J. Physiol. (1981), **317**, *pp.* 487–495
With 4 text-figures
Printed in Great Britain

PRE- AND POST-SYNAPTIC ACTIONS OF BOTULINUM TOXIN AT THE RAT NEUROMUSCULAR JUNCTION

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(*Received 11 December 1980*)

SUMMARY

1. Extensor digitorum longus muscles of rats were paralysed with local, non-lethal doses of botulinum toxin Type A (BoTx). At 2 and 7 days after toxin injection, the nerve–muscle preparations were excised and end-plate currents analysed at 23 °C by dual-micro-electrode voltage clamp.

2. At 2 days after BoTx injection, the growth time of miniature end-plate currents (m.e.p.c.s) increased from a rather narrow range with a mean of 0·59 to a mean of 1·35 ms with a large variability between m.e.p.c.s. End-plate currents (e.p.c.s) were reduced compared to unpoisoned muscle. The decay phase of m.e.p.c.s and e.p.c.s, the growth phase of e.p.c.s and the voltage sensitivity of m.e.p.c.s were unchanged.

3. At 7 days after BoTx injection, the findings were similar to 2 days except that the time constant of the decay phase of m.e.p.c.s and e.p.c.s was about twice as long as normal and that the voltage sensitivity of m.e.p.c.s was increased.

4. The acetylcholine null potential (about 0 mV) was unchanged after treatment with BoTx.

5. The increase in the growth time of m.e.p.c.s compared to e.p.c.s following the injection of BoTx suggests that the poisoning, besides blocking quantal release, affects the time course of spontaneous but not that of evoked release. After BoTx poisoning the trans-synaptic diffusion of a majority of spontaneously released transmitter quanta seems to occur more slowly or from areas more distant from the highest concentration of the post-synaptic receptor than that of evoked release.

6. The increase in the decay phase of m.e.p.c.s and e.p.c.s and its increased voltage sensitivity observed in muscles poisoned for 7 days with BoTx suggest the appearance at the end-plate of a population of new receptors with a prolonged ion channel opening time similar to that previously described for extrajunctional receptors after denervation and for junctional receptors during development.

INTRODUCTION

Botulinum toxin (BoTx) has both pre- and post-synaptic effects at the neuromuscular junction. Its primary action is on the nerve terminal, where it blocks release of acetylcholine (see review by Howard & Gundersen, 1980) without affecting impulse

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conduction down the nerve or at the nerve terminal (Harris & Miledi, 1971). BoTx does not completely abolish acetylcholine release. Miniature end-plate potentials (m.e.p.p.s) of low-frequency and end-plate potentials (e.p.p.s) of low amplitude can be recorded from BoTx-treated muscles (Harris & Miledi, 1971; Cull-Candy, Lundh & Thesleff, 1976).

In addition, BoTx produces denervation-like effects in the muscle, such as an increase in extrajunctional sensitivity to acetylcholine (Thesleff, 1960). Since extrajunctional receptors have channel kinetics different from those at the end-plate of innervated muscle (Dreyer, Müller, Peper & Sterz, 1976; Sakman, 1978; Gage & Hamill, 1980), there existed the possibility that similar alterations may occur at the end-plate after denervation.

To circumvent the problem of locating and analysing end-plate receptors after denervation. BoTx was used to produce denervation-like effects. Using this technique, the neuromuscular junction remains morphologically intact and miniature end-plate currents (m.e.p.c.s) and end-plate currents (e.p.c.s) can be analysed by voltage-clamp technique. Furthermore, a study of the growth time of such currents provides information about the kinetics of transmitter–receptor interactions in BoTx poisoning.

METHODS

All experiments were performed *in vitro* on the extensor digitorum longus (e.d.l.) muscle of male (Wistar) rats (150–200 g). Toxin injection and surgical procedures were done using diethyl ether for anaesthesia.

