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MINIATURE END-PLATE POTENTIALS IN RAT SKELETAL MUSCLE POISONED WITH BOTULINUM TOXIN

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SUMMARY

1. Spontaneous transmitter release, recorded as miniature end-plate potentials (m.e.p.p.s), was studied in rat extensor digitorum longus (e.d.l.) and soleus muscles partially or completely paralysed by botulinum toxin type A (BoTx). Normal unpoisoned muscles were examined for comparison.

2. Analysis of m.e.p.p.s in both normal and BoTx-poisoned muscles confirmed the presence of two populations of potentials. One population, which comprised about 96 % of the m.e.p.p.s recorded at non-poisoned end-plates, was characterized by a uniform time course and a mean time-to-peak of 0·5–0·7 ms. These potentials had a shape and time-to-peak similar to that of quantal end-plate potentials (e.p.p.s) evoked by nerve stimuli. These were designated 'fast m.e.p.p.s'. The other population of m.e.p.p.s was characterized by a slower, more variable rise-time, the time-to-peak exceeding 1·1 ms, and generally a larger amplitude. These were designated 'slow m.e.p.p.s'.

3. In both partial and complete paralysis by BoTx the frequency of fast m.e.p.p.s was reduced by more than 90 % and the reduction lasted several weeks. After 6–10 days of poisoning the frequency of slow m.e.p.p.s gradually increased. The highest frequency of slow m.e.p.p.s (0·4 Hz) was recorded in the partially paralysed soleus muscle, the frequency being about ten times that at unpoisoned end-plates. In both partially paralysed muscles slow m.e.p.p. frequency returned towards normal 28 days after poisoning.

4. A significant correlation ($r = 0·67$) was observed between the quantal content of e.p.p.s and the frequency of fast m.e.p.p.s in partially paralysed e.d.l. muscles. No significant correlation was observed between quantal content of e.p.p.s and the frequency of slow m.e.p.p.s. To further study if muscle activity influenced the appearance of slow m.e.p.p.s, partially paralysed soleus muscles were directly stimulated *in vivo* during the first 11–13 days following BoTx poisoning, using a stimulation pattern which inhibits nerve terminal sprouting and the appearance of denervation changes. This procedure did not alter the frequency of slow m.e.p.p.s as compared to unstimulated poisoned controls.

5. It is concluded that enhancement of slow m.e.p.p. frequency in muscles poisoned

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with BoTx is related to the blockade of evoked Ca^{2+}-dependent quantal transmitter release. However, additional factors influence this type of spontaneous and Ca^{2+}-insensitive release of acetylcholine since there is a great variability between fibres and a time lag between the disappearance of fast m.e.p.p.s and the activation of slow m.e.p.p. frequency.

INTRODUCTION

Botulinum toxin type A (BoTx) is a presynaptically acting neurotoxin with a specific and long-lasting effect at cholinergic nerve endings (for recent reviews, see Simpson, 1981; Sellin, 1981). At the neuromuscular junction its actions are characterized by a block of impulse-evoked release of acetylcholine (ACh) and by a marked reduction in the frequency of spontaneous miniature end-plate potentials (m.e.p.p.s).

Several investigators have shown that early during BoTx poisoning small-amplitude m.e.p.p.s appear, causing a leftward shift in the distribution of m.e.p.p. amplitudes (Harris & Miledi, 1971; Boroff, Del Castillo, Evory & Steinhardt, 1974; Kriebel, Llados & Matteson, 1976). Recently it has been shown that in the extensor digitorum longus (e.d.l.) muscle of the rat there appears, after several days of poisoning, a new kind of m.e.p.p. which possesses characteristics different from these so-called sub-m.e.p.p.s. These m.e.p.p.s have a more variable, but generally much longer, time-to-peak than m.e.p.p.s at unpoisoned junctions, and their amplitude is frequently larger, shifting the distribution of m.e.p.p. amplitudes towards the right (Sellin & Thesleff, 1981; Colméus, Gomez, Molgó & Thesleff, 1982; Thesleff, Molgó & Lundh, 1983). Fundamentally, these m.e.p.p.s differ from those at unpoisoned junctions by being unaffected in frequency by procedures which alter extra- or intracellular Ca^{2+} concentrations. Also, the temperature dependence of the relase processes producing m.e.p.p.s in poisoned and unpoisoned muscles is quite different. In unpoisoned muscles m.e.p.p. frequency is reduced with temperature with a Q_{10} of 2–3, while in BoTx-poisoned muscles the reduction is much more marked, with a Q_{10} of about 12. Furthermore, despite the presence of these abnormal m.e.p.p.s, transmitter quanta released by nerve stimuli at poisoned junctions cause end-plate potentials (e.p.p.s) with a uniformly fast time-to-peak similar to that of m.e.p.p.s at unpoisoned junctions. Therefore, unlike at normal junctions, e.p.p.s and m.e.p.p.s in poisoned muscles are not caused by identical transmitter release mechanisms (Cull-Candy, Lundh & Thesleff, 1976; Thesleff *et al.* 1983; Thesleff & Molgó, 1983).

