor nerve terminals

As demonstrated by Brooks (1954) the ACh stores are not depleted in muscles fully paralysed by BTX, since passing strong direct currents through the bath elicits ACh release. The presence of ACh in nerve endings blocked by BTX was confirmed by Thesleff (1960), who reported that

Fig. 7. Influence of increasing the extracellular concentration of calcium ions on the e.p.p.s recorded from a junction almost fully blocked by BTX. Each of the frames shows the effects of ten consecutive stimuli applied to the nerve. *A* and *B* were taken in normal Ringer solution (1·8 mM calcium.). In *A* only two out of the ten stimuli gave rise to e.p.p.s. In *B* the ratio was seven failures to three e.p.p.s. Records *C*, *D* and *E* were taken immediately after the calcium concentration in the bath had been increased to 21 mM. In *C* all the stimuli gave rise to e.p.p.s. Only one failure was observed in *D* and three failures in *E*. Soon after *E* was taken, however, a complete and irreversible block of the junction occurred. Notice increased latency in the high calcium concentration. Calibrations in *E*, for all records: vertical 0·5 m*V*; horizontal 1 msec.

mechanical injury to the motor nerve terminals by the micro-electrode caused a high frequency discharge of m.e.p.p.s and by Harris & Miledi (1971) who obtained slight increases in some, but not all BTX treated

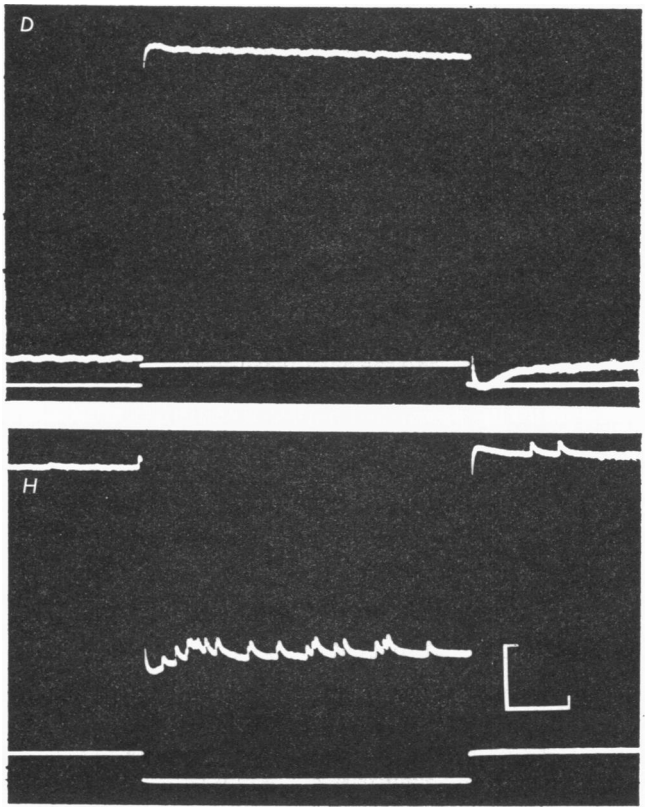

Fig. 8. Effects of depolarizing (*D*) and hyperpolarizing (*H*) current pulses applied to the motor nerve terminals of a preparation fully blocked by BTX. The upper trace on each record is the potential across the end-plate membrane. The lower trace monitors the current flowing between the nerve electrode and a bath electrode (see Methods). The large deflexions, simultaneous with the current pulses, in the membrane potential trace, are artifacts due to the current flowing in the bath. Whereas depolarizing pulses of any strength and duration were ineffective in causing transmitter release, hyperpolarization of the terminals had an effect similar to that observed in normal preparations; i.e. it gave rise to the release of transmitter packets (see text for details). Record *H* was one of a series of pulses and a few residual m.e.p.p.s were seen between the current pulses. One of such m.e.p.p.s is seen in record *H* interrupted by the beginning of the hyperpolarizing pulse. Calibrations in *H*, vertical, 2 mV for voltage and 20 μA for the current; horizontal, 5 msec.

fibres upon raising the potassium concentration which presumably depolarized the nerve terminals.