A powdered preparation of *Clostridium botulinum* toxin type A, with a mouse LD_{50} of about 0·05 μg/kg, was dissolved in a buffer solution as described by Ambache (1949). BoTx was given in a single injection of 0·25 ml (about six mouse LD_{50} doses) subcutaneously into the anterolateral region of the right hind leg, superficial to the distal part of the tibialis anterior muscle. The amount of toxin given produced complete paralysis of the lower leg within 18 h. Electrophysiological examination has shown that with this amount of toxin and mode of administration spontaneous quantal release of acetylcholine, recorded as m.e.p.p.s, is reduced to less than 10 % of normal and nerve-evoked release to less than 1 % of normal (Cull-Candy *et al.* 1976). In some experiments we compared the effect on neuromuscular transmission of the crude BoTx used in the present study with that of the isolated and purified neurotoxic component (mol. wt. about 150 000) of BoTx type A obtained through the courtesy of Dr J. Oliver Dolly, Department of Biochemistry, Imperial College, London. In equipotent doses, no difference was observed.

At 2 or 7 days after BoTx injection, the e.d.l. nerve–muscle preparations were excised under a continuous flow of oxygenated (95 % O_2 – 5 % CO_2) Krebs-Ringer solution having the following composition (mM): NaCl, 135; KCl, 5; $CaCl_2$, 4; $MgCl_2$, 1; Na_2HPO_4, 1; $NaHCO_3$, 15; glucose, 11. The pH of this solution was 7·2–7·3. The excised muscles were pinned through their tendons, stretched approximately to their resting lengths, placed in a temperature-regulated (23 ± 0·5 °C) chamber (25 ml) and suffused at a rate of 4 ml/min. After a 45 min equilibration period, m.e.p.c.s and e.p.c.s were analysed using dual-micro-electrode voltage clamp.

The voltage recording micro-electrodes were filled with 3 M-KCl and had tip resistances of $5–10 \times 10^6$ Ω. The current passing micro-electrodes were filled with 2 M-K citrate and were bevelled to a tip resistance of about $2·5 \times 10^6$ Ω. Current was measured through an agar bridge Ag–AgCl bath electrode coupled to a current-to-voltage converter. The time constant of the voltage-clamp circuit was < 100 μs.

End-plates were localized by following fine nerve branches and the micro-electrode was inserted in an apparent end-plate region. Localisation was considered acceptable when the m.e.p.c.s had a rise time of less than 1·0 ms. The current-passing micro-electrode was inserted in the same fibre at an inter-electrode distance of less than 100 μm. Proper positioning of both micro-electrodes in the same fibre was accepted when current pulses injected through the current-passing micro-electrode

produced voltage deflexions of both oscilloscope traces. Noise reduction for resolution of m.e.p.c.s was accomplished by placing a 500 Hz filter (30% attenuation) in the recording circuit.

E.p.c.s were recorded similarly to m.e.p.c.s, but following nerve stimulation via a platinum wire–glass capillary suction electrode. In unpoisoned muscle, contraction during nerve stimulation was prevented either by increasing the Mg^{2+} concentration of the suffusate or by utilizing a modification of 'cut muscle' preparation (Barstad, 1962; Glavinović, 1979). In this case, the muscle fibres were crushed on both sides of the end-plate region using a fine-tipped forceps, leaving an intact area of about 1·5 cm. After an additional equilibration period (30 min) resting potential ranged from −20 to −40 mV. In some experiments transmitter release in the BoTx-treated muscles was enhanced by the addition of 2·5 μM-4-aminopyridine to the bathing medium. Data from e.p.c.s were considered acceptable when the maximum membrane potential variation was less than 2% of the clamped voltage. A 1500 Hz filter (5% attenuation) was routinely used for the recording of e.p.c.s.

