J. Physiol. (1976), **260**, *pp.* 177–203
With 14 *text-figures*
Printed in Great Britain

EFFECTS OF BOTULINUM TOXIN ON NEUROMUSCULAR TRANSMISSION IN THE RAT

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(*Received 20 January 1976*)

SUMMARY

1. Botulinum toxin (BoTx) type A partially blocks spontaneous transmitter release from nerve terminals in the rat. Miniature end-plate potentials (m.e.p.p.s) are present at all end-plates, initially with a low frequency but increasing with time after poisoning. Their amplitude distribution is at first skew with a predominance of very small m.e.p.p.s but, after a few days, larger than normal m.e.p.p.s appear.

2. Tetanic nerve stimulation, Black Widow Spider Venom, the Ca-ionophore A 23187 or mechanical damage to nerve terminals increases the frequency of m.e.p.p.s and alters the amplitude distribution of m.e.p.p.s towards a normal Gaussian one; the m.e.p.p. size approaches that seen at normal end-plates. This was seen at any time after poisoning.

3. Nerve stimulation gives rise to end-plate potentials (e.p.p.s) of low amplitude and high failure rate. Statistical analysis indicates that evoked release is quantal in nature and follows Poisson statistics, quantum size being initially very small, but after a few days approaching normal size. Short-term tetanic nerve stimulation reversibly increases the quantum content of e.p.p.s and during early stages of paralysis long-term (2 hr) stimulation causes an apparently permanent increase in quantum size.

4. Raising the extracellular Ca concentration from 2 to 16 mM increases the frequency of m.e.p.p.s in normal muscle but not in BoTx poisoned ones. K-free medium or ouabain, which are believed to raise the intracellular Ca concentration in nerve terminals, similarly increases m.e.p.p. frequency in normal but not in poisoned muscles. When the Ca-ionophore A 23187 is used together with high extracellular Ca (> 4 mM) massive release of transmitter occurs from poisoned terminals.

5. The extracellular Ca concentration which causes a certain level of transmitter release in response to nerve impulses is considerably higher

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at BoTx poisoned end-plates than at normal ones. The slope value for Ca dependence of transmitter release is about 1·5 compared with about 3 at normal end-plates.

6. Tetraethylammonium (TEA) greatly increases the amount of transmitter released by nerve impulses and restores neuromuscular transmission during all stages of poisoning, although it has no effect on spontaneous transmitter release. In the presence of TEA the power relation between Ca concentration and quantum content at the BoTx poisoned end-plate is similar to that seen at normal end-plates.

7. It is suggested that in BoTx poisoning the mechanism for transmitter release has a reduced sensitivity to Ca, and the level for activation by intracellular Ca is elevated. Once the intracellular concentration of Ca is raised to this level, by tetanic nerve stimulation, mechanical injury to nerve terminals, the Ca-ionophore or the prolongation of the nerve action potential with TEA, augmented transmitter release occurs, similar to that which occurs in normal nerve terminals at a lower level of Ca.

INTRODUCTION

Botulinum toxin (BoTx), produced by *Clostridium botulinum*, blocks acetylcholine (ACh) release from cholinergic nerve terminals (see review by Simpson, 1973). The toxin does not alter impulse conduction in the nerve or in the nerve terminals (Harris & Miledi, 1971) or the sensitivity to ACh in the end-plate region (Thesleff, 1960).

Recent studies have shown that BoTx does not completely abolish transmitter release from motor nerve terminals (Harris & Miledi, 1971; Spitzer, 1972; Boroff, del Castillo, Evoy & Steinhardt, 1974; Tonge, 1974). Spontaneous miniature end-plate potentials (m.e.p.p.s) of reduced frequency and amplitude persist even when neurally evoked end-plate potentials (e.p.p.s) are completely abolished. During onset of paralysis or when the nerve is lightly intoxicated (Thesleff, 1960; Boroff *et al.* 1974; Miledi & Spitzer, 1974), and occasionally during high frequency stimulation at later stages of BoTx paralysis (Tonge, 1974) e.p.p.s of a greatly reduced amplitude have been observed.

We have examined the properties of spontaneous and of neurally evoked release of transmitter from BoTx poisoned nerve terminals in rat skeletal muscle with emphasis on the importance of calcium, since this ion was observed to affect markedly the release process.

METHODS

The experiments were made on the extensor digitorum longus (EDL) muscle of male Sprague–Dawley rats (180–200 g). The muscle was poisoned by *Clostridium botulinum* toxin type A, dissolved in a buffer solution as described by Ambache

(1949). BoTx was given in a single injection of 0·25 ml. s.c. into the anterolateral region of the right hind leg, superficial to the distal part of the tibialis anterior muscle and the underlying EDL muscle. The amount of toxin given produced complete paralysis of the leg within 18 hr. The general condition of the animals was affected and muscular weakness was observed; by the third day about 10% of the animals died. Those which survived started to improve after the third day, but total paralysis of the injected leg remained during the whole observation period of up to 3 weeks. When the dose of BoTx was increased 20 times all animals died on the third day after the injection.

At various times after injection the muscle was removed from an animal under ether anaesthesia, placed in a constant temperature bath (35–37° C) and perfused with an oxygenated medium of the following composition (mM): NaCl, 135; $NaHCO_3$, 15·0; Na_2HPO_4, 1·0; KCl, 5·0; $CaCl_2$, 4·0; $MgCl_2$, 1·0; glucose, 11·0. When the ionic composition of the solution was altered, tonicity was maintained by adjustments of NaCl. The pH of the solution was 7·2–7·3.

EDL muscles from unpoisoned animals were used as controls. In some experiments the EDL muscle was denervated by sectioning the peroneal nerve at the knee of an animal under ether anaesthesia.

Electrical recording. Micro-electrodes filled with 2 M-K citrate acidified to pH 6·5 with citric acid were used for intracellular recording. Micro-electrodes of between 10 and 20 MΩ were selected for low noise. The noise level of the recording circuit was $\simeq 50\ \mu V$ making it possible to observe and measure m.e.p.p.s and e.p.p.s of low amplitude; the potentials were photographed from a Tektronix 502 oscilloscope and/or recorded on a polygraph (Mingograf 81). Extracellular recordings of end-plate activity were made with micro-electrodes filled with 4 M-NaCl.

Nerve stimulation. The nerve was stimulated with current pulses of 0·1 msec duration applied close to the point of nerve entry into the muscle by use of a glass capillary suction electrode. In a few experiments only a branch of the nerve was stimulated.

Localization of end-plates. This was done by following the fine superficial nerve branches and inserting the micro-electrode close to these sites. The rise time of m.e.p.p.s showed some variability after BoTx poisoning and was therefore not a reliable criterion for determining the proximity of the end-plate. For this reason the more consistent rise time of the e.p.p.s was used.

Abolishing fibrillation and mechanical movement of muscle. The presence of mechanical movement, resulting from fibrillation potentials in BoTx treated muscle (Josefsson & Thesleff, 1961), made intracellular recording difficult, particularly 6 days or longer after treatment. In experiments where nerve stimulation was not being used tetrodotoxin 10^{-6} M (Sankyo Co. Ltd, Tokyo) was routinely added to such muscles. In experiments where neurally evoked transmitter release was investigated mechanical movement was abolished by soaking the muscle in a medium containing Dantrolene sodium, 3 μg/ml. for 30 min, after which time the muscle was returned to normal medium (Ellis & Bryant, 1972). This was carried out only on muscles treated more than 6 days previously with BoTx.

Black Widow Spider Venom (BWSV). 1 drop of BWSV (from *Latrodectus mactans*) 5 glands/0·5 ml. in 0·9% NaCl was made up to 1 ml. with bathing solution and added to the muscle bath (5 ml.).

Ca-ionophore A 23187 (Eli Lilly Co.) was dissolved in ethanol and added to the bathing solution to give a final concentration of 10^{-5} M of the ionophore and 0·5% ethanol. In these experiments 0·5% ethanol was present as control in the bathing medium before the addition of the ionophore. This concentration of ethanol had only a slight effect on m.e.p.p. frequency (Gage, 1965).

Correction of m.e.p.p. and e.p.p. amplitudes. In comparing m.e.p.p.s and e.p.p.s from different fibres allowance was made for differences in resting potential according to the formula $70-15/E-15$, where E is the recorded resting potential (Katz & Thesleff, 1957). The fibres investigated had membrane potentials between -60 and -80 mV.

Analysis of transmitter release. The applicability of Poisson's theorem for transmitter release was tested by the method of failures (del Castillo & Katz, 1954) and also by comparing predicted and observed numbers of quantal components (Katz, 1966).