The purpose of the present study was to examine the presence of the different populations of m.e.p.p.s in BoTx-poisoned muscles in relation to the degree of neuromuscular block, muscle activity and to the type of muscle affected. The study was made on the 'fast' e.d.l. and the 'slow' soleus muscle of adult rats.

METHODS

All experiments were performed on the e.d.l. or soleus muscle of male Sprague–Dawley rats with a body weight of 150–250 g, with the exception of those involving direct electrical stimulation of soleus muscle *in vivo*, which were performed on rats of the Wistar strain. All control animals were weight matched.

Complete or partial paralysis by BoTx. Muscle paralysis was produced *in vivo* by the injection of crystalline *Clostridium botulinum* type A toxin (haemagglutinine plus neurotoxin) in a gelatine–phosphate buffer solution as described by Ambache (1949). No difference in action at the neuromuscular junction is observed between equipotent doses of this toxin and that of the isolated and purified neurotoxic component (Sellin & Thesleff, 1981). To produce complete paralysis of e.d.l. and soleus muscles, a single bolus of 0·25 ml containing about 60 pg of the neurotoxin was injected into the anterolateral region of the right hind leg, superficial to the e.d.l. muscle. As previously described (Cull-Candy *et al.* 1976), this amount of toxin paralysed the hind leg within 18 h, the paralysis lasting 3–4 weeks. Otherwise the animal appeared unaffected.

For partial paralysis, 0·25 ml containing about 2·4 ng neurotoxin was injected subcutaneously into the dorsal region of the rat. This dose of toxin and mode of application caused, within 24 h, generalized weakness of all muscles, affecting both hind legs equally. Doubling this dose was lethal.

To evaluate the degree of paralysis of e.d.l. and soleus muscles in the partially paralysed animals twitch and tetanic tensions were recorded *in vivo*. Unpoisoned animals were used as controls. Following anaesthesia induced by Inactin, 100–200 mg/kg body weight (Byk-Gulden, Constanz, F.R.G.; 5-ethyl-5-1-methyl-propyl-2-thio-sodium barbituric acid), administered intraperitoneally, the e.d.l. and soleus muscles were exposed and freed from surrounding tissue. Their distal tendons were cut and secured to a Grass FT 03 force displacement transducer while the knee joint was rigidly fixed. The muscles were stimulated indirectly through their nerves using supramaximal square voltage pulses of 50 μs duration. The nerve proximal to the stimulating electrode and branches of the scitatic nerve to other muscles were cut or crushed. Resting muscle tension was adjusted to produce maximal twitch response. Muscle tensions were recorded by displaying the transducer output on a Tektronix 5113 storage oscilloscope. In each experiment five single twitch responses produced by 0·5 Hz stimulation were averaged, and tetanic tension was recorded in response to 100 Hz stimulation. The rectal temperature of the animal was 36–37 °C.

Electrophysiological recording. At 2–28 days after poisoning with BoTx the rats were killed by diethyl ether and e.d.l. and soleus nerve–muscle preparations were excised under a continuous flow of oxygenated Krebs–Ringer solution. The excised muscles were pinned through their tendons, stretched approximately to their resting lengths, placed in a temperature-regulated (30 ± 1 °C) chamber (25 ml), and suffused at the rate of 4 ml/min with a bathing solution of the following composition (mM): NaCl, 135, KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; Na_2HPO_4, 1; $NaHCO_3$, 15; glucose, 11. The pH of this solution was 7·2–7·3. In solutions with altered Mg^{2+} or K^+ isosmolarity was maintained by adjusting Na^+. All solutions were continuously aerated with 95% O_2 + 5% CO_2.