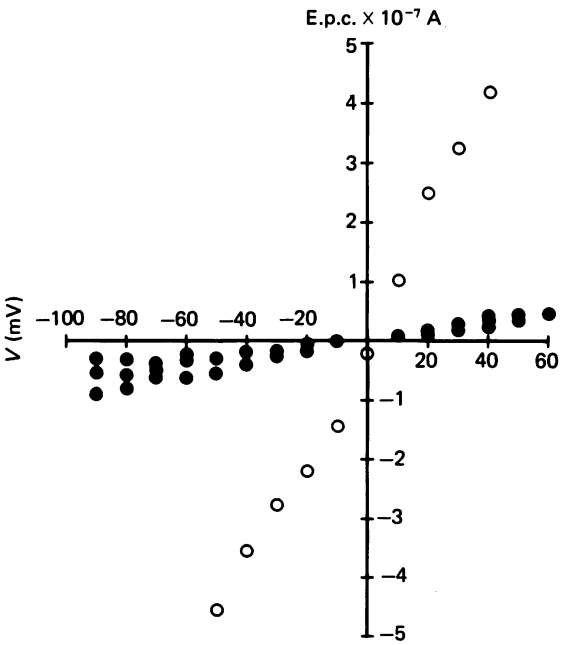

Fig. 1. End-plate currents (e.p.c.s) as a function of holding potential (V) recorded from a normal (○) muscle and a muscle which had been treated with BoTx for 7 days (●). Currents were recorded at 23 °C and contractions were prevented by using the crushed-fibre technique. Transmitter release was enhanced in the BoTx-treated muscle by the addition of 2·5 μM-4-aminopyridine to the suffusate.

RESULTS

In normal muscle, where transmitter release was not inhibited by high Mg^{2+}, large e.p.c.s were recorded in response to nerve stimulation (Fig. 1). The amplitude of e.p.c.s from normal muscle showed a linear relation to clamped voltage and had an acetylcholine null potential of $+0{\cdot}18 \pm 1{\cdot}78$ mV for the eight end-plates sampled. E.p.c.s recorded from BoTx-treated muscle were depressed to a similar degree at both 2 and 7 days after toxin injection (Fig. 1). However, the relationship of peak e.p.c. to clamped voltage remained linear and the acetylcholine null potential was unchanged compared to normal muscle at both 2 days ($-1{\cdot}64 \pm 2{\cdot}90$ mV, $n = 6$) and

7 days ($-0{\cdot}59 \pm 2{\cdot}03$ mV, $n = 9$) after toxin injection. The depression in peak e.p.c. of BoTx-treated muscles was similar to the depression observed in normal muscle suffused with 12 mM-Mg^{2+} Krebs-Ringer solution (Table 1).

Measurements of m.e.p.c.s were dictated by the resolving power of the recording system, which was unable to resolve consistently m.e.p.c.s of less than 2 nA. Accepting the exclusion of a population of small m.e.p.c.s, there was a slight increase in m.e.p.c. amplitudes after toxin injection (Table 1). BoTx poisoning had significant effects on

TABLE 1. Amplitude of e.p.c.s and m.e.p.c.s in normal and BoTx-treated muscles clamped at -80 mV

	Normal	BoTx (2 days)	BoTx (7 days)
e.p.c. (nA)	$49{\cdot}8 \pm 13{\cdot}6$	$58{\cdot}2 \pm 6{\cdot}8$	$65{\cdot}4 \pm 5{\cdot}2$
	(10)	(5)	(17)
m.e.p.c. (nA)	$6{\cdot}38 \pm 0{\cdot}40$	$7{\cdot}95 \pm 1{\cdot}04$	$8{\cdot}52 \pm 0{\cdot}47$
	(35)	(26)	(26)

* Values indicate means ±S.E. of means for the number of fibres shown within parentheses.
The normal muscles were suffused with a solution containing 12 mM-Mg^{2+} when e.p.c.s were recorded. Transmitter release in BoTx-treated muscles was enhanced by the addition of 2·5 μM-4-aminopyridine to the suffusate.

