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Presynaptic and postsynaptic effects of the venom of the Australian tiger snake at the neuromuscular junction

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Summary

1. Crude venom (TSV) from the Australian tiger snake (*Notechis scutatus scutatus*) has both presynaptic and postsynaptic effects at the neuromuscular junctions of toads.

2. TSV (50 μg/ml) rapidly blocked indirectly elicited muscle twitches without affecting the compound action potential in the sciatic nerve or twitches elicited by direct stimulation.

3. Low concentrations of the venom (1–10 μg/ml) reduced the amplitude of miniature endplate potentials (m.e.p.ps) and inhibited the depolarization of muscle fibres normally caused by carbachol. It was concluded that a fraction of the venom binds to acetylcholine receptors.

4. The frequency of m.e.p.ps was at first increased by TSV at a concentration of 1 μg/ml. Occasional, high frequency 'bursts' of m.e.p.ps were recorded in some preparations. The mean frequency of m.e.p.ps appeared to fall after several hours in the venom.

5. The quantal content of endplate potentials (e.p.ps) was reduced by the venom. With low concentrations (1 μg/ml), an initial increase in quantal content was often seen. When the quantal content was markedly depressed there was no parallel reduction in the amplitude of nerve terminal spikes recorded extracellularly, though a later fall in size and slowing of time course was often seen.

6. There was evidence that TSV eventually changed the normal Poisson characteristics of the spontaneous release of quanta and this may be correlated with electronmicroscopic changes in nerve terminals.

7. Tiger snake antivenene counteracted the postsynaptic, but not the presynaptic effects of TSV when they had developed.

Introduction

Increasing attention is being paid to toxins and venoms by physiologists and pharmacologists because many biotoxins have been found to have very specific and useful effects on excitable cells. For example, tetrodotoxin from puffer fish, and saxitoxin synthesized by dinoflagellates (Kao, 1966; Narahashi, 1972), and

maculotoxin from the blue-ringed octopus (Dulhunty & Gage, 1971 ; Gage & Dulhunty, 1973) all selectively block action potentials in nerve and muscle cells.

There are two families of snakes, Elapidae and Hydrophiidae, which have venoms that are known to contain neurotoxins (for reviews, see Lee, 1971, 1972). The venoms and neurotoxins of the banded krait, *Bungarus multicinctus*, and of the cobra species *Naja naja atra, Naja naja, Naja naja siamensis* and *Naja nivea*, all of the elapid family, have been studied in considerable detail by means of electrophysiological techniques (Lee & Chang, 1966 ; Meldrum, 1965 ; Lester, 1970 ; Eaker, Harris & Thesleff, 1971 ; Earl & Excell, 1972). All the toxic crude venoms and neurotoxins which have been tested block neuromuscular transmission, generally by binding with postsynaptic receptors so that acetylcholine is less effective in depolarizing the postsynaptic cell. This property of snake venoms has been exploited to investigate and isolate acetylcholine receptors (Barnard, Wieckowski & Chiu, 1971 ; Miledi, Molinoff & Potter, 1971 ; Miledi & Potter, 1971 ; Raftery, Schmidt, Clark & Wolcott, 1971 ; Berg, Kelly, Sargent, Williamson & Hall, 1972 ; Bosmann, 1972 ; Fambrough & Hartzell, 1972 ; Franklin & Potter, 1972 ; Hartzell & Fambrough, 1972 ; Karlsson, Heilbronn & Widlund, 1972 ; Meunier, Olsen, Menez, Fromageot, Boquet & Changeux, 1972 ; Raftery, Schmidt & Clark, 1972 ; Schmidt & Raftery, 1972). Only the venom from the krait, *Bungarus multicinctus*, has been found to contain a fraction (β-bungarotoxin) which acts on nerve terminals rather than on postsynaptic receptors (Lee & Chang, 1966).

Little work has been done to identify the neurotoxic actions of Australian snake venoms, some of which were thought to have a curare-like action at the neuromuscular junction (Kellaway & Holden, 1932). We have investigated the effects of the crude venom of the Australian tiger snake (*Notechis scutatus scutatus*, of the family Elapidae) on neuromuscular transmission to identify the sites at which it acts, in the hope that these will eventually be related to different fractions of the venom. Recently, Karlsson, Eaker & Rydén (1972) have described the separation of five separate neurotoxins from tiger snake venom (TSV). The efficacy of tiger snake antivenene in counteracting the separate effects of the venom has also been investigated. A preliminary report of some of the findings has been made (Datyner & Gage, 1973).

Methods

Isolated sciatic-sartorius nerve-muscle preparations from Queensland cane toads (*Bufo marinus*) were used in the experiments.