RESULTS

Spontaneous transmitter release

M.e.p.p. frequency and amplitude

End-plates were examined between 18 hr and 21 days following BoTx poisoning. M.e.p.p.s were recorded from practically all end-plates. Their frequency was initially low, but increased with time after poisoning (see Table 1). Similarly, their mean amplitude was initially small, increasing in size with time (Table 1). When end-plates were examined within 24 hr after BoTx treatment, m.e.p.p. amplitude histograms (Fig. 1*B*) showed a skew distribution with a predominance of small m.e.p.p.s (Harris & Miledi, 1971; Spitzer, 1972; Tonge, 1974). The mean amplitude was smaller than normal; however, a population of small m.e.p.p.s similar to those seen following BoTx treatment was sometimes present in normal muscle as shown by the histogram in Fig. 1*A*. The mean frequency $\pm$ S.D. of the small m.e.p.p.s in normal muscle was $0{\cdot}15 \pm 0{\cdot}10$/sec (in seven fibres) which is comparable to that observed during the early stage of BoTx poisoning (Table 1). Increasing the dose of BoTx twentyfold did not cause, in 24 hr, a change in m.e.p.p. frequency or amplitude more pronounced than that obtained with the usual dose (Table 1).

Few, or no normal size m.e.p.p.s were seen after 1 day, but 2 days after

TABLE 1. Frequency and amplitude of m.e.p.p.s at various times after BoTx poisoning. The values are means $\pm$ S.D. of means in individual fibres and the numbers in parentheses denote the number of fibres and muscles examined, respectively. The Table also includes data obtained 1 day after poisoning with a dose of BoTx 20 times larger.

Days after BoTx	Frequency (sec^{-1})	Amplitude (mV)
Control	$7{\cdot}0 \pm 1{\cdot}47$ (6; 4)	$0{\cdot}6 \pm 0{\cdot}18$ (10; 8)
1	$0{\cdot}1 \pm 0{\cdot}06$ (10; 5)	$0{\cdot}3 \pm 0{\cdot}08$ (11; 6)
1 (20 × dose)	$0{\cdot}1 \pm 0{\cdot}06$ (5; 3)	$0{\cdot}3 \pm 0{\cdot}06$ (4; 3)
2	$0{\cdot}2 \pm 0{\cdot}11$ (10; 8)	$0{\cdot}4 \pm 0{\cdot}12$ (13; 9)
3–4	$0{\cdot}2 \pm 0{\cdot}06$ (10; 7)	$0{\cdot}6 \pm 0{\cdot}23$ (11; 8)
5–6	$0{\cdot}9 \pm 0{\cdot}43$ (10; 5)	$1{\cdot}3 \pm 0{\cdot}45$ (6; 5)
7–9	$0{\cdot}6 \pm 0{\cdot}16$ (9; 7)	$1{\cdot}5 \pm 0{\cdot}46$ (7; 7)
12–14	$0{\cdot}6 \pm 0{\cdot}11$ (10; 4)	$2{\cdot}4 \pm 0{\cdot}38$ (5; 4)

toxin the proportion of m.e.p.p.s with normal, or slightly larger than normal amplitude increased. The shift in amplitude distribution continued with time so that by 5–6 days after BoTx treatment 24 % of the m.e.p.p.s (eight fibres) had amplitudes larger than 2 mV in contrast with normal

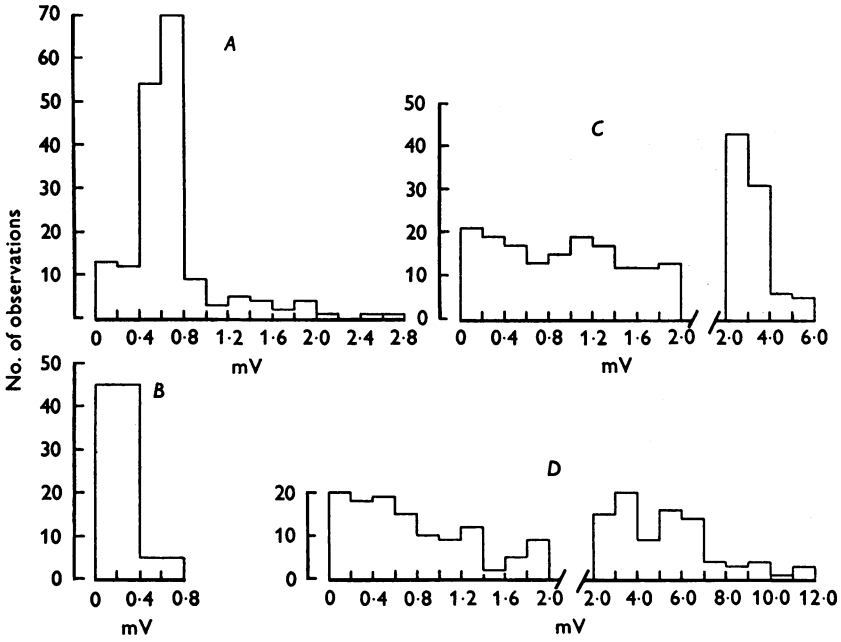

Fig. 1. Amplitude distribution of m.e.p.p.s at single end-plates. *A*, control muscle; *B*, 1 day after BoTx poisoning; *C*, 6 days after poisoning; *D*, 14 days after BoTx.

end-plates at which 2 % of the m.e.p.p.s (six fibres) were above 2 mV. After 12–14 days the m.e.p.p.s often reached values as large as 10 mV (Fig. 1*D*). Fig. 2 shows examples of m.e.p.p.s recorded in a control muscle and muscles taken 1, 9 and 14 days respectively after BoTx treatment.

At all end-plates poisoned with BoTx m.e.p.p.s showed a large variability in their time course. With the micro-electrode positioned such that some m.e.p.p.s had 1 msec rise time, other m.e.p.p.s had rise times which varied between 1 and 10 msec. There was no apparent correlation between amplitude and rise time. On occasion m.e.p.p.s had humps on the rising or falling phases suggesting they were composed of more than one quantum.

Origin of m.e.p.p.s

To determine the site of origin of m.e.p.p.s EDL-muscles were denervated the day before poisoning with BoTx. Visually identified end-plates

were investigated for the presence of m.e.p.p.s 48 hr after BoTx treatment. No electrical activity was found during observation periods lasting at least 5 min at each end-plate. This indicates that the m.e.p.p.s originate from the motor nerve and also seems to eliminate the possibility that they arose from Schwann cells (see Bevan, Miledi & Grampp, 1973).

It seems that the m.e.p.p.s seen after BoTx are caused by ACh-quanta

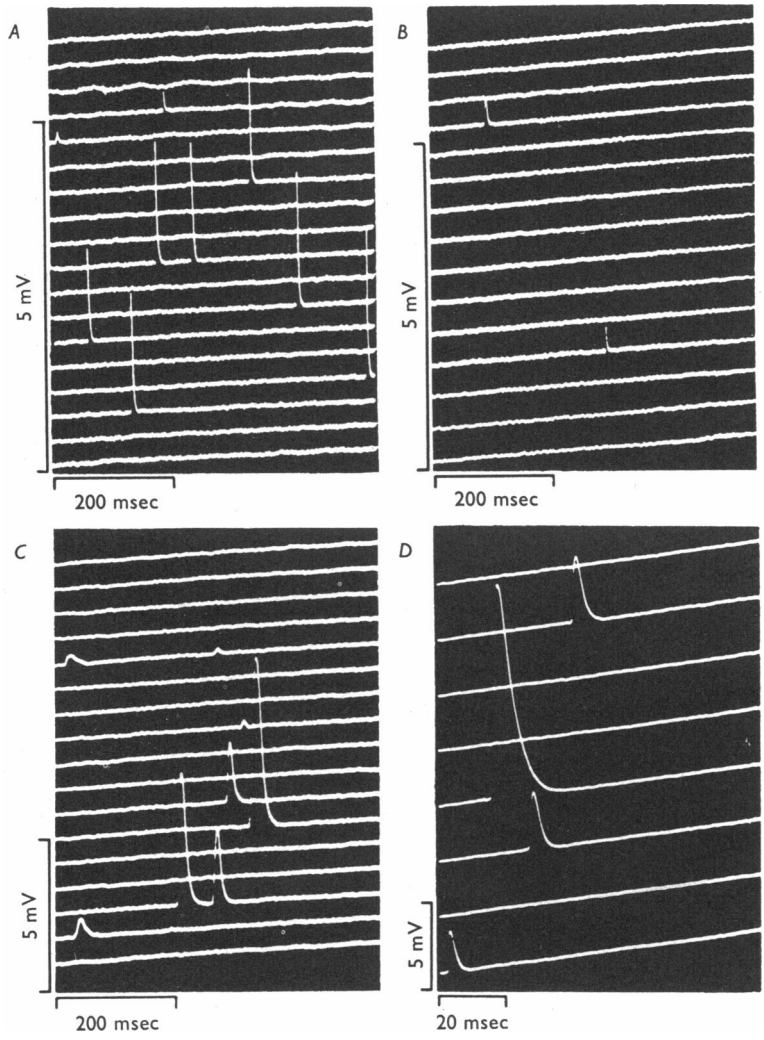

Fig. 2. Examples of m.e.p.p.s recorded in *A*, normal muscle, *B*, 1 day after BoTx poisoning, *C*, 9 days after BoTx, and *D*, 14 days after poisoning.

because D-tubocurarine 10^{-6} w/v, when added to the perfusing medium, completely abolished all spontaneous activity.