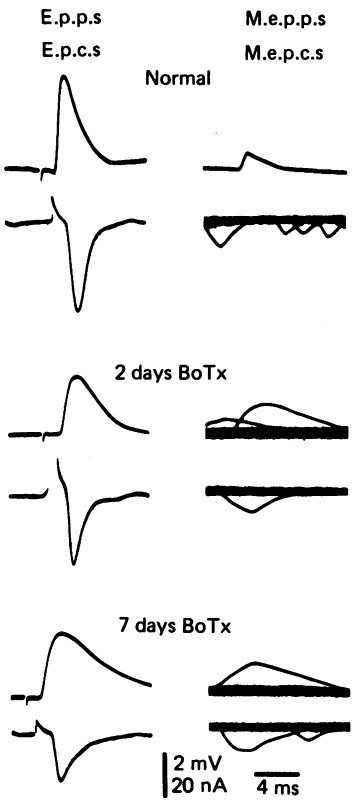

Fig. 2. Examples of e.p.p.s, m.e.p.p.s., e.p.c.s, and m.e.p.c.s recorded from normal muscle and from 2- and 7-day BoTx-poisoned muscles. The current recordings were made at a holding potential of -80 mV. For the recording of e.p.c.s transmitter release was depressed in normal muscles by the addition of 12 mM-Mg^{2+} to the suffusate.

the time course of end-plate currents. Examples of such potentials and currents in normal and 2- and 7-day BoTx-poisoned muscles are shown in Fig. 2. The manner and extent of these effects on the growth and decay phases of e.p.c.s and m.e.p.c.s were dependent on the length of time after toxin injection (Fig. 2 and Table 2).

The mean time-to-peak of the growth phase of m.e.p.c.s recorded from normal end-plates, clamped at −80 mV, was 0·59±0·03 ms. A frequency histogram of m.e.p.c.s from normal muscle indicated little variability in the growth time (Fig. 3).

TABLE 2. Time course of m.e.p.c.s and e.p.c.s recorded from normal and BoTx-treated muscles voltage-clamped at −80 mV

	Growth time (ms)	τ of decay phase (ms)
	m.e.p.c.	
Normal	0·59±0·03*	1·26±0·09
	(35)†	(35)
2 days BoTx	1·35±1·06	1·34±0·10
	(11)	(11)
7 days BoTx	1·28±0·09	2·52±0·21
	(9)	(9)
	e.p.c.	
Normal‡	0·55±0·03	1·35±0·15
	(10)	(10)
2 days BoTx	0·65±0·03	1·59±0·09
	(5)	(5)
7 days BoTx	0·71±0·05	2·60±0·19
	(17)	(17)

* Mean ±S.E. of mean.

† Number of end-plates sampled.

‡ Normal muscles were suffused with 12 mM-Mg^{2+} Krebs–Ringer solution to reduce the amplitude of the e.p.p.

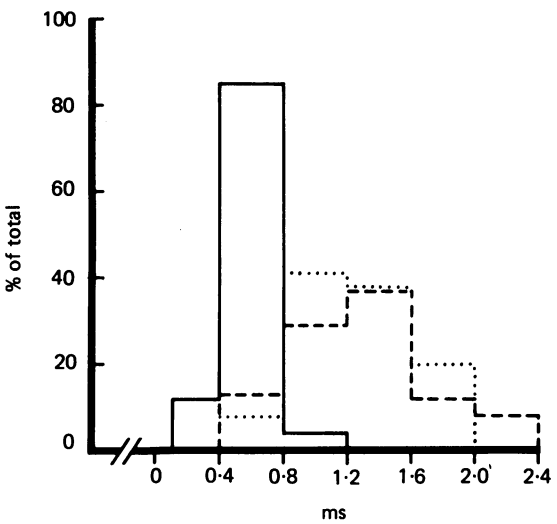

Fig. 3. Frequency histograms of the growth phase of m.e.p.c. recorded from normal (continuous lines), and 2 day (dashed line) and 7 day (dotted line) BoTx-treated muscles. The holding potential was at −80 mV and the data were normalized as percentage of total fibres sampled.