Solutions

The standard Ringer solution contained (mM): NaCl, 115 ; KCl, 2·5 ; $CaCl_2$, 1·8 ; Na_2HPO/NaH_2PO_4 buffer, 3 (pH=7·1–7·3). In order to reduce the quantal content of endplate potentials (e.p.ps), in some experiments the concentration of $CaCl_2$ was lowered to 0·9 mM and $MgCl_2$ was added to give a concentration of 5 or 10 mM. When miniature end plate potential (m.e.p.p.) frequency was low, the NaCl concentration was sometimes increased by 50% to make the solution hypertonic and hence increase m.e.p.p. frequency. In other experiments, (+)-tubocurarine chloride (D.H.A.) was added to solutions to give the concentrations indicated in the text. Carbachol solutions contained carbamylcholine chloride (Farmer Hill) at a concentration of 12·5 μg/ml.

Tiger snake venom and antivenene

Dried tiger snake venom was obtained from Eric Worrell's Australian Reptile Park, P.O. Box 192, Gosford, N.S.W. 2250. The venom was dissolved in standard Ringer solution at a higher concentration (100 or 200 μg/ml) and then diluted in the solution being used in the experiment to give the appropriate concentration. As snake venom components have been reported to be allergenic (Mendes, Cintra and Corrêa, 1960) a respirator was worn to guard against venom inhalation when the dried powdered venom was being weighed out.

Tiger snake antivenene (Commonwealth Serum Laboratories) was dialysed against distilled water to remove the tricresol contained as a germicide.

Electrophysiology

The sciatic nerve was stimulated through a pair of platinum electrodes with a grounded wire between, and the compound action potential was recorded through another pair of electrodes closer to the muscle. Glass capillary microelectrodes were used for both intracellular and extracellular recording from endplate regions of surface muscle fibres. The former were filled with 3M-KCl and had resistances of 5–20 MΩ; the latter were filled with 4M-NaCl and had resistances less than 3 MΩ (this was often achieved by scraping or breaking their tips). The nerve was stimulated with brief (0·2 ms) voltage pulses from an isolated stimulator (Devices Isolated Stimulator, 2533). Signals were led via a high-input-impedance, capacity-neutralized amplifier to an oscilloscope where they were photographed. When small extracellular signals were being recorded, they were amplified further ($\times$400) and fed through an analog-to-digital converter to a computer (PDP 8/I, Digital Equipment Company) to give a progressive sum which was displayed on an oscilloscope and photographed. This method gave a better signal to noise ratio.

Analysis of results

The methods and rationale of the statistical analysis of the quantal content of e.p.ps have been described in detail by Katz (1969) and Martin (1966). The quantal contents (m) of e.p.ps were calculated from three equations:

$$m = \frac{\text{mean e.p.p. amplitude}}{\text{mean m.e.p.p. amplitude}} \quad \ldots\ldots(1)$$

$$m = \ln \frac{\text{number of stimuli}}{\text{number of 'failures'}} \quad \ldots\ldots(2)$$

$$m = (1/\text{co-efficient of variation of e.p.ps})^2 \quad \ldots\ldots(3)$$

The last two equations derive from the Poisson equation, and the fact the variance equals the mean in a Poisson distribution.

Where necessary, the amplitudes of e.p.ps and m.e.p.ps were corrected for changes

in resting membrane potential according to the formula

$$Vc = Vx. \frac{Vs - Veq}{Va - Veq}$$

where Vc=corrected e.p.p. or m.e.p.p. amplitude

Vx=uncorrected e.p.p or m.e.p.p. amplitude

Vs=standard value chosen for resting membrane potential

Va=actual resting membrane potential

Veq=equilibrium potential for the e.p.p. (−15 mV).

The values for the quantal content obtained using equations 1 and 3 were corrected for non-linear summation of unit potentials as described by Martin (1966).

The chi-square test was used to detect deviations of m.e.p.p. frequency from a Poisson distribution (cf. Gage & Hubbard, 1965) and a level of significance of 5% was used, the level being read from the tables (Pearson & Hartley, 1966).

Results

Muscle twitches

When nerve-muscle preparations were exposed to TSV at a concentration of 50 μg/ml in normal Ringer, twitches in response to stimulation of the nerve disappeared within 45 minutes. Muscle fibres closest to the entry of the main nerve trunks, where the muscle is thickest, were last affected. When nerve stimulation no longer caused a twitch, direct electrical or mechanical stimulation of the muscle still caused contraction. This indicated that action potentials could still be generated in the muscle. In order to analyse the reason for the loss of the twitch in more detail, electrical recordings were made from the sciatic nerve and surface fibres of sartorius muscles which were exposed to the venom.

