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Effects of an isolated toxin from Australian Tiger snake *(Notechis scutatus scutatus)* venom at the mammalian neuromuscular junction

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Summary

1. The acute effects of a purified toxin from Australian Tiger snake (*Notechis scutatus scutatus*) venom have been investigated at the mammalian neuromuscular junction.

2. The toxin was injected into the tail vein of mice. Death was due to respiratory paralysis.

3. The resting membrane potential, and action potential of muscle fibres in muscles from *in vivo* intoxicated animals were normal.

4. The frequency of miniature end plate potentials (m.e.p.p.s) from intoxicated nerve-muscle preparations was reduced, although m.e.p.p. amplitude was unaltered.

5. Nerve stimulation resulted in end plate potentials (e.p.p.s) of quantal amplitude; only rarely was the e.p.p. large enough to give rise to an action potential.

6. High (20 mM) K^+ did not increase m.e.p.p. frequency in intoxicated preparations.

7. The toxin was largely ineffective *in vitro*.

8. The similarities and differences between this toxin, β-bungarotoxin and botulinum toxin are discussed.

Introduction

The Australian Tiger snake (*Notechis scutatus scutatus*) is a member of the Elapid family. All of the Elapidae are poisonous, the tiger snake being more poisonous than most others (Minton, 1971). Death is due to respiratory depression.

Among the purified toxins isolated from the venom of the animal (Karlsson, to be published) are a neurotoxin, homologous to a neurotoxin of the Thailand cobra, which is a potent blocking agent of cholinergic receptors of the end-plate, and of the chronically denervated muscle membrane (Lester, 1970; Eaker, Harris & Thesleff, 1971), and the toxin which is the subject of this communication. Preliminary observations by one of us (E. K.) suggested it to be a neurotoxin with a different mode of action.

Methods

Male albino mice (NMRI strain, Anticimex, Stockholm) weighing 25–30 g were used throughout these experiments. The toxin (called notexin) is a basic protein

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having 119 amino acid residues (mol. wt. 13574) in one peptide chain cross-linked by seven disulphides (Karlsson, Eaker & Rydén, 1972). It was dissolved in normal saline before use.

10 μg of toxin in 0·2 ml solution was injected into the tail vein of the animal. Within 20 min, the animal experienced neuromuscular weakness, as evidenced by paralysis of the leg muscles, and severe respiratory distress, and at this point was usually killed by cervical dislocation. A hemidiaphragm (and in some cases the extensor digitorum longus muscles) was dissected out, and mounted in a constant flow bath which was continuously perfused with an oxygenated fluid (Liley, 1956) maintained at pH 7·2–7·4 and at 28–29° C.

Muscle fibre resting membrane potential, miniature end-plate potentials (m.e.p.p.s) and end-plate potentials in response to nerve stimulation (e.p.p.s) were recorded and measured using standard intracellular recording techniques. All amplitude measurements of m.e.p.p.s were corrected to a resting membrane potential value of −75 mV (Katz & Thesleff, 1957).

Action potentials were generated and recorded using a double microelectrode technique. The current passing electrode, filled with 2 M potassium citrate, was inserted into the same fibre as the recording electrode at a separation of 50–100 μm. To ensure optimal conditions for action potential generation, the muscle fibre was hyperpolarized to a local potential of −90 to −95 mV (Redfern & Thesleff, 1971). The time differential of the action potential was obtained using a CR circuit.

Results

In vivo *intoxicated muscles*

Resting membrane potential and action potential

In these experiments, extensor digitorum longus muscles (EDL) were removed from animals treated with the neurotoxin. The mean resting membrane potential, maximum rate of rise of potential, action potential threshold and action potential overshoot were measured and compared with values obtained from normal muscles. It can be seen in Table 1, that no differences were found between the two groups of muscle.

TABLE 1. *Resting membrane potential and characteristics of the action potential in muscle fibres of normal mice and mice treated with Tiger snake toxin*

	Normal muscles	Intoxicated muscles
Resting membrane potential (mV)	77·8± 0·81 (23)	78·3± 0·57 (21)
Action potential threshold (mV)	43·9± 0·99 (23)	43·1± 0·64 (21)
Action potential overshoot (mV)	46·9± 1·0 (23)	43·1± 1·5 (21)
Maximum rate of rise of action potential (V/s)	690 ±18 (23)	672 ±28 (21)

The muscle studied was the extensor digitorum longus muscle. The results are expressed as mean±S.E. Number of fibres studied is shown in parentheses. Each result is pooled from 2–3 muscles.