BoTx poisoning, however, resulted in more than a twofold increase in the time-to-peak of m.e.p.c.s as shown for 2 and 7 day poisoned muscles in Table 2. This increase was accompanied by an increase in the variability of this parameter (Fig. 3). In contrast to these changes in m.e.p.c.s, the growth phase of the e.p.c. in BoTx-treated muscles appeared to be unchanged compared to normal muscles (Table 2).

The decay phase of e.p.c.s and m.e.p.c.s was affected by BoTx poisoning but with a time course different from their growth phases (Table 2). The time constant of the

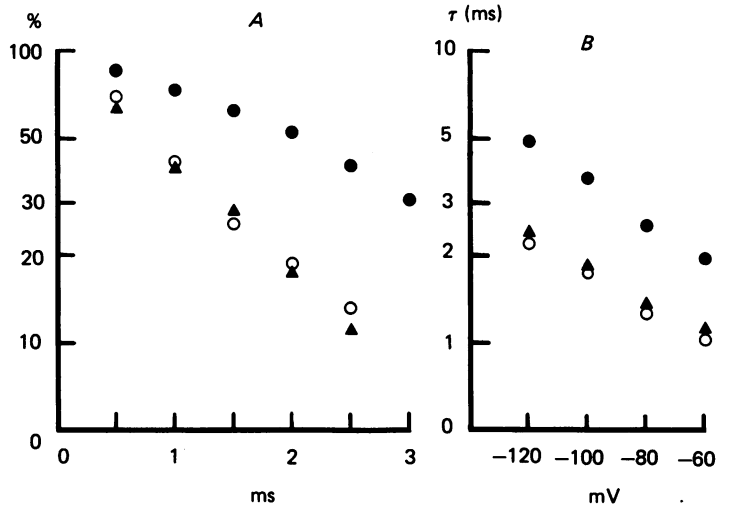

Fig. 4. *A*, the time course of the decay phase of m.e.p.c.s recorded from a single end-plate in normal and BoTx-treated muscles and plotted semilogarithmically as a function of time. The normal currents (○), 2 days after BoTx injection (▲) and 7 days after BoTx injection (●) were recorded from end-plates voltage-clamped at −80 mV. *B*, the mean time constant (τ) for the decay phase of m.e.p.c.s determined from eight to thirty-seven recordings in normal (○), 2 (▲) and 7 (●) day BoTx-treated muscles and plotted semilogarithmically as a function of holding potential.

decay phase was not different from normal at 2 days after toxin injection. However, at 7 days after toxin injection, the time constant of the decay phase was approximately twice that of normal muscle in both m.e.p.c.s and e.p.c.s. Although BoTx poisoning prolonged the growth phase of m.e.p.c.s, the decay phase could still be characterized by a simple exponential function (Fig. 4*A*).

The effect of membrane potential on the time constant (τ) of the decay phase of m.e.p.c.s for normal and BoTx-treated muscles is shown in Fig. 4*B*. The relationship between the membrane potential and the time constant of the decay phase has been described by Magleby & Stevens (1972) as:

$$\tau(V) = \tau(0)\exp(V/H),$$

where $\tau(V)$ is the time constant at clamped potential V, $\tau(0)$ is the time constant at zero potential, and H is the constant indicating the voltage sensitivity. That is, H is the change in membrane potential required to produce an e-fold change in the time constant. The above equation predicts an exponential decrease in τ as V goes in the

depolarizing direction. The results of the present experiment fulfilled this criterion as shown in Fig. 4*B*.

At 2 days after toxin injection, the time constant of the decay phase of m.e.p.c.s was unchanged from normal at all the voltages examined. However, at 7 days after toxin injection, the time constant of decay phase was increased compared to normal muscle at all voltages (Fig. 4*B*). This increase in the time constant was accompanied by a decrease in the calculated voltage sensitivity constant H, which was 75, 78 and 64 mV for normal, 2-day and 7-day BoTx-treated muscles respectively.

The growth phase of e.p.c.s and m.e.p.c.s from normal and BoTx-treated muscles was unaffected by changes in holding potential.