Action potentials and endplate potentials

It was found that compound action potentials could still be recorded extracellularly from a sciatic nerve, soaked for more than 6 h in TSV (50 μg/ml). Furthermore, in a nerve-muscle preparation, in which both the sciatic nerve and the muscle were exposed to venom (50 μg/ml), action potentials could be recorded extracellularly from the nerve more than 6 h after the muscle had stopped twitching. These observations show that the venom does not inhibit action potentials in the nerve trunk, although it is possible that the nerve sheath and other diffusion barriers might have prevented access of the venom to the surface membrane of the nerve fibres.

The above results indicate that the neuromuscular junction is the most likely site of venom action. In order to analyse this blockade of neuromuscular transmission in more detail, intracellular recordings were made from endplate regions of surface muscle fibres in preparations exposed to the venom.

Endplate potentials elicited by supramaximal stimulation of the nerve were recorded in a preparation exposed to solutions containing (+)-tubocurarine (1 μg/ml). When TSV (50 μg/ml) was introduced into the bath, e.p.ps rapidly declined in size and disappeared within several minutes. This result confirms the tentative conclusions drawn from the above experiments; namely, that TSV exerts its effects on neuromuscular transmission rather than on muscle contraction or conduction of action potentials in the nerve trunk. The rapidity of the effect indicated that lower concentrations of TSV could probably be used in subsequent experiments.

To distinguish between the possible sites of action of TSV at the neuromuscular junction further experiments were carried out. In particular, the possibility that the venom might have both pre- and post-synaptic actions, like the venom from *Bungarus multicinctus*, was tested.

Most of the experiments to be described were done in solutions containing magnesium ions and a lower than normal calcium ion concentration so that transmitter release was reduced, and e.p.p.s and m.e.p.ps could be recorded in the same muscle fibre.

Postsynaptic action of the venom

Miniature endplate potential amplitude. In the presence of TSV at a concentration of 10 μg/ml, the amplitude of both e.p.ps and m.e.p.ps fell rapidly. The decrease in m.e.p.p. amplitude was slower in 1 μg/ml TSV and a typical example of the time-course of the change with the lower concentration is shown in Figure 1. A solution containing TSV (1 μg/ml), was perfused through the bath during the 2 min marked by the column (Figure 1, TSV). The mean sizes of m.e.p.ps recorded during 5·5 min periods are shown by the points.

Changes in the amplitude of m.e.p.ps can, with few exceptions, be attributed to changes in postsynaptic properties (Katz, 1962). There are exceptions, however.

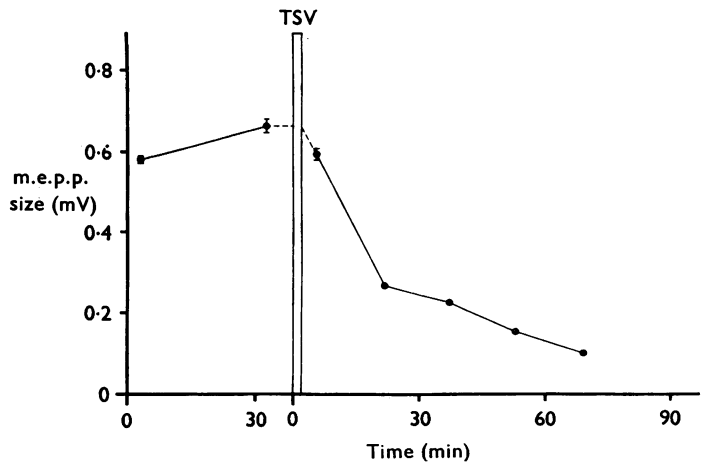

FIG. 1. The fall in miniature endplate potential (m.e.p.p.) size caused by tiger snake venom (TSV) (1 μg/ml). The vertical column, marked TSV, denotes the time during which the bath solutions were changed. Each point represents the mean size of m.e.p.ps occurring during a 5·5 min recording period. The vertical bars show 1 S.E.M. when this extends beyond the points.

For example if acetylcholine synthesis is inhibited, a reduction in m.e.p.p. amplitude can occur (Elmqvist & Quastel, 1965). To confirm that TSV did have a postsynaptic effect, the influence of the venom on the sensitivity of postsynaptic receptors to the acetylcholine analogue, carbamylcholine (carbachol) was tested.