These findings suggest that even if the brief depolarization produced by the presynaptic action potential is ineffective in releasing ACh, a prolonged depolarization of the motor nerve terminals may still be able to liberate transmitter packets. To investigate this possibility we studied the effects of applying depolarizing (cathodic) and hyperpolarizing (anodic) currents to the motor nerve terminals in preparations paralysed by BTX.

Our first experiments were carried out in fully blocked muscles but, contrary to our expectations, cathodic polarization of the terminals failed to elicit any ACh release. All our results were negative, regardless of the intensity and duration of the applied cathodic currents.

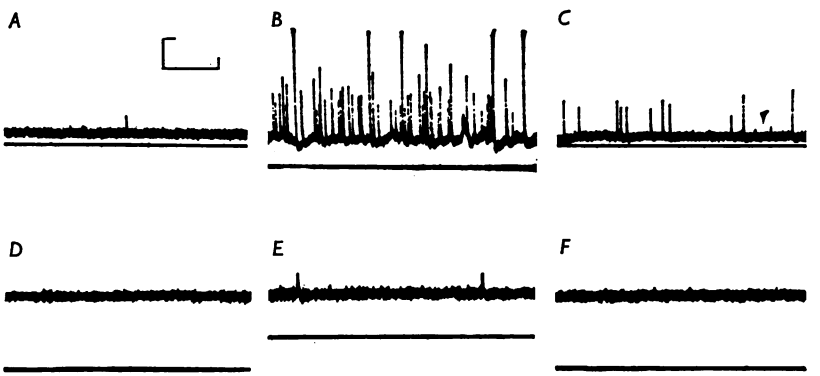

Fig. 9. Effects of hyperpolarization and depolarization of the motor nerve terminals in a junction almost fully blocked by BTX. The upper trace on each record shows the membrane potential of the muscle fibre recorded via a condenser-coupled amplifier. The lower trace monitors the current which flowed through the nerve electrodes (see Methods); in records *A*, *C*, *D* and *F* this trace shows the reference zero level. Record *A* shows the only spontaneous m.e.p.p. observed in this junction. Record *B* shows the effects of a hyperpolarizing current: a relatively high frequency discharge of large m.e.p.p.s. Record *C* shows part of the anodic after-discharge. Record *E* shows two of the few m.e.p.p.s elicited by terminal depolarization. Calibrations in *A* for all the records, vertical 200 μV and 20 μA; horizontal 1 sec.

To our surprise, however, we observed that anodic polarization of the terminals in the same fully blocked preparations evoked discharge of m.e.p.p.s, as shown in Fig. 8. The properties of this effect are similar to those observed in normal muscle (see del Castillo & Katz, 1954*c*); i.e. there is both a critical current density, or threshold strength, and a variable delay between the onset of the current pulse and the beginning of the transmitter discharge. In addition, the release of ACh packets is not

restricted to the duration of the current pulses, but continues as an after-discharge, for variable times.

One difference, however, was clear between the effects of nerve terminal hyperpolarization in preparations fully blocked by BTX and in normal, control muscles. In the latter, the discharges reach high frequencies that were described as 'bursts' (see Figs. 2, 3 and 4 of del Castillo & Katz, 1954*c*). In preparations fully blocked by BTX, however, the frequency of the m.e.p.p.s evoked by anodic currents is usually lower and individual m.e.p.p.s can be clearly identified.