Tetrodotoxin 10^{-6} M, which blocks action potentials (Kao, 1966), had no action on m.e.p.p. amplitude distribution. This seems to exclude the no possibility that the large m.e.p.p.s resulted from spontaneous action potentials in the nerve terminal and the subsequent phasic release of transmitter.

It has been shown that BoTx poisoning is followed by axonal sprouting of the motor nerve terminal (Duchen, 1970, 1971), and that regenerating nerve terminals may release m.e.p.p.s with an abnormal amplitude distribution, with more small events than normal (Dennis & Miledi, 1974*a*) or more large events than normal (Thesleff, 1966; Bennett, McLachlan & Taylor, 1973).

In an attempt to test if some part of the m.e.p.p. population at the BoTx poisoned end-plate resulted from the release of transmitter from sprouting nerve terminals, animals were given a second injection of BoTx 5–6 days after the first injection. If some of the m.e.p.p.s arose from transmitter release from nerve terminal sprouts, which formed after the first injection, or alternatively, from reinnervating nerve terminals, these m.e.p.p.s might be expected to disappear 24 hr after a second injection of BoTx. End-plates investigated in this manner showed the m.e.p.p. frequency and amplitude distributions normally seen 6–7 days after a single injection of BoTx. Transmitter release at this stage was therefore uninfluenced by a second dose of BoTx and the large amplitude m.e.p.p.s which appeared were apparently resistant to the toxin.

Influence of nerve stimulation

Nerve stimulation at frequencies of 20–100 Hz gave rise to increased frequencies of m.e.p.p.s at some end-plates in BoTx poisoned muscles as previously reported (Harris & Miledi, 1971; Spitzer, 1972). The increase started a few seconds after the beginning of stimulation and declined slowly after the end of the tetanic train. Sometimes stimulation caused bursts of m.e.p.p.s (Fig. 5*A*). When m.e.p.p. frequency was increased by stimulation the amplitude distribution of the m.e.p.p.s approached the normal type of distribution seen at end-plates.

The amplitude distribution of m.e.p.p.s 1–3 days after BoTx treatment underwent a more permanent change following several hours of stimulation at 15–30 Hz. Fig. 3 shows amplitude distributions of m.e.p.p.s recorded from the same end-plate before and after 4 hr of continuous stimulation of the nerve. The mean amplitude of the m.e.p.p.s was increased from 0·45 to 0·94 mV following stimulation. Unlike the m.e.p.p. distribution present during a short period of tetanic stimulation, the m.e.p.p. distribution

after prolonged stimulation did not revert to a skew distribution when stimulation ceased. Instead a class of larger m.e.p.p.s remained for at least 6 hr superimposed on the skew distribution. Differences between the amplitude distributions of the m.e.p.p.s occurring at stimulated and unstimulated end-plates in the same muscle were seen when a branch of the nerve had been stimulated tetanically for several hours. In this situation only the end-plates which had been stimulated changed their m.e.p.p. amplitude distribution to incorporate larger amplitudes.

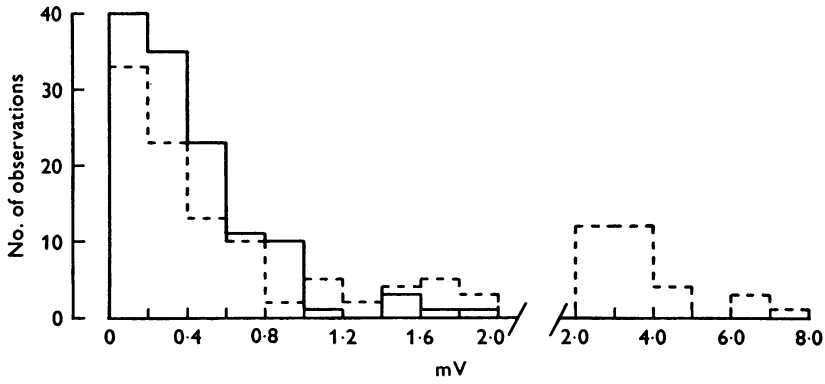

Fig. 3. Amplitude distribution of m.e.p.p.s at the same end-plate before (continuous line) and after (interrupted line) 4 hr of stimulation of the nerve at 15 Hz. The experiment was made 3 days after BoTx.

The amplitude distribution of m.e.p.p.s at BoTx poisoned end-plates was also changed by mechanical irritation of the nerve terminal with a micro-electrode. This increased the frequency of m.e.p.p.s and their amplitude distribution changed from a skew to a Gaussian distribution, the mean m.e.p.p. size approaching that seen at end-plates in unpoisoned muscles (Fig. 4*A*).

The effect of Black Widow Spider Venom (BWSV)

As BoTx treated terminals are refractory to several factors which increase the spontaneous release of transmitter from normal nerve terminals, such as increased osmolarity and increased potassium concentration (Harris & Miledi, 1971), it was of interest to investigate the action of BWSV. This venom causes a massive release of transmitter from nerve terminals with a corresponding depletion of synaptic vesicles in a wide variety of species (see Longenecker, Hurlbut, Mauro & Clark, 1970; Cull-Candy, Neal & Usherwood, 1973).

The application of BWSV in the bathing medium, to BoTx poisoned muscles and to control muscles, produces a much increased m.e.p.p.

frequency (B. A. Stamenović, B. D. Bošković & M. S. Cvetković, personal communication). We observed that following a delay of about 1–5 min the m.e.p.p. frequency in control and in BoTx (3–9 days) poisoned muscles reached levels which could not be counted (i.e. > 200/sec), interrupted by periods of near normal frequency. In the BoTx treated muscles the m.e.p.p. amplitudes were altered from the skew or bimodal distribution, before the increase, to a bell-shaped distribution after the m.e.p.p. frequency had begun to increase (see histogram in Fig. 4*B*).

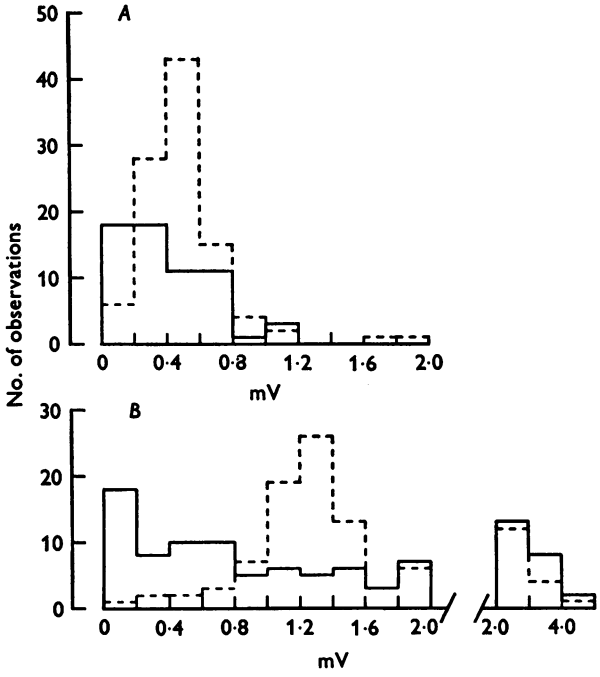

Fig. 4. Amplitude distributions of m.e.p.p.s in single fibres, (*A*) 2 days and (*B*) 8 days after BoTx are shown by continuous lines. The amplitude distribution was altered (interrupted lines) in *A* by mechanical injury of the nerve terminal by the micro-electrode and in *B* by the addition to the bathing medium of BWSV.