Potentials were recorded intracellularly by conventional 2 M-K-citrate-filled glass capillary micro-electrodes with resistances of $4–8 \times 10^6$ Ω. The potentials were displayed and photographed on analog and digital storage oscilloscopes (Tektronix 5223 and D13). The peak-to-peak noise level of the recording system was about 50 μV. After an approximate 15 min period for muscle bath equilibration, surface muscle fibres were examined for the presence of m.e.p.p.s or e.p.p.s at the junctional region localized with a dissection microscope. E.p.p.s were elicited by nerve stimulation (2–4 V, 50 μs) via a platinum wire–polyethylene capillary suction electrode. Recording was considered focal if m.e.p.p.s or e.p.p.s with a time-to-peak of less than 0·8 ms were observed.

In experiments not involving nerve stimulation, tetrodotoxin at a concentration of 5×10^{-7} M was added to the bathing solution to eliminate spontaneous action potential generation in nerve and muscle which occurred because of denervation-type changes following BoTx poisoning.

Analysis of synpatic potentials. All synaptic potentials were analysed for frequency, amplitude, and time-to-peak with the aid of an ABC 80 microcomputer with a Data Disc 82 double floppy disk unit as described in detail by Colméus *et al.* (1982). The present program has been modified to further improve accuracy of time-to-peak measurements. The system has a resolution of 20 μV in amplitude, 30 μs in time-to-peak, and 0·3 ms in the frequency measurements. The amplitude of m.e.p.p.s or e.p.p.s were corrected to a standard resting membrane potential of −75 mV and an ACh equilibrium potential of 0 mV (Katz & Thesleff, 1957; Sellin & Thesleff, 1981). Fibres with resting membrane potentials below −55 mV were excluded. In general, m.e.p.p.s were recorded and analysed during a period of 4 min at each end-plate. E.p.p.s were analysed from a train of 100 nerve stimuli at 1 Hz. Results are expressed as means ± S.E. of mean. Number of fibres and number of muscles examined are given within parentheses.

Quantal components of the evoked e.p.p.s were studied in e.d.l. and soleus muscles partially paralysed by BoTx, evoked release being reduced to a subthreshold level by the use of high Mg^{2+}

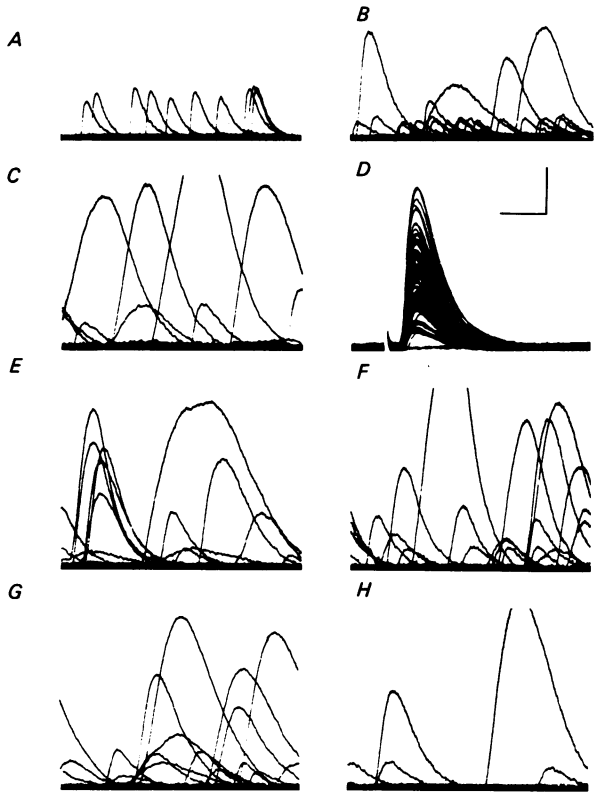

Fig. 1. Examples of m.e.p.p.s at unpoisoned (*A* and *B*) and at BoTx-poisoned end-plates (*C*, *E*, *F*, *G* and *H*). All records are superimposed oscilloscope tracings and, with the exception of records *C* and *D*, the calibrations are 1 mV and 4 ms. *A* and *B* show m.e.p.p.s from unpoisoned normal e.d.l. and soleus muscles respectively. Note in *A* a uniform population of fast m.e.p.p.s, while in *B* a mixed population of fast and slow m.e.p.p.s can be seen. *C* and *D* are m.e.p.p.s and e.p.p.s, respectively, from the same end-plate in a partially BoTx-paralysed e.d.l. muscle (12 days). Note the variable and prolonged time-to-peak of many of the m.e.p.p.s as compared to the fast, consistent time-to-peak of the e.p.p.s. Calibrations for record *C* are 1 mV and 2 ms and for record *D*, 2 mV and 2 ms. Records *E–H* illustrate typical recordings from two partially paralysed soleus muscles (*E* and *F*) and completely paralysed e.d.l. and soleus muscles, (*G* and *H*) respectively.