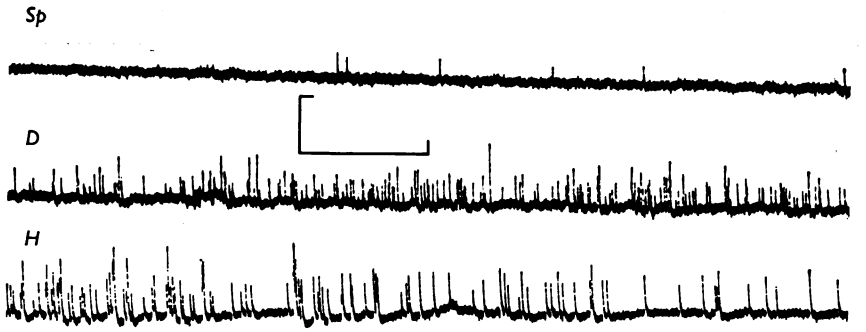

Fig. 10. M.e.p.p.s recorded from a neuromuscular junction partially blocked by BTX. Record *Sp* shows a sample of spontaneously released m.e.p.p.s, in the absence of current flow. *D* are those evoked by the depolarization of the motor nerve terminals, and *H* are those during the hyperpolarization of the nerve terminals. The voltage and time calibrations are the same for *Sp* and *D* (100 μV and 1 sec, respectively), but different for *H* (200 μV and 0·5 sec). The distribution of the amplitudes of all the m.e.p.p.s recorded during this experiment is given by the histograms *A*, *B* and *C* of Fig. 11.

Fig. 9 shows the result of a similar experiment on an almost but not quite fully blocked preparation; i.e. in a preparation where nerve stimulation still gave rise to detectable e.p.p.s as small as 0·3 mV. In this instance, the potential across the muscle membrane was recorded through a condenser-coupled amplifier and the flow of current was regulated by a manually operated potentiometer. Only one small spontaneous m.e.p.p. was observed in the absence of current flow during almost one minute (see Fig. 9*A*). Anodic current evoked a relatively high frequency of discharge of m.e.p.p.s, many of which were larger than 0·5 mV. Part of this discharge is shown in record *B* of the same Figure. After the anodic current was switched off, an after-discharge was seen to persist for a few seconds, as illustrated in record *C*. No further spontaneous m.e.p.p.s were observed (*D*). A cathodic current was then applied to the terminals and during the period of presynaptic depolarization only two m.e.p.p.s were discharged.

These are shown in record *E*. Record *F* shows the base line after the cathodic current was switched off.

Similar experiments were performed on preparations in which the block had developed to a lesser extent than in the cases just described with results intermediate between these and the effects observed in normal muscles, indicating a relationship between the degree of neuromuscular block induced by BTX and the effects of the cathodic currents. That is, in all those instances where nerve stimulation gave rise to e.p.p.s several millivolts in amplitude, nerve terminal depolarization was effective in eliciting the discharge of m.e.p.p.s. In the example of the effects of polarizing currents on a partially blocked preparation shown in Fig. 10, the flow of depolarizing current caused a high frequency (about 22/sec) discharge of small m.e.p.p.s. A hyperpolarizing current evoked a discharge of larger m.e.p.p.s, part of which is illustrated in record *H*. It should be emphasized, also, that in partially blocked preparations 'bursts' of m.e.p.p.s are often seen when the hyperpolarizing current is first switched on although the frequency of discharge of m.e.p.p.s decreases gradually even if the intensity of the current remains constant.

In spite of the clear correlation between the degree of block and the effectiveness of cathodic currents in causing transmitter liberation, we did not succeed in demonstrating a quantitative relationship between these two factors. It was difficult to follow the development of the neuromuscular block caused by the toxin by observing the effects of nerve terminal polarization. It must be emphasized that the cathodic current applied to the nerve while recording from one end-plate affects all adjacent end-plates to various degrees, greatly accelerating the rate of paralysis. In addition, the strength of the monitored currents gives only a qualitative indication of the magnitude of the depolarization, since this depends upon three factors: (i) the fraction of the applied current that penetrates into any given nerve fibre, (ii) the space constant of the nerve fibre and (iii) the distance between the oil–Ringer interphase and the junction.