Neurally evoked transmitter release

Characteristics of e.p.p.s

Following BoTx treatment e.p.p.s with a small amplitude were seen at practically all end-plates investigated, when the motor nerve was stimulated at 0·5 Hz; however, more than 50 % of the nerve impulses at each end-plate failed to release any transmitter in the presence of 4 mM-Ca. This also applied to animals examined 1 day after the injection of a dose

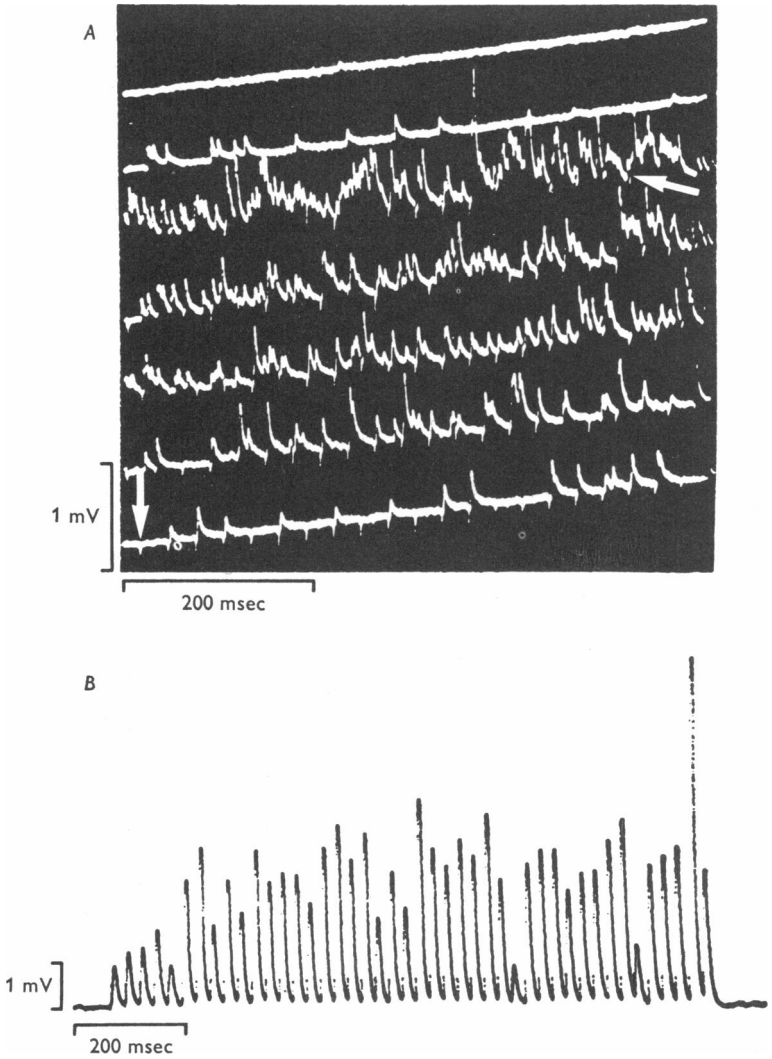

Fig. 5. E.p.p.s in response to nerve stimulation at 40 Hz in muscles 2 (*A*) and 5 (*B*) days after BoTx poisoning. In *A* (read from bottom to top) the nerve is stimulated during the period marked by arrows. The number of failures to release transmitter decreased during stimulation and simultaneously the m.e.p.p. frequency increased. Note that many e.p.p.s are of about the same amplitude as the smallest m.e.p.p.s. Five days after poisoning (*B*) nerve stimulation no longer causes failures to release transmitter and the e.p.p.s are markedly facilitated in amplitude during the period of stimulation.

of BoTx 20 times larger than usual (thirty fibres; three rats). The percentage of nerve impulses which failed to release transmitter decreased with time after poisoning, and after about 1 week this sometimes necessitated use of an increased Mg concentration in the bathing medium to obtain the 'single quanta e.p.p.s'.

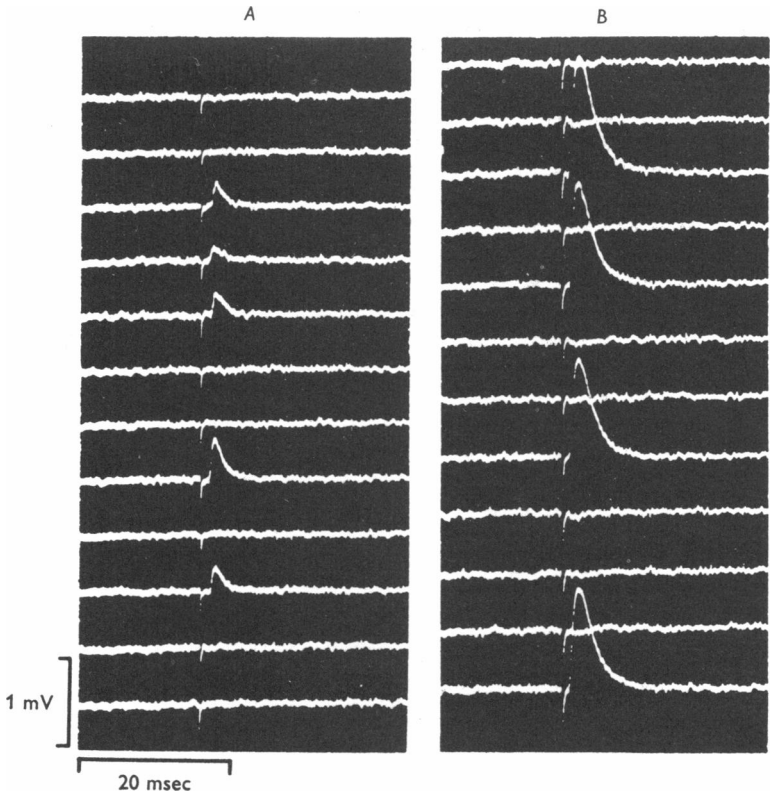

Fig. 6. E.p.p.s of single quanta (in both *A* and *B*) in response to stimulation at 0·5 Hz at the same end-plate in a muscle 1 day after BoTx poisoning. Between records *A* and *B* the nerve was stimulated at 15 Hz for 4 hr. Nerve stimulation caused an increase in e.p.p. amplitude without affecting the number of failures to release transmitter; compare records *A* and *B*. This effect appeared to be permanent, persisting for several hr after the end of stimulation.

When the nerve was stimulated at 5 Hz or more the number of failures to release transmitter was markedly reduced (Fig. 5) and the e.p.p.s in the beginning of the train showed facilitation, particularly during the later stages, i.e. 3 days or more after BoTx injection (Fig. 5*B*). E.p.p.s evoked by successive impulses appear to show stepwise fluctuation in amplitude

(Fig. 5*B*). This is similar to the situation described for Ca-deficient or Mg-blocked preparations (Fatt & Katz, 1952; del Castillo & Katz, 1954).

For the first 2 days after BoTx poisoning many e.p.p.s were of the same amplitude as the smallest *m.e.p.p.s* (Fig. 5*A* and 6*A*). By 2 or 3 days after poisoning the e.p.p.s were the size of m.e.p.p.s in normal muscle. Within 3 weeks after poisoning the e.p.p.s increased in size as could be expected from the reduction of the number of failures to release transmitter, and from the fibre atrophy following BoTx treatment (Jirmanová, Sobotková, Thesleff & Zelená, 1964).

As with spontaneous transmitter release, a second injection of BoTx given 4–9 days after the first one, did not alter the e.p.p. amplitude or the number of failures to release transmitter (forty fibres; four rats).

During the first day after BoTx treatment e.p.p.s were increased in amplitude by several hours of stimulation at 10–30 Hz, the very small e.p.p.s no longer occurring (Fig. 6*B*). The increase in amplitude persisted for at least 6 hr after the end of stimulation, and was not accompanied by any marked change in the number of failures of the nerve terminal to release transmitter, indicating that the larger e.p.p.s were still mainly *single* quanta. This change in amplitude distribution of e.p.p.s after prolonged tetanic stimulation has some similarity to the change in m.e.p.p. amplitudes which occurs at the same time (see above).

Unlike the m.e.p.p.s, the e.p.p.s had a relatively constant time course (Fig. 6).

Extracellular recording of e.p.p.s

Because of the large number of failures, it was important to show that the nerve terminals conducted a spike in response to every stimulus. Fig. 7 shows a typical result from experiments made with extracellularly positioned micro-electrodes. The nerve terminal spike only occasionally gave rise to an e.p.p. Failure of the nerve terminal spike was seen only in response to high frequency stimulation, i.e. > 50 Hz.

Quantal components of e.p.p.s

When evoked transmitter release from normal nerve terminals is blocked by high Mg so that more than 50 % of the impulses fail to release transmitter, most of the e.p.p.s are made up of single quanta (del Castillo & Katz, 1954). In this situation amplitude histograms of e.p.p.s and m.e.p.p.s are almost coincident.