(1–5 mM). Mean quantal content (m) was computed by the use of the direct method and either the failure method or the indirect variance method as described by Del Castillo & Katz (1954) and Martin (1966). The two latter techniques are appropriate since it has been shown that evoked transmitter release in BoTx-poisoned muscles is in satisfactory agreement with Poisson statistics (Cull-Candy *et al.* 1976) and since quantal content in our recording conditions is less than 20.

Direct stimulation of partially paralysed soleus muscles in vivo. Two Teflon-coated multistranded steel wires (o.d. 0·011, AS 632 Cooner Sales, Chatsworth, CA, U.S.A.) were implanted into the right leg of four 140–180 g male Wistar rats anaesthetized with chloralhydrate and pentobarbitone (Equithesin 0·4 ml/100 g). The distal ends of the wires, with the insulation removed, were placed across the soleus, one end proximally and dorsally and the other end distally and ventrally. Both wires were run under the skin to the head and into flexible silicone tubes (o.d. 0·6 mm) which were fixed to the skull with screws and dental cement. The wires and the protecting tubes were connected

to rotating contacts from a stimulator above the rats which could move freely in wide buckets. 7 days later BoTx was injected subcutaneously to produce partial paralysis of leg muscles and direct stimulation of the right soleus started. The stimulation consisted of sixty pulses at 100 Hz every 60 s (mean frequency 1 Hz). Each stimulus was bipolar, 0·2 ms in duration and 5–10 mA in each direction. The muscles were stimulated *in vivo* for 11–13 days and examined electrophysiologically *in vitro* 1 day later. Controls consisted of animals without treatment, with injection of BoTx only and with implanted sham electrodes (no stimulation) and injections of BoTx, two animals in each group.

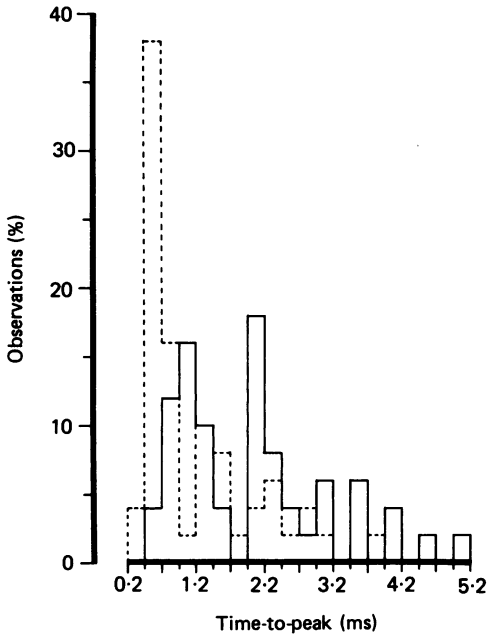

Fig. 2. Histograms for time-to-peak of m.e.p.p.s recorded from an e.d.l. partially BoTx paralysed for 8 days. Continuous line indicates m.e.p.p.s recorded from a fibre in a bathing solution containing 5 mM-K^+. The same fibre was then superfused with a high-K^+ (15 mM) solution (dotted line). Fifty consecutive m.e.p.p.s were recorded in each condition and their mean frequency was increased by K^+ from 0·28 to 0·40 Hz. Resting membrane potential was −73 mV in normal solution and −58 mV in 15 mM-K^+. Note that the proportion of fast-rising m.e.p.p.s increases in the presence of high K^+.

RESULTS

Characterization of m.e.p.p.s

Observing m.e.p.p.s in unpoisoned normal and in BoTx-poisoned muscles reveals the presence of two populations of m.e.p.p.s. One is characterized by a rather uniform and fast time-to-peak while the other population has a variable and prolonged time-to-peak. Typical examples of such m.e.p.p.s are shown in Fig. 1.

Fast-rising m.e.p.p.s resemble in their time course evoked quantal e.p.p.s as shown in Fig. 1. Furthermore, high K^+ (15 mM) selectively increases this portion of m.e.p.p.s, as demonstrated by the time-to-peak histograms in Fig. 2. Therefore, this type of m.e.p.p. presumably reflects the normal Ca^{2+}-dependent release mechanism for ACh quanta. We designate this m.e.p.p. population as 'fast m.e.p.p.s'.