Receptor sensitivity to carbachol. In untreated preparations, carbachol (12·5 μg/ml) depolarized endplate regions of surface muscle fibres by 15–30 mV, whereas in the presence of TSV at concentrations of 1 μg/ml or above, the depolarization was eventually prevented. One of these experiments is illustrated in Figure 2. The columns show the average depolarization (with respect to the original average resting membrane potential shown by the broken horizontal line, Fig. 2) measured in 20 or more fibres, during the times shown by the width of each column. Before TSV, the carbachol caused a depolarization of $22{\cdot}1 \pm 2{\cdot}6$ mV (mean ± 1 S.E.M., 21 fibres). In the presence of TSV (1 μg/ml), carbachol had progressively less effect on membrane potential and had no effect after about 2 h (Figure 2). When higher concentrations of TSV were used (for example, 10 μg/ml), the postsynaptic response to carbachol was blocked within 30 minutes. Results obtained in one of the experiments with 10 μg/ml TSV are shown in Figure 3. The effect of carbachol was clearly blocked very rapidly by TSV: it can be concluded that some fraction of the venom binds to acetylcholine receptors.

Presynaptic actions of the venom

Frequency of miniature endplate potentials. TSV (1 μg/ml) almost invariably caused an increase in the frequency of m.e.p.ps. (This was not seen with higher

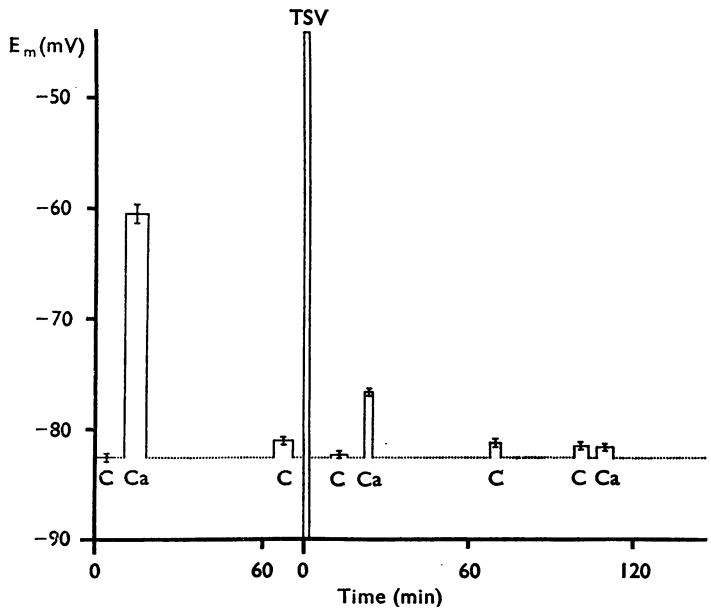

FIG. 2. The effect of tiger snake venom (TSV) (1 μg/ml) in preventing the depolarization of endplate regions by carbachol (12·5 μg/ml). The dotted horizontal line shows the mean membrane potential (E_m) at the beginning of the experiment. Each vertical column then shows the mean membrane potential of 20 or more fibres either in carbachol (Ca) or standard Ringer (C). The vertical bars show 1 S.E.M. After the narrow vertical column labelled TSV, all solutions contained TSV (1 μg/ml).

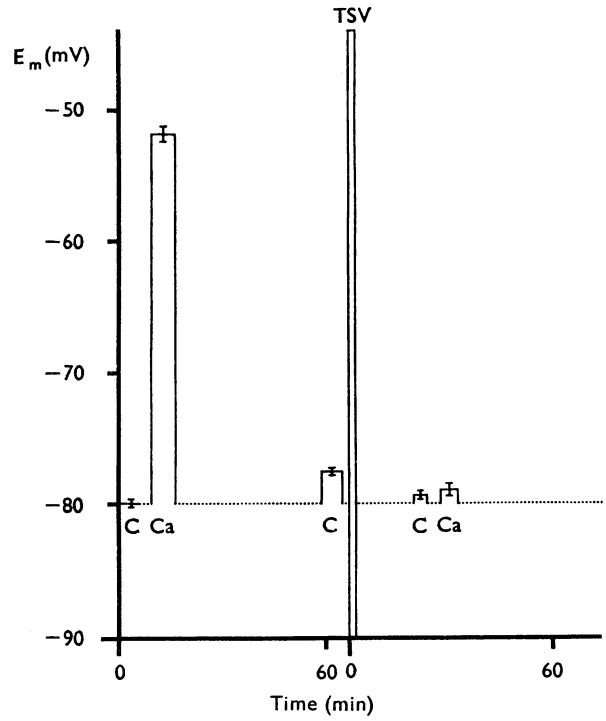

FIG. 3. The effect of tiger snake venom (TSV) (10 μg/ml) in preventing the depolarization caused by carbachol (12·5 μg/ml). The symbols and conventions are as described for Figure 2.

concentrations probably because of the postsynaptic effect of the venom.) In addition, occasional ' bursts ' of m.e.p.ps were seen in some, but not all, preparations. During these bursts, m.e.p.p. frequency could rise to more than a hundred times the basal frequency. (These rises in frequency were observed less often when calcium and magnesium concentrations were normal.) Eventually, the frequency of countable m.e.p.ps tended to fall but it is not possible to place too much weight on this observation because m.ep.ps became very small and were often difficult to distinguish from the baseline noise.