DISCUSSION

An analysis of m.e.p.c.s and e.p.c.s by voltage clamp can be useful in elucidating the effects of BoTx poisoning on transmitter–receptor interactions at the end-plate, particularly those affecting the time course of these currents. Gage & McBurney (1975) showed that the growth and decay phases of m.e.p.c.s were governed by processes with different characteristics. They demonstrated that the growth phase was non-exponential, relatively insensitive to changes in clamp potential and had a low Q_{10}. In contrast, the decay phase was exponential, voltage-sensitive, and had a high Q_{10}. They suggested that the growth phase was determined by the rate of arrival of acetylcholine at the post-synaptic receptors, while the factors controlling the decay phase were related either to the dissociation time of acetylcholine from its receptor (Adams & Sakmann, 1978) or to a conformational change in a macromolecule with a dipole moment in the post-synaptic membrane (Magleby & Stevens, 1972).

The present data on the growth time of m.e.p.c.s and e.p.c.s suggest that transmitter release in BoTx-treated muscles may derive from two different regions of the nerve terminal. M.e.p.c.s and e.p.c.s with a growth time of less than 0·8 ms, which is similar to that seen in unpoisoned muscles, probably result from quanta released from 'active zone' areas opposite the crests of the end-plate folds where the greatest concentrations of receptors exist (Couteaux & Pécot-Dechavassine, 1970; Heuser, Reese & Landis, 1974). Another type of release gives rise to m.e.p.c.s but not to e.p.c.s, with a much slower growth phase 0·8–2·4 ms. It seems possible that this latter type of quantal release occurs from areas at a greater distance from the greatest density of post-synaptic receptors or from areas from which diffusion is slowed compared to transmitter release from 'active zone' regions. In either case, miniature e.p.c.s would be slow rising and could have rounded peaks. This does not occur in e.p.c.s because nerve-evoked release is voltage-dependent and apparently always occurs at 'active zone' regions. In this context it is of interest that Gage & McBurney (1975) observed, at the normal toad neuromuscular junction, a small population of m.e.p.c.s with a growth time between 0·5 and 5 ms, compared with 50–300 μs for the majority of m.e.p.c.s suggesting, also at unpoisoned terminals, the presence of spatially separated areas for spontaneous quantal transmitter release.

The marked prolongation of the decay phase of m.e.p.c.s and e.p.c.s seen 7 days after BoTx-treatment indicates the appearance in the end-plate of receptor complexes with an increased channel open time. Several investigators have reported that the

receptors which are inserted extrajunctionally, following denervation, or at the end-plate, during embryonic development, have a single channel lifetime which is more prolonged than the receptors at the innervated adult end-plate (Dreyer *et al.* 1976; Sakmann, 1978; Gage & Hamill, 1980; Fischbach & Schuetze, 1980). Loring & Salpeter (1980) reported that, following denervation, the apparent half-time of receptor turnover at the end-plate is only 2–3 days, compared to 10 days in the innervated end-plate and about 1 day in the extrajunctional membrane of denervated muscles. One interpretation was that the end-plate after denervation contains a dual population of receptors; the original receptors with a slow turnover time and new ones with a turnover rate similar to that of extrajunctional receptors (Levitt, Loring & Salpeter, 1980). Our results suggest that 2–7 days of botulinum poisoning induces the appearance in the end-plate of receptors with an increased channel life-time, i.e. similar to those in the extrajunctional membrane, and that these are responsible for the prolongation of the decay phase of m.e.p.c.s and e.p.c.s.

The possibility that the prolongation of the decay phase resulted from a repetitive binding of acetylcholine to its receptor because of diminished cholinesterase activity cannot be excluded but appears less likely since BoTx-poisoned nerve terminals, under appropriate conditions, release transmitter quanta without a shift in the amplitude distribution towards higher values (Spitzer, 1972; Boroff, del Castillo, Evoy & Steinhardt, 1974; Cull-Candy *et al.* 1976). Furthermore, there was an increase in the voltage sensitivity of the decay phase of m.e.p.c.s indicative of altered receptor binding properties (Magleby & Stevens, 1972) while such an effect is not seen with cholinesterase inhibition (Katz & Miledi, 1973).