The second population of m.e.p.p.s, with a slow time-to-peak, has no resemblance

TABLE 1. Mean amplitude and time-to-peak for m.e.p.p.s and e.p.p.s in e.d.l. and soleus muscles

	Fast m.e.p.p.s		Slow m.e.p.p.s		E.p.p.s, time-to-peak (ms)
	Amplitude (mV)	Time-to-peak (ms)	Amplitude (mV)	Time-to-peak (ms)	
Control normal					
E.d.l. (39/5)	0·49±0·034	0·47±0·010	0·60±0·120	1·43±0·086	—
Soleus (43/5)	0·36±0·017	0·60±0·012	0·55±0·113	1·78±0·167	—
Partially paralysed (10–14 days)					
E.d.l. (27/4)	0·53±0·054	0·54±0·029	1·03±0·156	1·57±0·112	0·56±0·019 (57/13)
Soleus (38/4)	0·46±0·049	0·70±0·028	1·43±0·178	2·13±0·159	0·74±0·036 (32/8)
Completely paralysed (10–14 days)					
E.d.l. (30/4)	0·43±0·057	0·74±0·029	1·54±0·230	2·43±0·201	—
Soleus (31/4)	0·42±0·070	0·79±0·023	1·34±0·187	2·37±0·194	—

The figures are from control normal muscles and from muscles partially and completely paralysed by BoTx. Values are mean±S.E. of mean and number of end-plates/number of muscles studied are given within parentheses. Fast m.e.p.p. was considered any m.e.p.p. with a time-to-peak of less than 1·1 ms and slow m.e.p.p. any m.e.p.p. with a time-to-peak longer than that value, see text. The values for e.p.p.s were obtained from end-plates with a quantal content of less than 10.

to evoked e.p.p.s (Fig. 1) and is unaffected by high K^+ (Fig. 2). Time-to-peak seems a more reliable criterion for separating the two groups than m.e.p.p. amplitude, which may be small or large in both populations as shown in several of the records in Fig. 1. We designate this population of m.e.p.p.s as 'slow m.e.p.p.s'.

A separation into fast and slow m.e.p.p.s generally presented no difficulty. In unpoisoned muscles the low frequency and obvious dissimilarity between slow m.e.p.p.s and fast m.e.p.p.s made it possible to count these events by hand. Subsequent analysis of time-to-peak showed that using twice the mean time-to-peak as a criterion to distinguish slow m.e.p.p.s from normal m.e.p.p.s agreed well with hand counting.

As shown in Table 1, m.e.p.p.s in unpoisoned muscles and e.p.p.s in partially BoTx-paralysed muscles were characterized by time-to-peak values of about 0·5–0·7 ms. On this basis we classified m.e.p.p.s with times-to-peak not exceeding twice that value, i.e. less than 1·1 ms, as fast m.e.p.p.s, while m.e.p.p.s with rise times exceeding 1·1 ms were classified as slow m.e.p.p.s. Mean resting membrane potentials of unpoisoned e.d.l. and soleus muscle fibres were $70·8 \pm 0·75$ and $72·1 \pm 0·71$ mV respectively.

By this classification it is possible to observe in normal, unpoisoned muscles a small population of slow m.e.p.p.s appearing at an average frequency of $0·04 \pm 0·005$ Hz in e.d.l. and of $0·03 \pm 0·003$ Hz in soleus. However, the prevalence of slow m.e.p.p.s in normal e.d.l. and soleus muscles is widely variable among different fibres. In some fibres they are not detected at all, even during prolonged periods of recording, while in other muscles or fibres their frequency may be quite high. An example of the latter type of fibre is shown in Fig. 1*B*, the frequency of slow m.e.p.p.s in that soleus fibre being 0·18 Hz. The average fast m.e.p.p. frequency in unpoisoned fibres was $1·9 \pm 0·15$ and $1·3 \pm 0·10$ Hz for e.d.l. and soleus muscles respectively.

Muscles partially paralysed by BoTx

To evaluate the degree of neuromuscular blockade in e.d.l. and soleus muscles of rats receiving a subcutaneous injection of a standard dose of BoTx (see Methods), isometric twitch and tetanic contractions were recorded 4–12 days after poisoning. As shown by the graphs in Fig. 3, the tetanic and twitch contractile forces of the e.d.l. muscle are reduced by about 10 % and 40 % respectively by the fourth day after toxin injection and the muscle remains paralysed at about this level for at least an additional 8 days.