In four experiments, the effect of TSV on m.e.p.ps in solutions containing no calcium ions but 2 or 4 mM $MgCl_2$, was tested. TSV (1 μg/ml) still caused the usual fall in m.e.p.p. amplitude but no significant rise in the frequency of m.e.p.ps was observed in three of the experiments. There was an increase in m.e.p.p. frequency in the fourth experiment but the microelectrode was found to have moved and it is thought that this was probably the cause of the different result.

Quantal content of endplate potentials. In view of the effect of TSV on acetylcholine receptors, the reduction in amplitude of e.p.ps caused by the venom must have been caused, at least partly, by a curare-like, postsynaptic effect of the venom. Further experiments were done to see if there was any deficiency of evoked transmitter release in preparations exposed to venom. In fourteen preparations exposed to TSV (1 μg/ml) an eventual reduction in quantal content was seen. In thirteen of these there was an early, transient increase in quantal content, preceded in eleven cases by a definite temporary fall in quantal content

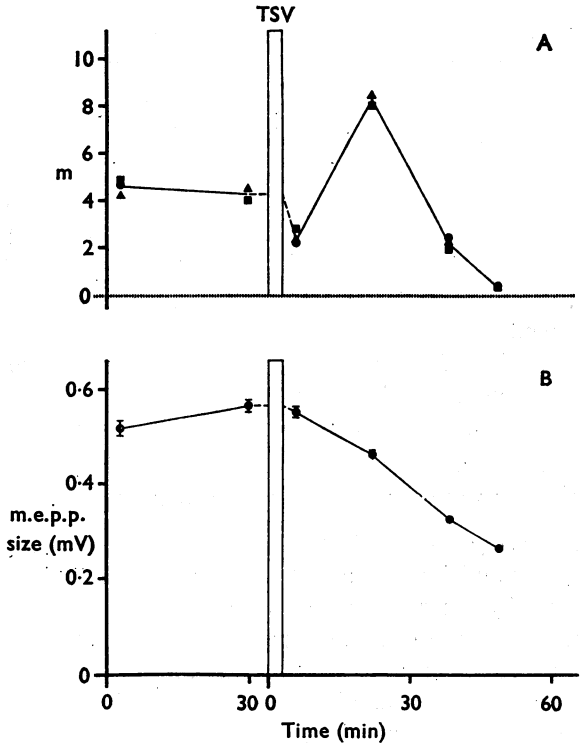

FIG. 4. The effect of tiger snake venom (TSV) (1 μg/ml) on the quantal content (m) of endplate potentials (e.p.ps) (A) and the size of miniature endplate potentials (m.e.p.ps) in the same muscle fibre (B). The vertical columns show the introduction of the solution containing TSV. In A, quantal contents calculated (for at least 100 e.p.ps), from equations 1, 2 and 3 in **Methods**, are shown as triangles, circles and squares respectively. In B, the points show mean m.e.p.p. sizes and the vertical bars show 1 S.E.M. when larger than the points.

below the control level. In the fourteenth experiment the quantal content fell throughout. The results of a typical experiment are shown in Figure 4A. Quantal contents calculated from the ratios of mean e.p.p. and m.e.p.p. amplitudes, the number of 'failures', and the co-efficient of variation (see **Methods**), are shown as triangles, circles and squares respectively. There was the initial fall in quantal content, followed by an increase, and then a progressive, gradual disappearance of e.p.ps. Changes in m.e.p.p. amplitude in the same fibre are shown in Fig. 4B, to illustrate the concomitant postsynaptic effect.

When TSV, at a concentration of 0·1 μg/ml, was used in a similar experiment, the quantal content of e.p.ps was still rising after two hours. With 0·5 μg/ml, the quantal content of e.p.ps fell after about 2 h, there having been a previous increase. In one experiment, small e.p.ps were recorded without twitches in a preparation in standard Ringer. Within 30 min of adding TSV to the bath at a concentration of 1 μg/ml, the mean quantal content was markedly reduced and 'failures' were seen for the first time. This effect was even faster than in solutions containing magnesium. In other preparations exposed to TSV (1 μg/ml) in Ringer solution containing 173 mM NaCl, the decline in amplitude of e.p.ps was associated with an increased fluctuation in size, again indicating a decrease

in quantal content caused by the venom. Thus the inhibition of transmitter release by TSV is not seen only under conditions of reduced transmitter release in the presence of abnormal magnesium and calcium concentrations.