Statistical properties of the m.e.p.p.s released by polarizing currents

In spite of these uncertainties a clear and interesting difference was observed between the m.e.p.p.s released by cathodic and anodic currents. The 'anodic' and 'cathodic' m.e.p.p.s differ both in their average amplitudes and in the distribution of their amplitudes. The fact that anodic polarization of the terminals releases relatively large m.e.p.p.s is apparent in the experiment of Fig. 9, although in this instance the number of spontaneous and 'cathodic' m.e.p.p.s is not statistically significant.

However, the histograms of the m.e.p.p.s recorded in the experiment

illustrated in Fig. 10 reveal clearly such differences (see Fig. 11). It can be seen that the 'anodic' m.e.p.p.s are larger than the 'cathodic' ones and that their amplitudes are distributed in an approximately gaussian fashion in marked contrast to the skewed distribution of the amplitudes of the m.e.p.p.s released by cathodic current.

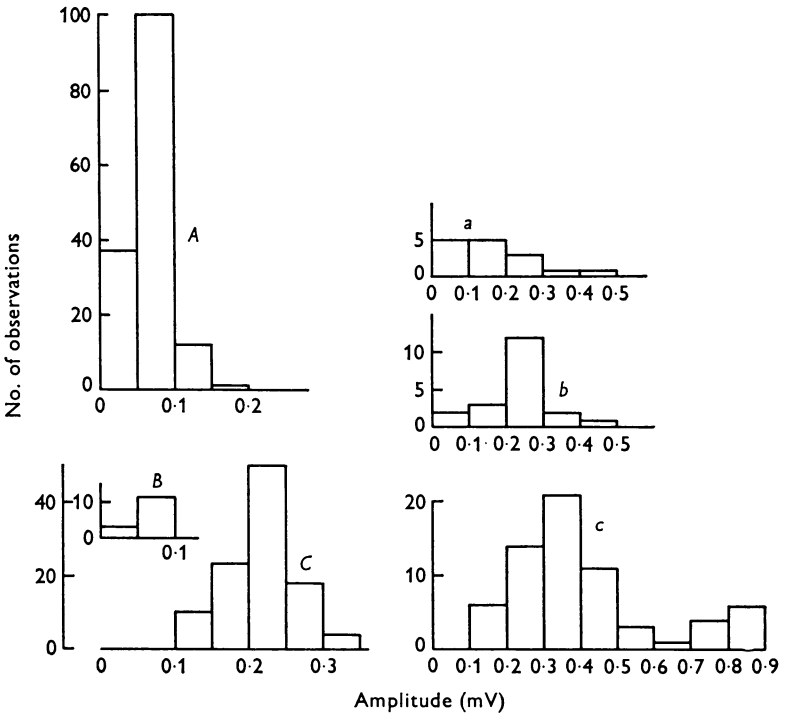

Fig. 11. Histograms showing the distribution of the amplitudes of m.e.p.p.s recorded from two neuromuscular junctions partially blocked by BTX. Histograms *A*, *B* and *C* are from the same junction. *B* are the spontaneously released m.e.p.p.s; *A* are those released during depolarization of the terminals and *C* are those elicited by terminal hyperpolarization (see part of the record in Fig. 10). Histograms *a*, *b* and *c* are from another junction. *a* shows the distribution of the amplitudes of the m.e.p.p.s released by nerve terminal depolarization; *c* are the 'anodic' m.e.p.p.s and *b* are the m.e.p.p.s released as an anodic after-discharge.

The histograms *a*, *b* and *c* of the same Fig. 11 show the m.e.p.p.s recorded in a junction of another partially blocked preparation. In this instance no spontaneous m.e.p.p.s were recorded during the observation period. The distribution of m.e.p.p.s during the anodic after-discharge is nearly normal by contrast with the skewed distribution seen during cathodic

current and they are of larger mean amplitude. Similar results were obtained in four other preparations.

It can be concluded, therefore, that the motor nerve terminals blocked by BTX contain two different populations of transmitter packets, *a*, those that are released spontaneously, by cathodic polarization and, presumably, under the influence of the nerve impulse and *b*, those that are discharged only during anodic polarization.

The first population is clearly affected by the toxin. Under exposure to BTX the average amplitude of these packets becomes progressively smaller. Eventually, the release of transmitter quanta by presynaptic depolarization becomes fully blocked.