The soleus muscle is more severely paralysed, with tetanic contraction being reduced by 40–50 % and twitch contraction by about 65 %. One reason for this may be that the safety margin of transmission is lower in a 'tonic' soleus muscle than in a 'phasic' e.d.l. muscle (Sterz, Pagala & Peper, 1983). Also, the number of synaptic impulses is much higher in the slow soleus than in the fast e.d.l. muscle (Henning & Lømo, 1984), and this higher activity might enhance the blocking effect of BoTx (Hughes & Whaler, 1962). This difference in the degree of paralysis induced by BoTx in e.d.l. and soleus muscles of the same animal offers an opportunity to study the appearance and relative prevalence of the two m.e.p.p. populations in relation to the degree of neuromuscular impairment.

Frequency of fast m.e.p.p.s and slow m.e.p.p.s

The upper graphs in Fig. 4 illustrate changes in fast m.e.p.p. (*B*) and slow m.e.p.p. (*A*) frequencies during partial paralysis of e.d.l. and soleus muscles. Typical records obtained from such muscles are shown in Fig. 1. In both muscles the fast m.e.p.p. frequency is initially reduced by the toxin, for e.d.l. to 8·7% (17/2) and for soleus to 2·2% (13/2) of normal. In the e.d.l., which is paralysed to a lesser degree, fast m.e.p.p. frequency rapidly recovers and reaches or even exceeds normal frequency

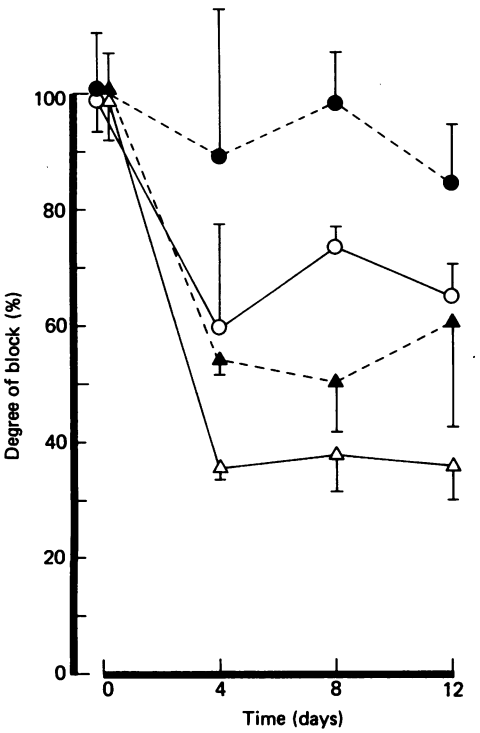

Fig. 3. The graphs illustrate the degree of neuromuscular block in the e.d.l. (circles) and soleus (triangles) muscles of rats that received a single subcutaneous injection of BoTx. Open symbols, continuous lines are for single twitch tensions (0·5 Hz), and filled symbols, dotted lines are for tetanic tensions (100 Hz). Values are expressed as a percentage of the tension recorded in unpoisoned normal animals, and each point is the mean ± S.E. of mean of three or four muscles.

24–28 days after poisoning. In the soleus muscle fast m.e.p.p. frequency remains low, with only a slight increase with time. Slow m.e.p.p. frequency is altered in an opposite way. In the e.d.l., with only 10–30% paralysis, there is only a small, about twofold ($P < 0{\cdot}01$), increase from control in slow m.e.p.p. frequency, while in the more effectively paralysed soleus muscle slow m.e.p.p. frequency gradually increases about tenfold. In both muscles slow m.e.p.p. frequency returns towards normal 28 days after poisoning.

To further study the possibility of a connexion between type of m.e.p.p. observed and degree of neuromuscular blockade we looked for a correlation between the

frequency of each kind of m.e.p.p. and the quantal content of evoked e.p.p.s at the same end-plate. A significant correlation ($r = 0{\cdot}671$; 70/15) was observed between the frequency of fast m.e.p.p.s and the mean quantal content of the e.p.p. in e.d.l. muscle fibres. No significant correlation is observed between slow m.e.p.p. frequency and e.p.p.s in either of the two muscles.