Fig. 8 shows amplitude histograms of e.p.p.s and m.e.p.p.s from BoTx poisoned end-plates 1, 5 and 14 days after BoTx treatment, when the number of failures was more than 50 %. Amplitude distributions of the spontaneous and the evoked events do not coincide, but the overlap is

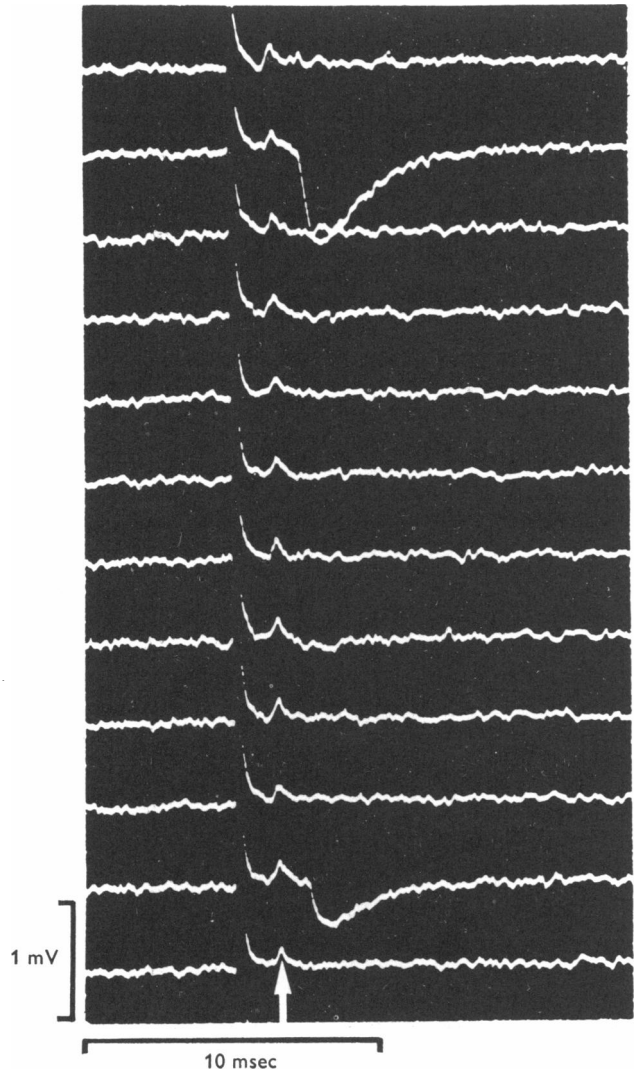

Fig. 7. Extracellular recording from an end-plate in a muscle 4 days after poisoning with BoTx. The nerve is stimulated at 0·5 Hz, the shock artifact being in each instance followed by a nerve terminal spike as marked by the arrow in the bottom tracing. Occasionally, the nerve impulse gave rise to an e.p.p. as shown by the potentials present in two of the tracings.

greatest in the early stages of poisoning. The mean amplitude of m.e.p.p.s is smaller than that of the e.p.p.s, and the m.e.p.p. histogram always contained a larger porportion of both small and large events than the e.p.p. histogram (Fig. 8). In this situation the e.p.p.s could not be made up of

quantal components identical with the m.e.p.p.s and follow Poisson statistics, unless only a part of the population of spontaneously released transmitter packets were available for release by nerve impulses. As the

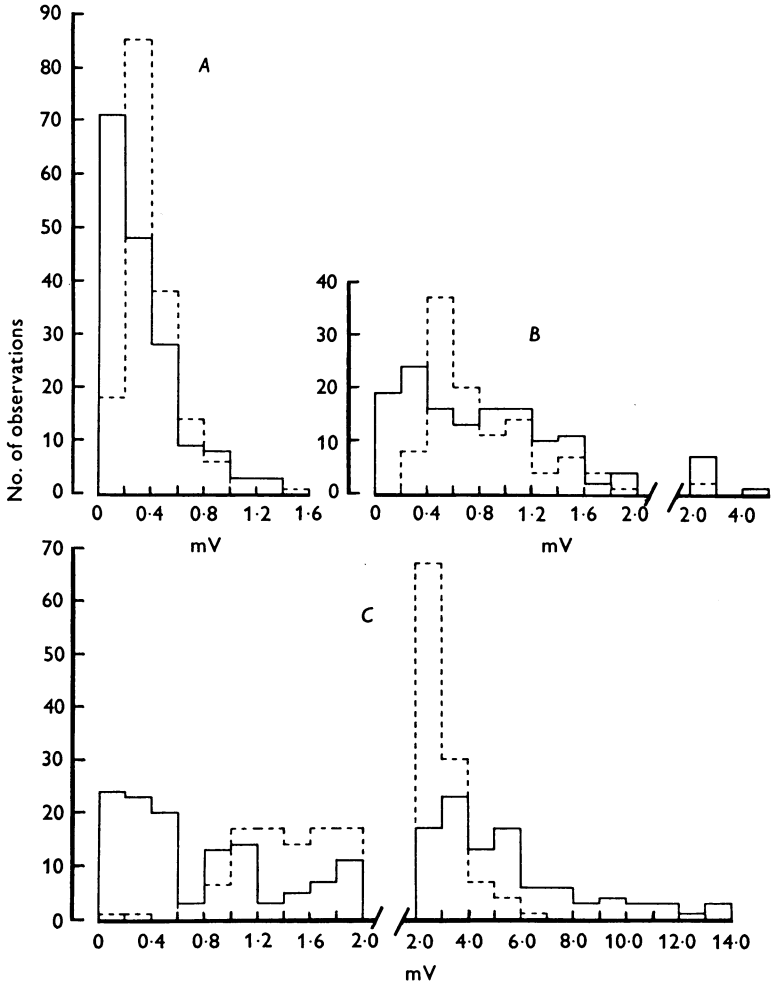

Fig. 8. Amplitude distributions of m.e.p.p.s (continuous line) and e.p.p.s (interrupted line). Graphs *A*, *B*, *C* from muscles 1, 5 and 14 days after BoTx respectively. E.p.p. amplitudes were obtained from end-plates where the number of failures to release transmitter was higher than 50 %. In *C* an increased Mg concentration was used to obtain a high percentage of failures.

m.e.p.p. population released during the action of BWSV has an amplitude distribution similar to the e.p.p.s of single quanta seen later than 1 day after poisoning, these m.e.p.p.s were used as quantum size in statistical

analysis of evoked transmitter release later than day 1. As shown in Table 2*A* values are obtained which are in satisfactory agreement with Poisson statistics.

If it is assumed that Poisson's theorem applies to the transmitter release at BoTx poisoned end-plates, it is possible to calculate the predicted number of responses containing 0, 1, 2, 3, ..., quantal components, i.e. n_0, n_1, n_2, n_3, ..., $n_1 = mn_0$, $n_2 = (m/2)n_1$, $n_3 = (m/3)n_2$, etc. If m is calculated from m = ln (number of impulses/number of failures), and quantum size, q, from q = mean e.p.p./m, q is obtained without the use of m.e.p.p.s (Katz, 1966). If the e.p.p. amplitude histograms are divided into successive groups centred around amplitudes of multiples of q, approximate values are obtained for observed number of e.p.p.s. As shown in Table 2, there is, in general, satisfactory agreement between predicted and observed values for n during all stages after poisoning. However, in some end-plates a small discrepancy from Poisson statistics was observed in that the number of large e.p.p.s was slightly higher than predicted.

TABLE 2. Analysis of neurally evoked transmitter release at single end-plates of BoTx poisoned muscles for fit with Poisson statistics. Two different methods were used for testing. In Table *A* mean quantum content, m, is calculated from

$$m = \frac{\text{mean e.p.p. size } (A)}{\text{mean m.e.p.p. size during BWSV } (B)} \quad \text{and} \quad m = \ln \frac{\text{number of impulses } (C)}{\text{number of failures } (D)}$$

(del Castillo & Katz, 1954).

Table *B* shows the number of e.p.p.s predicted by the Poisson theorem containing 0, 1, 2, 3, ... quantal components (n_0, n_1, n_2, n_4 ...) over the number of observed, quantal components of e.p.p.s. For details see text

A

Days after BoTx	*A*/*B* (mV)	*C*/*D*	*m* (*A*/*B*)	*m* (ln *C*/*D*)
2	0·203/0·643	852/565	0·32	0·41
4	0·093/0·301	323/218	0·31	0·39
4	0·228/0·268	447/169	0·85	0·97
5	0·179/0·351	430/247	0·51	0·55
5	0·130/1·001	597/502	0·13	0·17

B

	Calculated *q* (mV)	*n* predicted/observed				
		n_1	n_2	n_3	n_4	n_5
1	0·27	118/120	42/36	10/13	2/2	0/1
2	0·18	138/130	50/52	12/15	2/5	0/2
2	0·15	85/85	17/14	2/5	0/0	
5	0·32	150/146	43/43	8/10	1/1	0/1
5	0·62	75/72	27/28	7/9	1/1	0/1

Effects of Ca on transmitter release

Spontaneous release

Fig. 9 illustrates m.e.p.p. frequencies recorded from fibres in normal and in BoTx poisoned muscles when the extracellular Ca was altered between 2 and 16 mM. Normal muscle showed increased m.e.p.p. frequencies with elevation of the Ca concentration, as previously reported for the rat phrenic nerve-hemidiaphragm preparation (Hubbard, Jones & Landau, 1968). In BoTx treated muscles there was no increase in the rate of m.e.p.p.s with increased levels of Ca. The amplitude distribution of m.e.p.p.s in BoTx poisoned muscles was not affected by these changes in Ca concentrations.

Neurally evoked release

Small e.p.p.s consisting of single or a few quanta occurred at nearly all end-plates investigated in 2 mM-Ca in BoTx poisoned muscles, but at this level of extracellular Ca about 95 % of the nerve impulses failed to release transmitter when the nerve was stimulated at 0·5 Hz. Reducing the Ca concentration below 2 mM increased the number of failures. Increasing the Ca concentration increased the number of e.p.p.s and their mean quantum content and this effect remained steady at the new level. It was therefore of interest to compare the Ca requirement for release of transmitter from BoTx poisoned nerve terminals with that of normal nerve terminals.