Spontaneous transmitter release : frequency

The mean frequency of m.e.p.p.s in 58 normal unpoisoned muscle fibres was 1·12/s±0·06 (S.E.), with a range of 0·23/s to 2·4/second. This frequency was 5–10 times higher than the mean frequency of m.e.p.p.s in 36 muscle fibres from neurotoxin poisoned muscles (0·25/s±0·03 ; range 0·03/s–0·67/s) (Table 2).

TABLE 2. *Frequency of miniature end plate potentials (m.e.p.p.s/s) in muscle fibres of normal mice and mice treated with Tiger snake toxin*

	Normal muscles	Muscles intoxicated *in vivo*	Muscles intoxicated *in vitro*
Normal bathing solution	1·12±0·06 (58)	0·25±0·03 (36)	0·74±0·07 (17)
High (20 mM) K^+ bathing solution	44·3 ±5·8 (16)	0·47±0·09 (16)	—

The muscle studied was the diaphragm. Frequency is expressed as mean±S.E. Number of fibres studied is shown in parentheses. The results for each experimental procedure are pooled from several muscles.

Amplitude

The mean amplitude of m.e.p.p.s recorded from normal muscle fibres did not differ from the mean amplitude of m.e.p.p.s recorded from neurotoxin poisoned muscle fibres (Fig. 1).

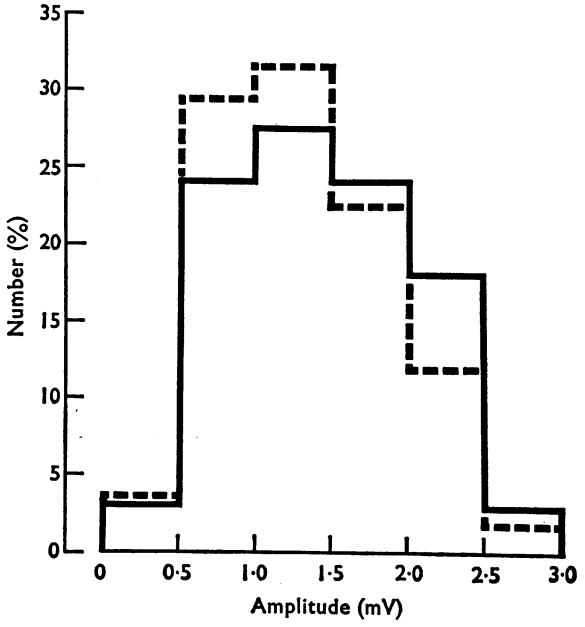

FIG. 1. Mean amplitude of miniature end-plate potentials (m.e.p.p.s) in 58 normal muscle fibres (solid line) and in 36 toxin-poisoned muscle fibres (broken line) presented in histogram form. Each m.e.p.p. was corrected to a resting membrane potential of −75 mV. The potentials were recorded from isolated diaphragm muscles.

Effects of potassium on the frequency of m.e.p.p.s

Since it is well established that the rate of release of m.e.p.p.s is controlled by the membrane potential of the nerve terminal (Katz & Miledi, 1967), it was of interest to determine the effects of high potassium concentration on the frequency of m.e.p.p.s in the neurotoxin-poisoned muscle fibres. A potassium concentration of 20 mM increased the rate of m.e.p.p.s from the normal mean of 1·12/s to a mean of 44·3/second. No such increase was noted in the poisoned muscles (Table 2).

End-plate potentials

The observation that this neurotoxin caused a reduction in the spontaneous release of transmitter, and also that most poisoned muscles failed to respond with a twitch to nerve stimulation, led us to examine the response of the muscle to nerve stimulation in more detail. In this situation, nerve stimulation was either ineffective or gave rise to e.p.p. of a few millivolts amplitude. Repetitive stimulation produced a series of e.p.p.s of varying, and presumably quantal amplitude. In only a few cases, was the amplitude of the e.p.p. sufficiently great to give rise to an action potential (Fig. 2).