This study was supported by grants from the Swedish Medical Research Council (14X-3112) and the Muscular Dystrophy Association of America. L.C.S. was supported by a postdoctoral fellowship (IF32NSO5935-02) from NINCDS of the National Institute of Health, U.S.A. The authors thank Ms B. Hansson for her technical assistance and Dr J. Molgó for his valuable advice during the voltage-clamp experiments.

REFERENCES

Adams, P. R. & Sakmann, B. (1978). A comparison of current–voltage relations for full and partial agonists. *J. Physiol.* **283**, 621–644.

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

Barstad, J. A. B. (1962). Presynaptic effect of the neuromuscular transmitter. *Experientia* **18**, 579–580.

Boroff, D. A., del Castillo, J., Evoy, W. H. & Steinhardt, R. A. (1974). Observations on the action of type A botulinum toxin on frog neuromuscular junctions. *J. Physiol.* **240**, 227–253.

Couteaux, R. & Pécot-Dechavassine, M. (1970). Vesicules synaptiques et pouches au niveau des 'active zones' de la jonction neuromusculaire. *C. r. hebd. Séanc. Acad. Sci., Paris* D **271**, 2346–2349.

Cull-Candy, S. G., Lundh, H. & Thesleff, S. (1976). Effects of botulinum toxin on neuromuscular transmission in the rat. *J. Physiol.* **260**, 177–203.

Dreyer, F., Müller, K. D., Peper, K. & Sterz, R. (1976). The M. omohyoideus of the mouse as a convenient mammalian muscle preparation. A study of junctional and extrajunctional receptors by noise analysis cooperatively. *Pflügers Arch.* **367**, 115–122.

Fischbach, G. D. & Schuetze, S. M. (1980). A post-natal decrease in acetylcholine channel open time at rat end-plates. *J. Physiol.* **303**, 125–137.

Gage, P. W. & McBurney, R. N. (1975), Effects of membrane potential, temperature and neostigmine on the conductance change caused by a quantum of acetylcholine at the toad neuromuscular junction. *J. Physiol.* **244**, 385–407.

GAGE, P. W. & HAMILL, O. P. (1980). Lifetime and conductance of acetylcholine activated channels in normal and denervated toad sartorius muscle. *J. Physiol.* **298**, 525–538.

GLAVINOVIĆ, M. I. (1979). Voltage clamping of unparalysed cut rat diaphragm for study of transmitter release. *J. Physiol.* **290**, 467–480.

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

HEUSER, J. E., REESE, T. F. & LANDIS, D. M. D. (1974). Functional changes in frog neuromuscular junctions studied with freeze-fracture. *J. Neurocytol.* **3**, 109–131.

HOWARD, B. D. & GUNDERSEN, C. B. (1980). Effects and mechanisms of polypeptide neurotoxins that act presynaptically. *A. Rev. Pharmacol. Toxicol.* **20**, 307–336.

KATZ, B. & MILEDI, R. (1973). The binding of acetylcholine to receptors and its removal from the synaptic cleft. *J. Physiol.* **231**, 549–574.

LEVITT, T. A., LORING, R. H. & SALPETER, M. M. (1980). Neuronal control of acetylcholine receptor turnover rate at a vertebrate neuromuscular junction. *Science, N.Y.* **210**, 550–551.

LORING, R. H. & SALPETER, M. M. (1980). Denervation increases turnover rate of junctional acetylcholine receptors. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2293–2297.

MAGLEBY, K. L. & STEVENS, C. F. (1972). The effect of voltage on the time course of end-plate currents. *J. Physiol.* **223**, 151–171.

SAKMANN, B. (1978). Acetylcholine-induced ionic channels in rat skeletal muscle. *Fedn Proc.* **37**, 2654–2659.

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

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