When higher concentrations of TSV (5 or 10 μg/ml) were used, no increase in quantal content was seen in four of five experiments, only a rapid fall within 15 minutes (Figure 5). (In the one other experiment a brief drop in quantal content was followed by a transient increase in quantal content and a subsequent progressive fall.) In three other experiments with TSV (10 μg/ml), e.p.ps disappeared too rapidly for any analysis of quantal content to be made.

In all of these experiments, quantal contents calculated by the three methods (see **Methods**) agreed very closely. This indicates that Poisson statistics still applied: the probability of release of a quantum of transmitter must still have been very low in the presence of the venom.

The reduction in quantal content of e.p.ps could be due to a change in the amplitude of presynaptic action potentials or a direct effect on excitation-secretion coupling. Experiments were done to distinguish between the two possibilities.

Extracellular recording. If a microelectrode is positioned close to an endplate region, currents generated by action potentials in the presynaptic terminal (nerve terminal spikes) and by the resultant e.p.p., can be recorded extracellularly (Katz & Miledi, 1965a, b). If the endplate current (e.p.c.) is affected by an agent but the nerve terminal spike is not, then excitation-secretion coupling may have been changed. On the other hand, disappearance of the nerve terminal spike indicates that the agent has an effect, directly or indirectly, on depolarization-activated sodium conductance in the nerve terminal. It is then necessary to do other experiments to determine whether excitation-secretion coupling is affected also.

In solutions containing curare, or magnesium with a reduced calcium concentration, nerve terminal spikes and e.p.cs were recorded. Because of the small

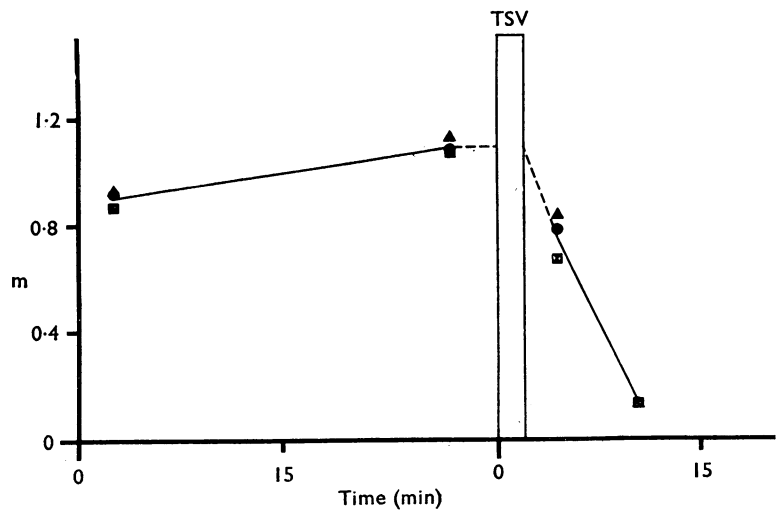

FIG. 5. The time course of the effect of tiger snake venom (TSV) (5μg/ml) on the quantal content (m) of endplate potentials (e.p.ps). Each point was calculated from at least 90 e.p.ps. The symbols and conventions are as described for Figure 4.

size of these signals, twenty consecutive responses were summed to increase the signal to noise ratio (see **Methods**). A record obtained in this way in Ringer solution containing (+)-tubocurarine (1·5 μg/ml) is shown in Figure 6A. The initial fast downward deflection is the nerve terminal spike, and the later, slower, downward deflection is the e.p.c. The record shown in Fig. 6B was obtained in the period from 2 to 6 min after exposure to 1 μg/ml TSV (in curare-Ringer). The e.p.c. had increased in amplitude without a perceptible change in the nerve