In contrast, the second population has a larger average size and an apparently normal amplitude distribution. Units of this type were released by anodic currents in all the preparations that we have examined, even in those fully blocked by the toxin. These packets seem to be analogous to those released following high frequency nerve stimulation in Harris & Miledi's (1971) experiments.

It is likely that BTX decreases the size of this population without affecting its statistical properties. This is suggested by the gradual reduction in the frequency of the m.e.p.p.s released during anodic polarization since, as we have mentioned, in fully blocked preparations the m.e.p.p.s occurred as single events instead of appearing as 'anodic bursts'.

Attempts were made to find out whether the transmitter packets that are driven to the presynaptic membrane by the hyperpolarizing currents could be liberated under the influence of membrane depolarization or by the nerve impulse. For that purpose the motor nerve of fully blocked preparations was stimulated both during the flow of anodic currents of various intensities and at various intervals after the anodic current was switched off. The results of these experiments were negative.

M.e.p.p.s released by terminal damage in fully blocked preparations

The results of the polarization experiments just described provide an explanation for Brooks' (1954) observation that ACh can be released from preparations fully blocked by BTX by passing strong d.c. currents through the muscle bath. It is likely that the transmitter was released in those experiments under the influence of anodic, rather than cathodic, polarization.

Our results, however, do not support the most simple explanation that would account for Thesleff's (1960) observation of m.e.p.p. discharge following damage to the nerve terminals. The thought that such a release was a consequence of membrane depolarization is not in agreement with our observations, since depolarization by currents of any intensity con-

sistently failed to discharge m.e.p.p.s during the advanced stages of the block.

As the statistical properties of the m.e.p.p.s released by cathodic and anodic currents are different, it was interesting to see to which type belong the m.e.p.p.s released by nerve terminal damage in fully blocked preparations. Although such discharges were frequently observed, the high

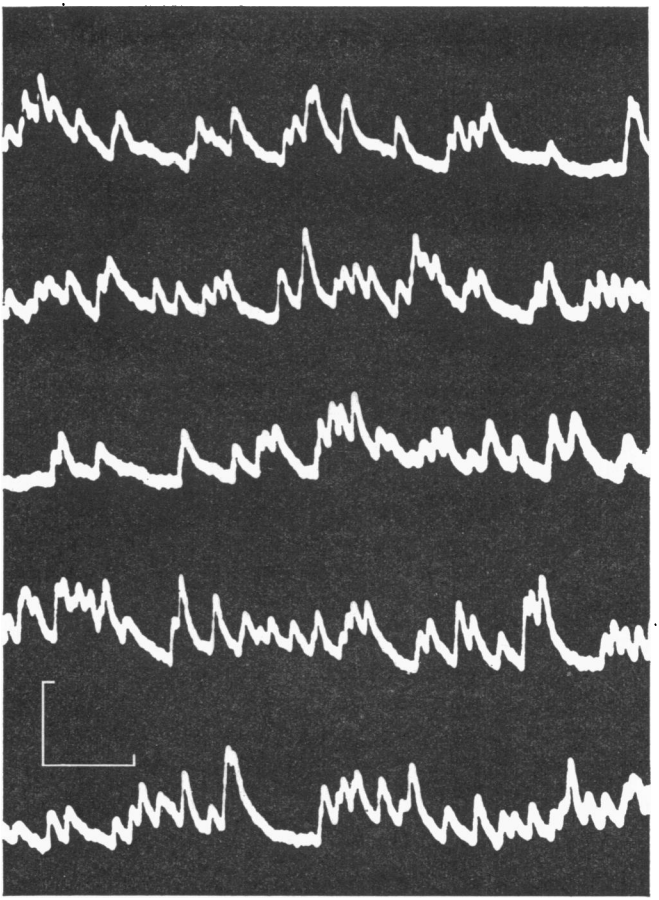

Fig. 12. High frequency discharge of m.e.p.p.s due to mechanical damage produced by the micro-electrode during impalement of a muscle fibre in a preparation fully blocked by BTX. In spite of the high frequency, the amplitudes of most m.e.p.p.s could be measured (see histogram of Fig. 13). Calibrations: vertical 0·5 μV; horizontal, 20 msec.