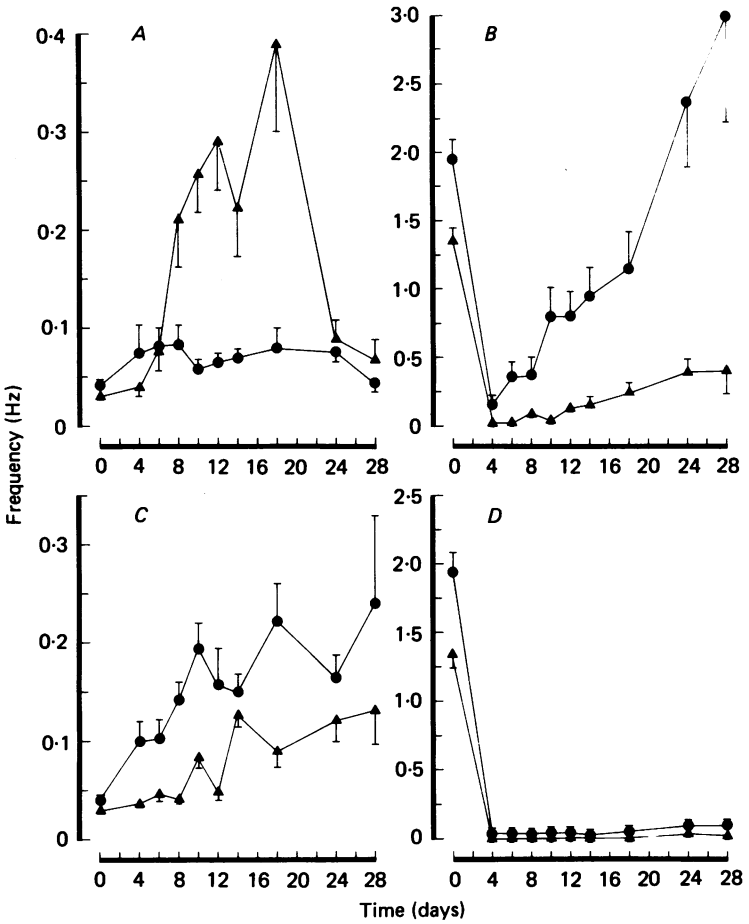

Fig. 4. The frequency of fast m.e.p.p.s and slow m.e.p.p.s in e.d.l. (circles) and soleus (triangles) muscles at 4–28 days after BoTx poisoning, with control data appearing at day 0. The values are means ± S.E. of mean for one to five muscles. Upper graphs are from partially paralysed muscles; lower graphs are from completely paralysed muscles. *A* and *C* give the data for slow m.e.p.p. frequencies, while *B* and *D* give the fast m.e.p.p. frequencies.

Muscles completely paralysed by BoTx

The lower graphs in Fig. 4 illustrate the dramatic drop in frequency of fast m.e.p.p.s in both e.d.l. and soleus muscles and an almost complete lack of recovery of this parameter during the 28 days of observation after poisoning (Fig. 4*D*). At the same time, the frequency of slow m.e.p.p.s gradually increases in both muscles, the increase

being more marked in e.d.l. than in soleus muscles (Fig. 4*C*). Of particular interest is that the increase in slow m.e.p.p. frequency in the completely paralysed soleus muscle is less than in a partially paralysed muscle (Fig. 4*A*). However, there is a large variation in the frequency of slow m.e.p.p.s between fibres, as shown by the S.E. of means.

In completely paralysed muscles the decay time of m.e.p.p.s is markedly slowed compared to normal (Fig. 1*G* and *H*), this presumably resulting from an increase in membrane time constant (Nicholls, 1956), the appearance at the end-plate of ACh receptors with a prolonged ion-channel open time (Sellin & Thesleff, 1981) and a reduction in acetylcholinesterase activity (Drachman, 1972; Cangiano, Lømo, Lutzembergen & Sveen, 1980).

Effects of muscle activity on m.e.p.p.s in partially paralysed soleus muscle

The observation that slow m.e.p.p. frequency is higher in a partially paralysed than in a completely paralysed soleus muscle (see Fig. 4) prompted us to investigate the possibility that muscle activity influences this type of transmitter release. Experiments were therefore made on four partially paralysed soleus muscles which had been directly stimulated at 100 Hz for a period of 0·6 s every 60 s up to 11–13 days after the injection of BoTx. Similar patterns of stimulation have been shown to prevent the appearance of denervation changes and nerve sprouting in the soleus muscle (Lømo & Westgaard, 1975; Brown, Goodwin & Ironton, 1977). The contralateral poisoned but unstimulated muscle served as control.

Slow m.e.p.p. frequency was $0{\cdot}09 \pm 0{\cdot}01$ Hz (21/4) for stimulated muscles and $0{\cdot}09 \pm 0{\cdot}02$ Hz (23/4) for unstimulated controls, i.e. no difference was seen. Normal m.e.p.p. frequency, however, differed significantly ($P < 0{\cdot}05$) between stimulated and unstimulated muscles, being $0{\cdot}06 \pm 0{\cdot}01$ and $0{\cdot}36 \pm 0{\cdot}10$ Hz respectively. This difference possibly reflects a more effective blockade by BoTx of the stimulated muscle (see Hughes & Whaler, 1962).