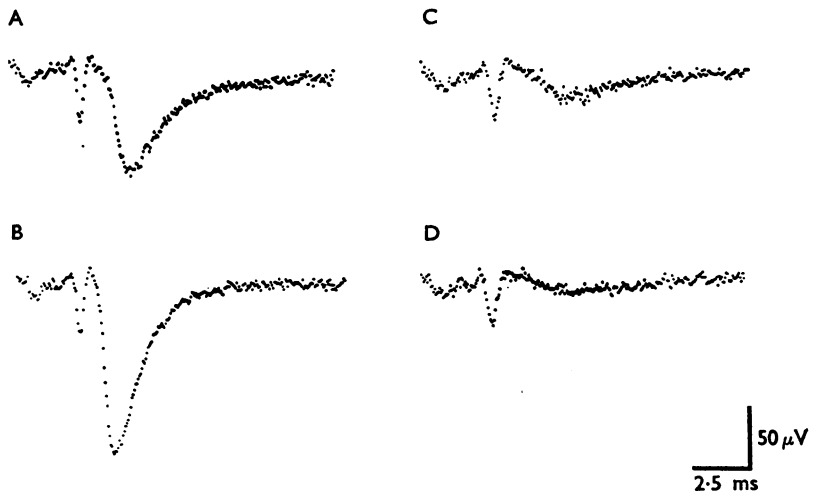

FIG. 6. Averaged extracellular recordings of nerve terminal spikes and endplate currents in solutions containing (+)-tubocurarine, 1·5 μg/ml. A: control. Subsequent records were obtained after the addition of tiger snake venom (TSV) (1 μg/ml) and were obtained at 2–6 min (B), 49–53 min (C), and 69–73 min (D). For further details, see text.

terminal spike. At 49–53 min (Fig 6C) and 69–73 min (Fig. 6D) in TSV, the e.p.c. amplitude had decreased markedly but the nerve terminal spike could still be recorded, relatively unchanged (there is a small reduction in the amplitude of the spikes which may be a real effect of the venom). There was usually an eventual decrease in the size, and a slowing of the time course of nerve terminal spikes. As these changes became obvious later than the large reduction in e.p.c. amplitude, they probably do not cause it.

The reduction in e.p.c. amplitude was probably not caused by a reduction in the amount of transmitter available for release. This was indicated in experiments in which the sciatic nerve was stimulated at frequencies of 50 to 100 Hz as illustrated in Figure 7.

Endplate currents evoked by two stimuli 20 ms apart (Fig. 7A) were recorded in Ringer solution (containing (+)-tubocurarine, 1·5 μg/ml). Facilitation of the second e.p.c. is evident. After 35–36 min in TSV (1 μg/ml) the e.p.cs were smaller (Fig. 7B), but facilitation was still evident, and the presynaptic spikes were unchanged. Three stimuli (at 100 Hz) were used after 72–73 min, and the repetitive stimulation effectively increased the amplitude of e.p.cs, the first of which was even smaller than before. At 99–100 min after exposure to TSV (Fig. 7D) the e.p.cs were even smaller, and facilitation was still evident, but the presynaptic spikes were broader and smaller than before. However, the three spikes were equal in amplitude and this suggests there was no ' drop-out ' of action potentials, even at this high frequency of stimulation.

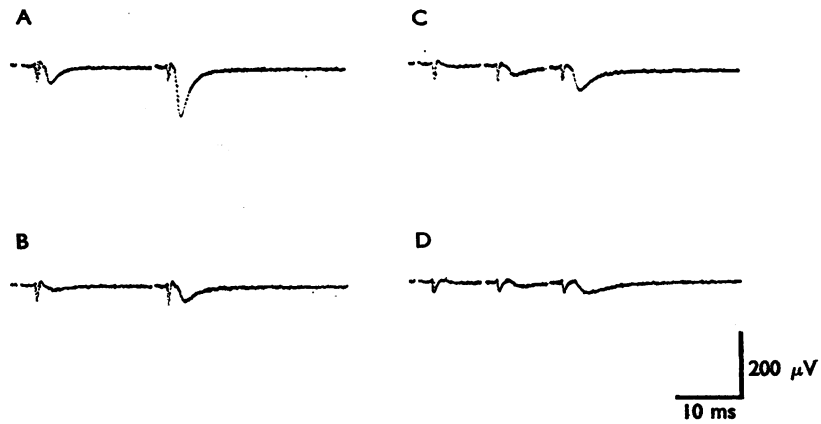

FIG. 7. The effect of repetitive stimulation on endplate currents reduced in size by tiger snake venom (TSV) (1 μg/ml). The solutions and methods were as described for Figure 6. A: control. Subsequent records were obtained after the addition of TSV, at 35–36 min (B), 72–73 min (C) and 99–100 min (D).

There was no change in the time interval between stimulus artefact and the peak of the nerve terminal spike during the fall in amplitude of e.p.cs, but there was a definite increase in the interval between the presynaptic spike and the e.p.c. This can be seen in Figure 6. In addition, as the e.p.c. fell in amplitude, its rise-time increased whereas the decay time increased only slightly.

These observations support the hypothesis that TSV has a direct effect on excitation-secretion coupling. There also seems to be a later-developing effect on action potentials in presynaptic terminals, though difficulty in maintaining a constant recording position may have been responsible for some of the changes.