In order to compare the two types of terminals over the same range of Ca concentrations it was necessary to reduce the neurally evoked transmitter release from the normal nerve to approximately the same level as that released from BoTx poisoned terminals. Muscles were therefore studied at a fixed level of Mg, normal muscles at 20 mM and BoTx treated muscles at 1 mM. A range of three or four different Ca concentrations was chosen (2–16 mM). At each Ca concentration records were taken of the responses to approximately 200–350 stimuli. Despite the high concentrations of divalent cations no failure of the nerve terminal spike was observed in control experiment with external recording, as previously described. Fig. 10 shows mean quantum content, m, determined over a range of Ca concentrations plotted on double logarithmic co-ordinates. The mean $\pm$ S.D. value for Ca dependence of transmitter release at normal end-plates (three fibres, 3 muscles) lay in a line with a slope of $2{\cdot}8 \pm 0{\cdot}15$. This compares with values of 2·7 for rat neuromuscular junction (Hubbard *et al.* 1968), 4 for mouse neuromuscular junction (Cooke, Okamoto & Quastel, 1973), 2·7 for the squid giant synapse (Katz & Miledi, 1970) and 4 for frog neuromuscular junction (Dodge & Rahamimoff, 1967; Dennis & Miledi, 1974*b*). The fact that we obtained comparable figures to those previously described, using about double the concentration of both Mg and Ca, is not unexpected if

the antagonism between these cations is competitive as is believed (Katz, 1969). Transmitter release at end-plates poisoned with BoTx also demonstrated a Ca dependence (Fig. 10), but this relationship differed in two distinct ways from that of normal end-plates.

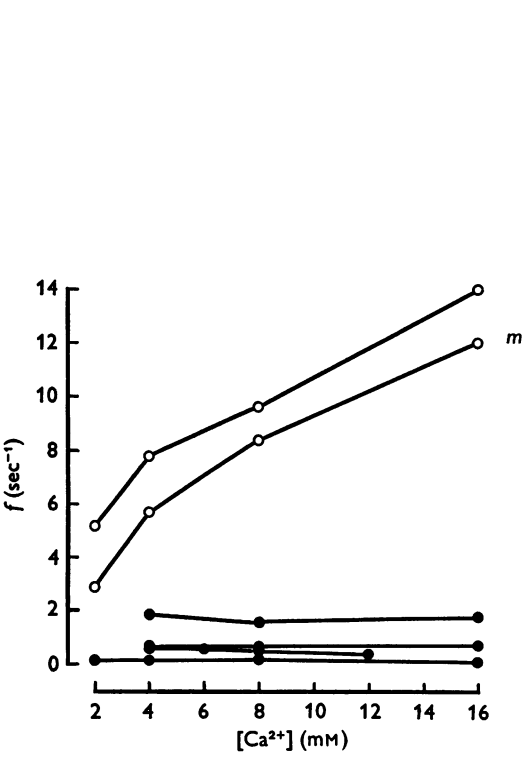

Fig. 9

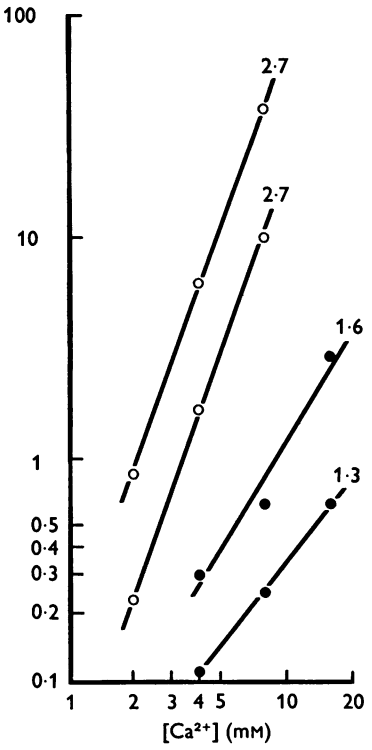

Fig. 10

Fig. 9. Mean m.e.p.p. frequencies recorded in different extracellular Ca concentrations (abscissa) at two end-plates in a normal muscle (open circle), and at four end-plates in muscles 2–4 days after BoTx poisoning (filled circles).

Fig. 10. Mean quantum content, *m*, at various extracellular Ca concentrations at two representative normal end-plates (open circles) and at two BoTx poisoned end-plates (filled circles) 2 and 4 days after poisoning. The values from normal muscles were obtained in the presence of 20 mM-Mg, (see text). The slope of each line is shown by the adjacent number.

Firstly, the extracellular concentration of Ca which allowed a certain level of transmitter release in response to a nerve impulse was considerably higher at BoTx poisoned end-plates than at normal end-plates.

Secondly, at BoTx poisoned end-plates (six fibres, six muscles) the

mean ± S.D. slope value for Ca dependence of transmitter release was 1·3 ± 0·18 compared with about 3 at normal end-plates (Fig. 10).

These results indicate that in BoTx poisoned muscles transmitter release is less affected by the external concentration of Ca than normal muscle but give no information about the mechanism of this effect. Conceivably BoTx could reduce the influx of Ca into the nerve terminal, or decrease the efficacy of intracellular Ca at eliciting transmitter release, or both.

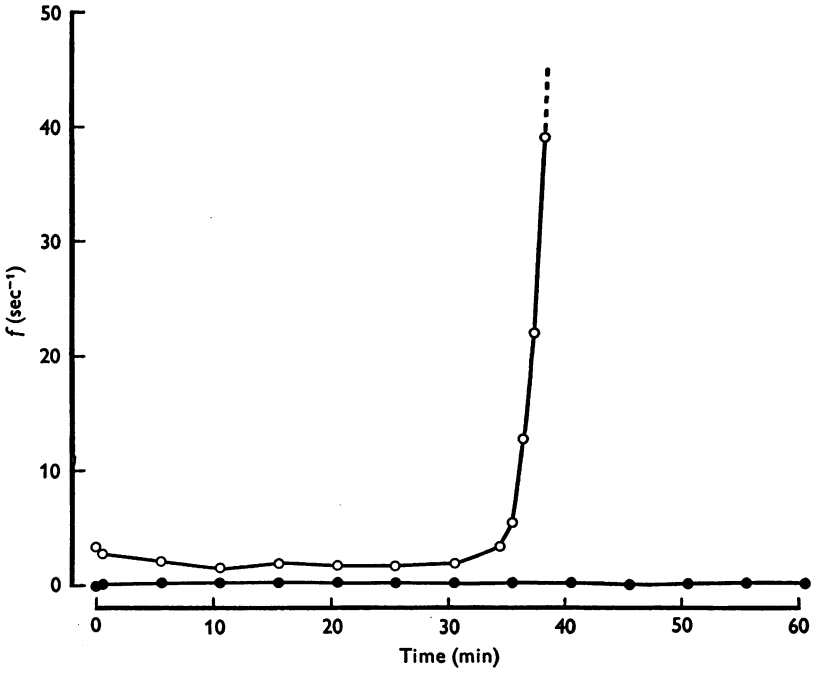

Fig. 11. Open circles show m.e.p.p. frequencies at an end-plate in a normal muscle in the presence of K-free medium added to the muscle bath at zero time. Filled circles are similar values from a BoTx poisoned muscle.

K-free medium or ouabain

Exposure of motor nerve terminals to cardiac glycosides or to K-free solution is known to cause a marked increase in m.e.p.p. frequency after a delay of 30–60 min (Elmqvist & Feldman, 1965; Birks & Cohen, 1968). The increased m.e.p.p. frequency is probably caused by inhibition of the Na–K exchange pump which has been suggested to lead to an increase in intracellular Ca (Baker & Crawford, 1975).

Following removal of potassium the m.e.p.p. frequency in normal muscle rose to a high level after a delay of 30–40 min, while transmitter release from BoTx poisoned terminals showed no significant change (Fig. 11). A

similar effect was observed in K-free medium following removal of extracellular Ca and the addition of 1 mM-EDTA to the solution. In this condition the increase in transmitter output is presumably caused by Ca released from intracellular storage sites.

The addition of ouabain (0·2 mM) to the bathing medium in the presence of 2 mM-Ca produced results similar to those seen in K-free medium. After a delay of 30–40 min the m.e.p.p. frequency at end-plates in normal muscle rose rapidly to a level which could not be measured, while the m.e.p.p. frequency at BoTx poisoned end-plates was not significantly altered.

These results support the idea that transmitter release in BoTx poisoned terminals, in comparison with normal terminals, has a lowered sensitivity to the level of intracellular Ca.

The Ca-ionophore A 23187

An antibiotic A 23187 is believed to act as a Ca-ionophore allowing the passage of Ca across biological membranes. In addition it may release Ca from intracellular binding sites (Reed & Lardy, 1972; Wong, Wilkinson, Hemill & Horng, 1973; Russell, Hansen & Thorn, 1974).