DISCUSSION

The present study has confirmed previous observations (Cull-Candy *et al.* 1976; Colméus *et al.* 1982; Thesleff *et al.* 1983) that there are two populations of spontaneous m.e.p.p.s in BoTx-poisoned muscles. One population, here called fast m.e.p.p.s, corresponds to evoked quantal release of ACh, and another has quite different properties and is here called slow m.e.p.p.s. Normal fast m.e.p.p. frequency and quantal content of evoked e.p.p.s are reduced by BoTx, indicating that the toxin blocks the Ca^{2+}-dependent transmitter release mechanism (Cull-Candy *et al.* 1976). This is further supported by the present observation that high K^+ (15 mM), which depolarizes the nerve terminal, selectively increases the proportion of fast m.e.p.p.s. Slow m.e.p.p. frequency, on the other hand, is enhanced during BoTx poisoning and is not influenced by nerve depolarization and other procedures affecting the Ca^{2+} concentration of the nerve terminal (Thesleff *et al.* 1983), indicating a Ca^{2+}-insensitive release mechanism for ACh. Furthermore, the study showed that slow m.e.p.p.s are not unique for BoTx-poisoned muscles but also occur at a low frequency in unpoisoned, normal muscle (see also Liley, 1957; Jansen & van Essen, 1976;

Cull-Candy, Miledi & Trautmann, 1979; Colméus *et al.* 1982; Heinonen, Jansson & Tolppanen, 1982). In this respect it is interesting that we observed that in normal muscle the frequency of slow m.e.p.p.s varies greatly between muscles, and between muscle fibres. A similar variability between fibres is also seen in muscles paralysed by BoTx, despite the fact that in this condition the over-all slow m.e.p.p. frequency is much higher. It indicates a variability between neurones or the possibility that local factors exert an influence on this type of transmitter release.

Of particular interest is the observation that slow m.e.p.p. activity is more marked in the partially paralysed soleus muscle than when the muscle is completely paralysed. However, in the less severely paralysed e.d.l. there is only a minor increase in slow m.e.p.p. frequency. This pointed to the possibility that muscle activity somehow influences the presynaptic transmitter release responsible for slow m.e.p.p.s. Experiments with direct *in situ* stimulation of partially paralysed soleus muscles, however, failed to alter the frequency of slow m.e.p.p.s, an observation which indicates that muscle activity is in itself not important for the induction and maintenance of that type of activity.

The pattern used for direct stimulation of soleus was such that it prevents the appearance of denervation-like changes in the muscle and nerve sprouting induced by the toxin or by partial denervation (Brown *et al.* 1977; Lømo & Westgaard, 1975). Therefore, our findings indicate that neither post-synaptic denervation changes nor nerve sprouting are primarily connected with the enhancement of slow m.e.p.p. frequency. Similarly, Thesleff *et al.* (1983) showed that X-ray irradiation of BoTx-poisoned muscles, a procedure known to inhibit nerve sprouting (Gomez, Duchen & Hornsey, 1982), failed to interfere with the appearance of slow m.e.p.p.s.

BoTx does not immediately enhance the frequency of slow m.e.p.p.s. Its effect is gradual and it takes 5–10 days before maximal activation is obtained. Upon recovery from paralysis slow m.e.p.p. activity is slowly reduced to normal values. At present we can only conclude that enhancement of slow m.e.p.p. frequency in muscles poisoned with BoTx is related to the blockade of evoked quantal transmitter release, but that in addition other factors influence this type of spontaneous, Ca^{2+}-insensitive, release mechanism for ACh.

M.e.p.p.s resembling slow m.e.p.p.s have been observed at end-plates with regenerating nerve terminals (Bennett, McLachlan & Taylor, 1973; Colméus *et al.* 1982) and in chickens during early development (Ding, Jansen, Laing & Tønnesen, 1983). Moreover, it is possible that the recently described spontaneous release of ACh quanta from growth cones of embryonic neurones in culture (Hume, Role & Fischbach, 1983; Young & Poo, 1983) corresponds to slow m.e.p.p.s in BoTx-poisoned muscles. The above observations suggest that the transmitter release mechanism responsible for slow m.e.p.p.s is present in neurones before the formation of functional synaptic contacts. BoTx blocks evoked transmitter release, i.e. it produces a state of functional disconnexion between nerve and muscle. Speculatively, this disconnexion might be the event which reactivates in the terminal a more primitive, embryonic type of ACh release.

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