frequency of the m.e.p.p.s made it difficult to measure the amplitudes of individual events. We were, however, able to do so in one instance where accidental damage to the motor nerve terminal in a fully blocked prepara-

tion gave rise to a relatively low frequency discharge. Part of this discharge is shown in Fig. 12 and the histogram of the m.e.p.p. amplitudes that could be measured is shown in Fig. 13. Although the distribution shows a certain skewness to the left, the classes above 150 μV are well represented. This histogram is clearly different from those obtained in partially blocked preparations and from the histograms of the m.e.p.p.s released in these preparations during the flow of cathodic current.

We conclude, therefore, that the population of transmitter quanta liberated by nerve terminal damage resembles that of the 'anodic' m.e.p.p.s. Our observations suggest also that the release of transmitter packets by terminal damage is not due, at least primarily, to a depolarization of the presynaptic membrane.

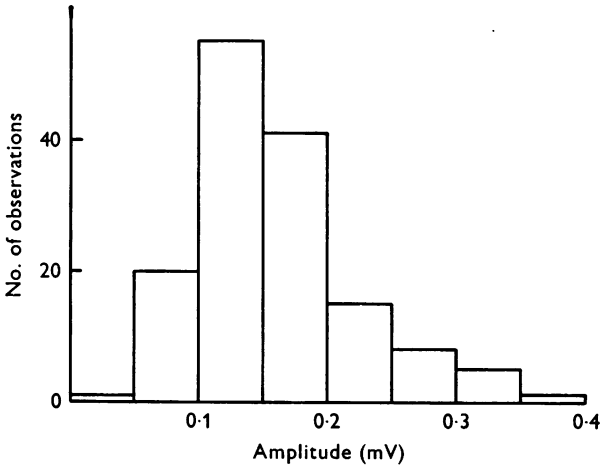

Fig. 13. Histogram of amplitudes of the m.e.p.p.s elicited by mechanical injury of the nerve terminals in a junction fully blocked by BTX. See text and records of Fig. 12.

DISCUSSION

As has been the case with most papers dealing with the action of BTX on skeletal muscle published during the last quarter of a century, the work described above poses more questions that it has answered. However, a number of facts concerning the onset and properties of the neuromuscular block produced by this toxin have become well established in the course of our experiments.

BTX seems to attach itself irreversibly to the nerve terminals long before the first signs of synaptic block become apparent. Neither repeated washing with Ringer solution nor treatment of the preparations with specific antibodies seems to affect development of the paralysis (Lamanna,

1959) the rate of which is accelerated by factors that increase transmitter liberation. These include nerve stimulation (Hughes & Whaler, 1962) and high Ca^{2+} concentrations (Simpson, 1973). Apparently, myoneural junctions exposed to BTX can only release a limited number of transmitter packets before becoming fully blocked.

The first detectable electrophysiological change in BTX-treated preparations is a progressive reduction in the average size of the m.e.p.p.s. The skewness to the left of the amplitude histograms of the m.e.p.p.s is clearly due to a predominance of small m.e.p.p.s. As the average amplitude of the spontaneous potentials approaches the noise level of the recording system it is likely that large numbers of m.e.p.p.s escape detection because of their very small size. In many experiments we were certain of the presence of positive-going deflexions, similar in shape to the larger m.e.p.p.s, but not definite enough to be identified as such and be included in the statistical treatment.

The spontaneously released transmitter packets seem to be analogous to those that give rise to the e.p.p.s elicited by nerve stimulation. This is suggested both by the correlation of the m.e.p.p.s with the e.p.p.s at the very early stages of the block and by the similarity of the amplitude histograms of the m.e.p.p.s released by cathodic currents to those of the spontaneous m.e.p.p.s.