At normal end-plates in the presence of 4 mM extracellular Ca the application of the ionophore (10^{-5} M) led, within 10–20 min, to a massive release of transmitter as observed by the appearance of m.e.p.p.s at frequencies above 200/sec. In BoTx poisoned muscles a similar application failed to cause a high frequency burst of transmitter and only after 60 min or longer did the m.e.p.p. frequency rise. Increasing the Ca concentration above 4 mM enhanced the effect of the ionophore in BoTx poisoned muscles and transmitter release comparable to that observed in normal muscle occurred. As shown by the histograms in Fig. 12*A*, the amplitude distribution of m.e.p.p.s, under these conditions of release, changed from a skew to a Gaussian distribution. When Ca (15–20 mM) was applied by microperfusion, through a pipette with a diameter of 50–100 μm, to the end-plate of a BoTx poisoned muscle fibre in the presence of the ionophore, a large increase in m.e.p.p. frequency occurred, and when the pipette was withdrawn the frequency returned to control values as shown in Fig. 12*B*.

Another way of studying the effects of the ionophore was to apply it to the muscle in Ca-free medium and in the presence of 1 mM-EGTA. This concentration of EGTA should reduce the external Ca level to less than 10^{-8} M (Hubbard *et al.* 1968; Miledi & Thies, 1971). Normal muscles, which had a low frequency of m.e.p.p.s in the absence of extracellular Ca, demonstrated a gradual increase of m.e.p.p. frequency when the ionophore (10^{-5} M) was applied and presumably released Ca from intracellular binding sites (Fig. 13). The frequency gradually rose, within 15–60 min, to a level that could not be accurately measured, i.e. above 200/sec and this was

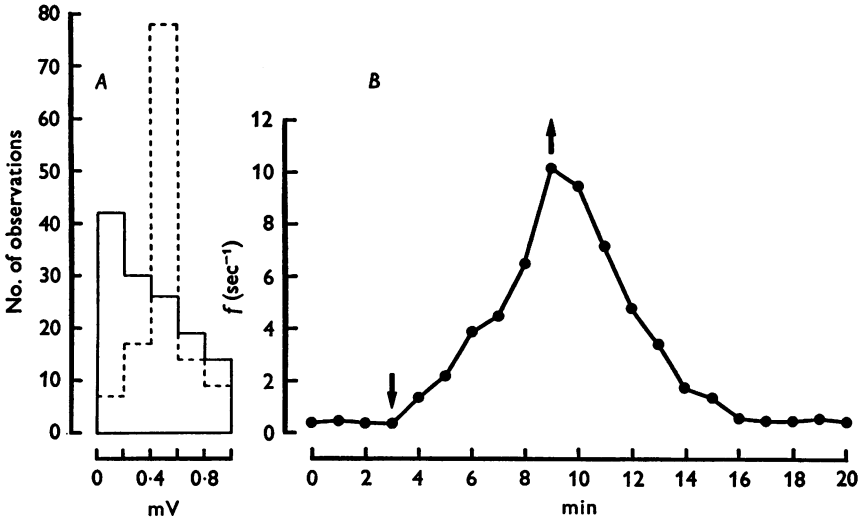

Fig. 12. *A*, amplitude distributions of m.e.p.p.s in a 3 days BoTx poisoned muscle before (continuous line) and in the presence of the Ca-ionophore A-23187 and 10 mM extracellular Ca (interrupted line). *B*, the frequency of m.e.p.p.s at a BoTx poisoned end-plate in the presence of the Ca-ionophore and 4 mM-Ca. Between the arrows the end-plate was superfused with 20 mM-Ca from a micropipette.

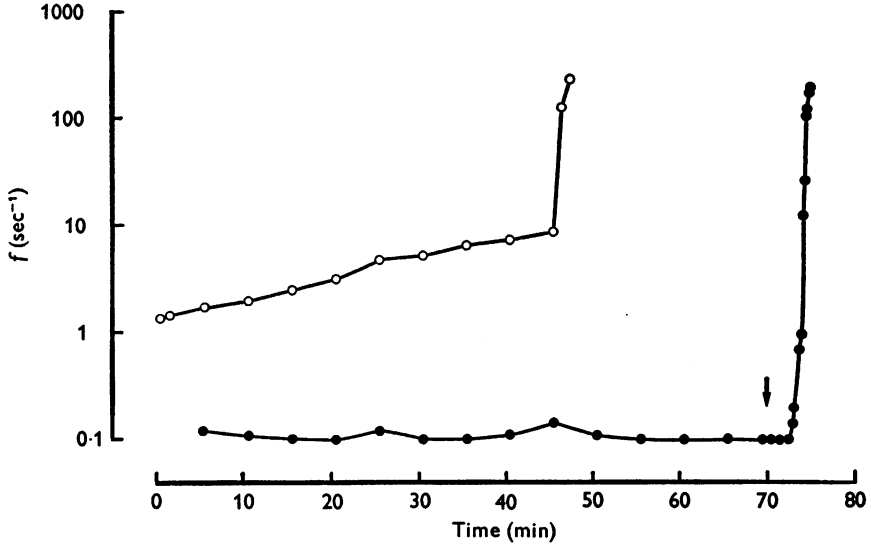

Fig. 13. M.e.p.p. frequencies at normal (open circle) and at a BoTx poisoned (filled circle) end-plate in a Ca-free medium with 1 mM-EGTA and 10^{-5} M-Ca-ionophore. At the normal end-plate the m.e.p.p. frequency gradually rose to a high value, while at the BoTx poisoned one no change occurred until EGTA was removed and 5 mM-Ca added to the bathing medium at the time shown by the arrow.

maintained for approximately 15 min. Finally, m.e.p.p.s decreased in both frequency and amplitude to very low levels. BoTx poisoned muscles showed no increase in m.e.p.p. frequency even following long treatment with the ionophore (in some experiments 2 hr). Removal of EGTA followed by the application of 5 mM-Ca produced an immediate increase in m.e.p.p. frequency, the frequency reaching levels similar to those observed in control muscles (Fig. 13). The accelerated release of m.e.p.p.s from BoTx treated muscles was readily reversed by removal of the extracellular Ca. The addition of Mg, 10 or 20 mM, did not noticeably reduce the accelerated release of transmitter.

Effects of tetraethylammonium (TEA)

TEA increases the amount of Ca which enters the nerve terminal during the presynaptic spike (Katz & Miledi, 1967). TEA 0·2–2·0 mM failed to alter m.e.p.p. frequency at BoTx poisoned end-plates and, similarly, elevating the level of Ca from 4 to 10 mM or of K from 5 to 20 mM failed to increase m.e.p.p. frequency in the presence of TEA (2 mM), showing that the drug has no obvious effect on spontaneous transmitter release in BoTx poisoning.

When neurally evoked transmitter release was studied, TEA markedly increased the amplitude of the e.p.p. and abolished failures of the nerve impulse to release transmitter (see Fig. 14, insert). In a typical fibre from a 3 day BoTx poisoned muscle, 0·2 mM-TEA increased the mean quantum content of e.p.p.s 6 times, 0·4 mM 12 times and 0·6 mM 23 times. With 0·4–0·6 mM-TEA a previously completely paralysed muscle started to twitch vigorously in response to single nerve stimuli, isometric twitch tensions of 10–15 *g* being recorded. This effect was obtained at any time after poisoning. A detailed account of the effects of TEA will be published elsewhere.

To study the Ca dependence of evoked release in the presence of TEA experiments were made with high Mg to reduce transmitter release to levels below that capable of generation of muscle twitches. Fig. 14 shows the power relation between Ca concentration and mean quantum content of e.p.p.s in the presence of 1 mM-TEA in BoTx poisoned (15 mM-Mg), and in control muscles (20 mM-Mg). At normal end-plates TEA had only a slight effect on the value for Ca dependence, the mean slope remaining at about 3 ($3{\cdot}7 \pm 0{\cdot}55$, three fibres), while in BoTx poisoned muscles (six fibres) the slope was altered from about 1·5 to a mean $\pm$ S.D. value of $5{\cdot}0 \pm 1{\cdot}57$ (compare with Fig. 10).