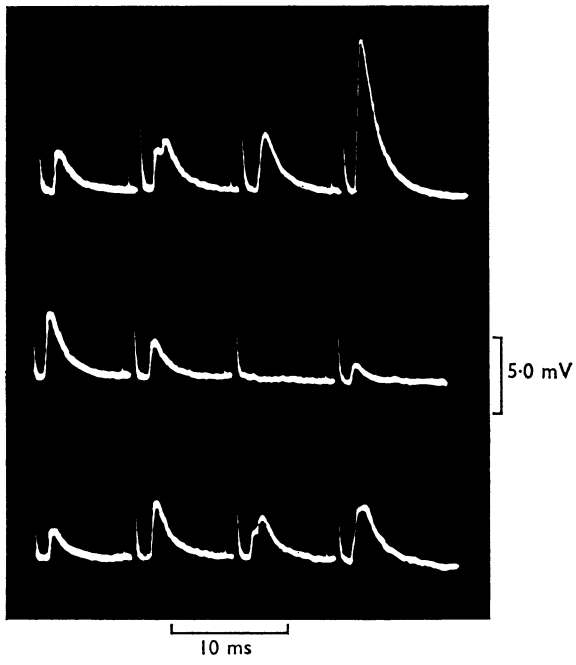

FIG. 2. Twelve consecutive end-plate potentials recorded from a muscle fibre end-plate in a toxin-poisoned muscle. The frequency of stimulation was 60 Hz. Note the irregularity in end-plate potential amplitude, and the occasional failure. Some potentials were observed with 'notches' in the rising phase that may have been due to an asynchronous release of several quanta of transmitter. Record retouched for reproduction.

In vitro *intoxicated muscles*

Resting membrane potential

E.D.L. muscles were dissected and incubated for periods of 1–2 h in either the normal bathing fluid, or bathing fluid and toxin (1 μg/ml). It can be seen in Table 3 that the toxin caused a small but significant depolarization in the muscle fibres.

Transmitter release

In these experiments, diaphragm preparations were dissected and incubated in the presence of the toxin (1·0–2·5 μg/ml) for 1 h at either room temperature or at 37° C. The spontaneous release of transmitter was measured before and after incubation. There was a small reduction in the m.e.p.p. frequency after incubation

TABLE 3. *Mean muscle fibre resting membrane potential in muscles incubated for up to 2 h in either normal bathing fluid or bathing fluid and Tiger snake toxin*

	Resting membrane potential	
Time	Normal bathing fluid	Bathing fluid and toxin
0 h	79·1±0·53 (65)	—
1 h	75·0±0·85 (31)	69·2±1·41 (36)
2 h	72·5±0·75 (32)	64·1±1·16 (32)

The muscle studied was the extensor digitorum longus. The results are expressed as mean±S.E. Number of fibres studied is shown in parentheses. Each result is pooled from 2 muscles.

in the toxin, but this was not as marked as in the *in vivo* experiments (Table 2). Even incubation in the presence of the toxin and high (20 mM) concentrations of K^+ failed to affect the release of the transmitter.

Discussion

The observation of particular interest concerning the action of this toxin is that its *in vivo* administration results in a reduction in the frequency of spontaneous transmitter release. Since the toxin does not reduce the mean amplitude of the m.e.p.p.s, it would seem that it has no post-synaptic activity. Furthermore, there is no effect on other properties of the muscle fibre membrane. This observed specific presynaptic activity is very similar to the effects of β-bungarotoxin, a toxin isolated from the venom of another elapid snake, *Bungarus multicinctus* (Chang & Lee, 1963), and to acute botulinum intoxication (Burgen, Dickens & Zatman, 1949; Brooks, 1956).

The depression of spontaneous transmitter release is accompanied by a similar depression of transmitter release following nerve stimulation. This particular action could be due either to the nerve impulse being unable to invade the nerve terminal, or to the disorganization of some other step in the mechanisms responsible for the release of transmitter. Our observation that high K^+ is largely ineffective in increasing the rate of spontaneous transmitter release in neurotoxin poisoned fibres suggests that there is an impairment in the release mechanism which is independent of nerve terminal depolarization.

It is of interest that the toxin was not as effective *in vitro*, even at relatively high concentrations, as *in vivo*. This lack of effect did not appear to be a function of temperature (cf. botulinum toxin *in vitro;* Burgen *et al.*, 1949). However it is known that the presynaptic blocking effects of botulinum toxin and β-bungarotoxin are partially dependent upon the frequency of nerve activity (Hughes & Whaler, 1962; Lee & Chang, 1966; Simpson, 1971), and this may be true for Tiger snake toxin. It is also of interest that β-bungarotoxin, in contrast to Tiger snake toxin, first causes an increase in m.e.p.p. frequency, before m.e.p.p.s are finally abolished.

The mean muscle fibre resting membrane potential was reduced by incubation for 1–2 h in the presence of the toxin. This may be important, since preliminary observations suggest that the prolonged local application of the toxin *in vivo* has a direct myotoxic action. This action, which distinguished the Tiger snake toxin from β-bungarotoxin and botulinum toxin (Thesleff, 1960) is currently being examined.

Our results suggest that the acute effect of the purified toxin isolated from the venom of Australian Tiger snake is a reduction in both spontaneous and nerve impulse mediated transmitter release from motor nerve terminals.

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