Reduction of the amount of transmitter released from the nerve terminals appears to be responsible first for the reduction in the number of fibres that contribute to the twitch followed by a continuing decline in subthreshold e.p.p. amplitude.

Previous work on the effects of BTX on muscle emphasized the lack of transmitter liberation in fully paralysed preparations and suggested that the main action of the toxin is a block of the neurosecretory mechanisms of the presynaptic membrane. Such an impairment cannot be excluded by our experiments. However, we have no direct evidence in support of this possibility. On the contrary, our observations incline us to think that the main target of BTX is the process by which the synaptic vesicles are refilled following the liberation of their contents into the synaptic space. It appears that under the influence of the toxin the vesicles gradually lose their ability to store ACh. As a consequence, the average size of the transmitter packets decreases steadily until they become too small to be detected. However, the opening of the vesicles and the release of whatever amounts of transmitter they may still contain does not seem to be affected by the toxin. Experiments are in progress, using markers that can be seen under the electron microscope, to obtain direct information on this problem.

Even when most of the nerve impulses fail to produce a detectable

depolarization of the end-plate membrane, an increase in the Ca^{2+} concentration transiently restores the e.p.p.s. In addition, considerable facilitation was observed when paired stimuli were applied to the nerve at all stages of the block. If the occurrence of facilitation can be regarded as an indication of the normal operation of the neurosecretory mechanisms, this aspect of transmitter release does not seem to be influenced by BTX.

There are some similarities between the action of BTX and the effects of tetanus toxin on mammalian neuromuscular junctions (Duchen & Tonge, 1973). Tetanus toxin causes a leftward skewness of the amplitude distribution of m.e.p.p.s and some reduction in their frequency. However, the authors suggest that, because elevated extracellular potassium fails to restore normal release, the effect of this toxin is to impair coupling between terminal depolarization and transmitter release.

Too little is known about the process of vesicle refilling to explain how this process might be impaired by the BTX. If normal refilling involves the specific binding of ACh to a component of the vesicular membrane, the effects of BTX could be exerted at this level. Nevertheless, if the estimate that only ten molecules of BTX are needed to fully block a synapse is correct, it is unlikely that the toxin could act directly upon each released vesicle. A hypothesis would be that the toxin molecules attached to the motor nerve terminals induce a change in the membrane of the vesicles during the exocytotic phase of their fill-release cycle. Such a mechanism might bear some resemblance to the still unexplained process by which fertilization renders the egg membrane impregnable to penetration by further sperm cells. Here, a localized event evokes a change in the state of the surface of the entire cell.

Another puzzling problem posed by our observations concerns the physiological significance of the population of BTX-resistant transmitter packets that are present within the terminals even in the most advanced stages of the block. These packets are not released by depolarization of the presynaptic membrane, but they are liberated by anodic polarization; by high frequency nerve stimulation (Harris & Miledi, 1971), and, presumably, by mechanical damage to the nerve terminals. As in frog muscle, the leftward shift in the distribution of m.e.p.p. amplitudes caused by Type A BTX on rat diaphragm muscle is transiently restored to a normal distribution during tetanic stimulation of the nerve (Spitzer, 1972).

A plausible explanation is that some transmitter packets are not sensitive to BTX simply because they do not participate in the production of either m.e.p.p.s or e.p.p.s. They may be vesicles located in the innermost region of the terminals which would mix with, and slowly replace, the vesicles that are situated close to the membrane and are actively involved in the fill–release cycle.

However, in spite of these and other possible speculations, we need a much more complete picture of the normal processes of transmitter packing and vesicle recycling before attempting to explain the effects of BTX on the motor nerve terminals.

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REFERENCES

AMBACHE, N. (1949). The peripheral action of *Cl. botulinum* toxin. *J. Physiol.* **108**, 127–141.

AMBACHE, N. (1952). Effect of botulinum toxin upon the superior cervical ganglion. *J. Physiol.* **116**, 9*P*.