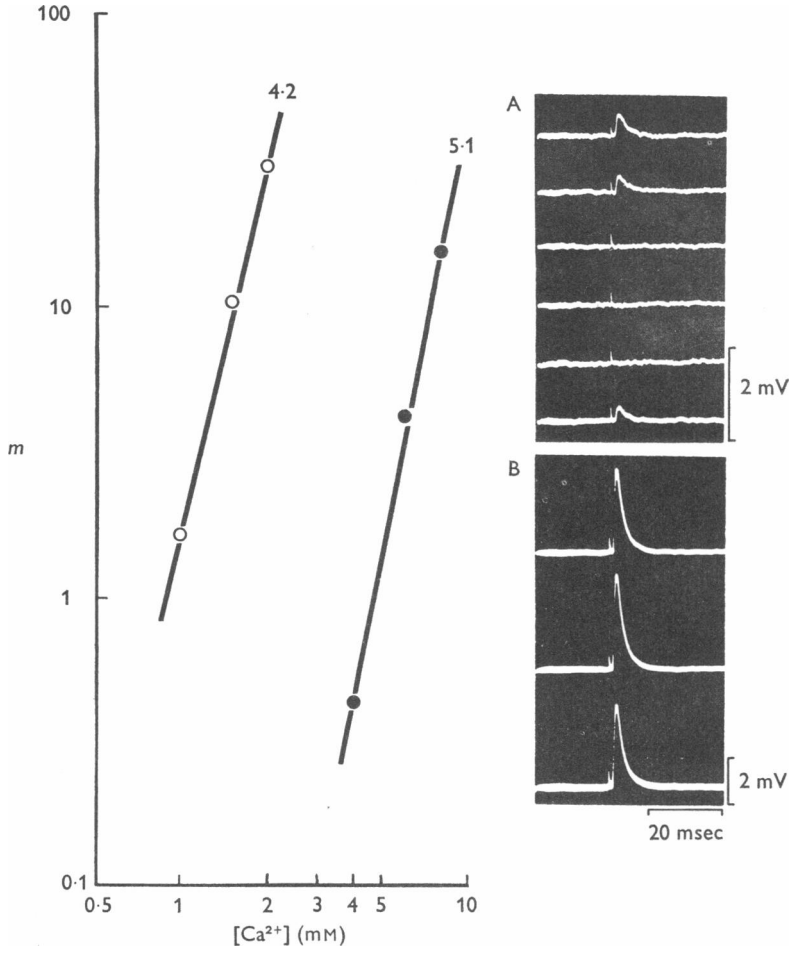

Fig. 14. Mean quantum content, m, at various extracellular Ca concentrations and in the presence of 1 mM-TEA; open circles from a representative end-plate in a normal muscle and filled circles from an end-plate in a BoTx muscle 4 days after poisoning. To reduce transmitter release below the level of generation of muscle twitches high concentrations of Mg were used, 15 mM in BoTx muscle and 20 mM in normal muscle. The numbers adjacent to each line indicate its slope. The inserts show representative records of e.p.p.s in response to nerve stimuli at 0·5 Hz at a 1 day BoTx poisoned end-plate before (upper record) and after (lower record) the addition to the bathing medium of 0·5 mM-TEA at 4 mM extracellular Ca. Note that the addition of TEA abolishes all failures of transmitter release and that the voltage calibrations of the two records are different.

DISCUSSION

After BoTx poisoning the initial drastic reduction in transmitter release is followed by a period of more gradual changes in the release process M.e.p.p.s which were small and infrequent early after poisoning (Harris & Miledi, 1971; Spitzer, 1973; Boroff *et al.* 1974; Tonge, 1974), increased in both frequency and amplitude throughout the period of study (18 hr–21 days). The increase in mean m.e.p.p. amplitude can only partly reflect the atrophy of the fibres that is observed after BoTx poisoning (Jirmanová *et al.* 1964; Katz & Thesleff, 1957).

After BoTx the m.e.p.p. amplitude distribution was at all times 'abnormal', consisting initially of a greater proportion of small m.e.p.p.s and later of small and large m.e.p.p.s. At any time after poisoning, it was possible to alter the amplitude distribution of m.e.p.p.s, to a distribution which approached that at normal end-plates, using procedures which enhanced spontaneous transmitter release, i.e. addition of the Ca-ionophore A23187 together with high Ca, addition of BWSV, short- or long-term tetanic nerve stimulation (Harris & Miledi, 1971; Spitzer, 1972), or nerve terminal damage (Boroff *et al.* 1974). These observations indicate that the action of BoTx on the m.e.p.p. amplitude distribution is presynaptic in origin. They further show that apparently normal packets of ACh exist in the nerve terminals after BoTx poisoning, although these are not usually spontaneously released.

Of particular interest was the finding that changes in the external Ca concentration between 2 and 16 mM affected the frequency of m.e.p.p.s in normal muscle, but not in poisoned muscle. Similarly, procedures which are believed to elevate the intracellular Ca concentration of nerve terminals, i.e. K-free medium or ouabain, increased the rate of m.e.p.p.s in normal muscle, but not in BoTx poisoned muscles. These observations indicate a reduced sensitivity of the transmitter release process to Ca in poisoned terminals. On the other hand, when a Ca-ionophore (A 23183) together with a raised extracellular Ca concentration (> 4 mM) were used in BoTx poisoned muscles, high frequency transmitter release was induced, similar to that at normal end-plates. At the same time an apparent normalization of the amplitude distribution of the m.e.p.p.s occurred, the histogram being altered from a skew or bimodal distribution towards a Gaussian one. The Ca-ionophore allows the passage of Ca ions through biological membranes (Reed & Lardy, 1972) and thereby the influx and accumulation of Ca in nerve terminals. This 'normalization' of m.e.p.p. amplitude distribution and frequency increase indicates that in BoTx poisoning the release mechanism is in principle intact, but requires a higher than normal level of intracellular Ca to be accelerated. Such a hypothesis

would also explain the finding that repetitive nerve stimulation, which raises intracellular Ca (Katz & Miledi, 1968; Rahamimoff, 1968), increased m.e.p.p. frequency and normalized their amplitude distribution in poisoned fibres. Mechanical injury to nerve terminals, which can be expected to cause the influx of Ca, similarly affected transmitter release.

Transmitter was released by nerve impulses at BoTx poisoned nerve terminals at all times after poisoning, but initially only with a high rate of failures even in the presence of 4 mM-Ca in the bathing medium. The question arose whether, apart from the reduced number of quanta/impulse, the impulse linked transmitter release, which still occurred, was fundamentally changed and no longer followed Poisson statistics. At any stage after BoTx poisoning the release was reasonably well described by Poisson statistics. At early stages (1–2 days) the quantum size of neurally evoked release was as small as the smallest m.e.p.p.s, but after that, sometimes even by 24 hr after treatment, it changed to a size similar to that seen in normal muscle. It was of interest that a similar change in the size of neurally released quanta could be induced by prolonged nerve stimulation *in vitro*. This suggests that nerve impulse activity *in vivo* may be the cause of the increase in quantum size observed about the second day after poisoning.

In the presence of 0·2–2·0 mM-TEA a drastic increase in the mean quantum content of e.p.p.s occurred during all stages of BoTx poisoning; the drug failed to affect spontaneous transmitter release showing that its action is confined to the release evoked by nerve impulses. At the same time the relationship between extracellular Ca concentration and mean quantum content of e.p.p.s changed towards normal, the slope value for Ca dependency being about 5 instead of about 1·5 (Figs. 10 and 14).

TEA prolongs the duration of the nerve terminal action potential by blocking the depolarization-induced increase in K conductance and this increases the amount of Ca which enters the terminal during the nerve impulse (Katz & Miledi, 1967; Kusano, Livengood & Werman, 1967). The observed effects with TEA are therefore compatible with the aforementioned hypothesis that the transmitter release process in BoTx poisoned terminals is functionally operative but has a low sensitivity to Ca. When, by the influx of Ca during the prolonged action potential, the intracellular level of Ca has reached a certain level, which may be much higher than in normal nerve terminals, the transmitter release process is activated and phasic release occurs with an almost normal power relation between Ca concentration and quantum content of e.p.p.s.

We have no definite explanation for the appearance of m.e.p.p.s and e.p.p.s of abnormal sizes after BoTx-treatment. Suggestions for the origin of small ACh quanta which are spontaneously released after BoTx treat-

ment have been proposed (Harris & Miledi, 1971) and these could be adapted to include *neurally evoked* release of small ACh packets. One possibility is that they represent gated release, i.e. a fractional depletion of the vesicles involved in the transmission process (see Pfenninger, 1973). It may be relevant that small m.e.p.p.s have been described at normal end-plates (Cooke & Quastel, 1973; Kriebel & Gross, 1974). Their frequency is less sensitive to procedures which generally increase the frequency of normal m.e.p.p.s and their probability of release by nerve impulses is low (S. Bevan, personal communication). Small m.e.p.p.s in untreated muscles occurred with a frequency similar to the small m.e.p.p.s seen in the early stages of BoTx poisoning. This is compatible with the concept that the small ACh quanta, released either spontaneously or by nerve stimulation, could, at normal and BoTx poisoned end-plates, have a similar origin with a low probability of release and be resistant to the action of BoTx.

The study was aided by a grant B70-14X-3112 from the Swedish Medical Research Council and by a grant from the Muscular Dystrophy Associations of America Inc. S. G. Cull-Candy was the recipient of a European Fellowship of the Royal Society and the Science Research Council of U.K. We are greatly indebted to Professor R. Miledi for his critical reading of the manuscript. The gift of Black Widow spider venom by Dr Nora Frontali, Instituto Superiore di Sanitá, Rome, and of the Ca-ionophore A-23187 by Eli Lilly Co. are gratefully acknowledged. Excellent technical assistance was provided by Miss B. Hansson.

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