BOROFF, D. A., DASGUPTA, B. R. & FLECK, U. S. (1968). Homogeneity and molecular weight of toxin of *Clostridium botulinum* type B. *J. Bact.* **95**, 1738–1744.

BOROFF, D. A. & FLECK, U. (1966). Statistical analysis of a rapid *in vivo* method for the titration of the toxin of *C. botulinum*. *J. Bact.* **92**, 1580–1581.

BROOKS, V. B. (1954). The action of botulinum toxin on motor nerve filaments. *J. Physiol.* **123**, 501–515.

BROOKS, V. B. (1956). An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials. *J. Physiol.* **134**, 264–277.

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

DASGUPTA, B. R., BOROFF, D. A. & ROTHSTEIN, E. (1966). Chromatographic fractionation of the crystalline toxin of *Clostridium botulinum* type A. *Biochem. Biophys. Res. Commun.* **22**, 750–756.

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

DEL CASTILLO, J. & KATZ, B. (1954*b*). Statistical factors involved in neuromuscular facilitation and depression. *J. Physiol.* **124**, 574–585.

DEL CASTILLO, J. & KATZ, B. (1954*c*). Changes in end-plate activity produced by pre-synaptic polarization. *J. Physiol.* **124**, 586–604.

DEL CASTILLO, J. & SÁNCHEZ, V. (1961). The electrical activity of the amphibian lymph heart. *J. cell. comp. Physiol.* **57**, 29–45.

DUCHEN, L. W. & TONGE, D. A. (1973). The effects of tetanus toxin on neuromuscular transmission and on the morphology of motor end-plates in slow and fast skeletal muscle of the mouse. *J. Physiol.* **228**, 157–172.

DUFF, J. T., WRIGHT, G. G., KLERER, J., MOORE, D. E. & BIBLER, R. H. (1957). Studies on immunity to toxin of *Clostridium botulinum*. A simplified procedure for isolation of type A toxin. *J. Bact.* **73**, 42–47.

ECCLES, J. C., KATZ, B. & KUFFLER, S. W. (1941). Nature of the 'end-plate potential' in curarized muscle. *J. Neurophysiol.* **4**, 362–387.

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

HUGHES, R. & WHALER, B. C. (1962). Influence of nerve-ending activity and of drugs on the rate of paralysis of rat diaphragm preparations by *Cl. botulinum* type A toxin. *J. Physiol.* **160**, 221–223.

KUPFER, C. (1958). Selective block of synaptic transmission in ciliary ganglion by type A botulinus toxin in rabbits. *Proc. Soc. exp. Biol. Med.* **99**, 474–476.

LAMANNA, C. (1959). The most poisonous poison. *Science, N.Y.* **130**, 763–772.

LAMANNA, C., MCELROY, O. E. & ECKLUND, H. W. (1946). The purification and crystallization of *Clostridium botulinum* type A toxin. *Science, N.Y.* **103**, 613–614.

SCHAEFER, H. & HAASS, P. (1939). Über einen lokalen Erregungsstrom an der motorischen end platte. *Pflügers Arch. ges. Physiol.* **242**, 364–381.

SHANKLAND, D. L., ROSE, J. A. & DONNINGER, C. (1971). The cholinergic nature of the cercal nerve-giant fiber synapse in the sixth abdominal ganglion of the American cockroach, *Periplaneta americana*. *J. Neurobiol.* **2**, 247–262.

SIMPSON, L. L. (1973). The interaction between divalent cations and botulinum toxin type A in the paralysis of the rat phrenic nerve–hemidiaphragm preparation. *Neuropharmacology* **12**, 165–176.

SIMPSON, L. L. & TAPP, J. T. (1967). Actions of calcium and magnesium on the rate of onset of botulinum toxin paralysis of the rat diaphragm. *Neuropharmacology* **6**, 458–492.

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

STOVER, J. H., FINGERMAN, M. & FORESTER, R. H. (1953). Botulinum toxin and the motor end-plate. *Proc. Soc. exp. Biol. Med.* **84**, 146–147.

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