Non-random release of quanta

In 1 μg/ml TSV, m.e.p.p. frequency initially conformed to a Poisson distribution (Gage & Hubbard, 1965) until the evoked release of quanta was considerably depressed. By then, the amplitude of m.e.p.ps was usually small (0·1–0·25 mV) although the frequency was sometimes still high. M.e.p.ps recorded in four experiments were analysed for a possible deviation from a Poisson distribution. In two experiments, a significant deviation was seen, but this could have been caused by a change in mean m.e.p.p. frequency seen during the time over which oscilloscope sweeps were photographed. In the third experiment, the chi-square level fell from 61–64% earlier in the experiment to 6–8% near the end of the experiment although the mean m.e.p.p. frequency was almost constant throughout the latter series. (During the observation period of 5·8 min, the mean frequency was 1·5/s during the first 2·9 min, and 1·7/s during the rest of the period). In the fourth set of results, there was a significant deviation from Poisson statistics (chi-square less than 2·5%) with only a slight change in mean frequency (in the observation period of 5 min, the mean frequency was 14·2/s during the first 2·5 min, and 15·0/s during the last 2·5 minutes). The frequency distribution had three peaks (Fig. 8) whereas, with a Poisson distribution, only one peak would be expected.

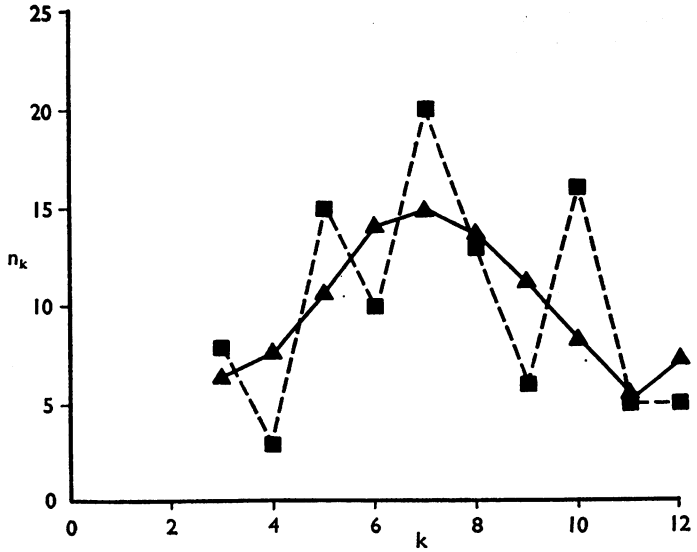

FIG. 8. Comparison of the observed (squares) and predicted (triangles) number of time periods (n_k) containing k miniature endplate potentials (m.e.p.ps), in a solution containing tiger snake venom (1 μg/ml). The class k=12 contains all k equal to or greater than 12. For further details, see text.

Effectiveness of antivenene

The standard treatment for the bite of a tiger snake includes the injection of antivenene, but there is some difference of opinion as to its effectiveness. We have tested the effect of antivenene at the neuromuscular junction.

When TSV (1 μg/ml) had caused the disappearance of m.e.p.ps and e.p.ps, the venom was washed out of the bath with Ringer solution. M.e.p.ps were found again at some endplates within an hour, but endplate potentials could be recorded in very few fibres.

The effectiveness of antivenene was compared with simple removal of TSV, either by washing out the venom with antivenene in the Ringer, or by adding antivenene to the bath still containing TSV. These procedures were started only after the effects of TSV were well developed and both m.e.p.ps and e.p.ps had disappeared. A better recovery than before was generally obtained. M.e.p.ps could be recorded at many endplates where they had disappeared but it was noticeable that their frequency was often higher than before exposure to the TSV. Full recovery was never obtained when antivenene was used in these ways.

On the other hand the effect of TSV (1 μg/ml) could be effectively neutralized by mixing it with antivenene (0·5 units/ml) before adding it to the bath. After 3 h in this solution, the amplitude and frequency of m.e.p.ps was unchanged.

When higher concentrations of antivenene were used in some experiments, an increase in the quantal content of e.p.ps was noted. The effect of the antivenene alone was tested at a concentration of 2·5 units/ml. Within an hour, the quantal content of e.p.ps had increased from 0·6 to 5·0. This effect was attributed to the presence of tricresol, a phenolic substance, which is added to the antivenene as a germicide during manufacture. It is known that phenols increase the quantal

content of e.p.ps (Otsuka & Nonomura, 1963). When tricresol was removed from the antivenene preparation by dialysis, the antivenene, even at a concentration of 5 units/ml, no longer increased the release of transmitter.

The experiments with low concentrations of antivenene (<1 unit/ml) were repeated with the dialysed material, but essentially the same effects were seen as before; presumably, the tricresol concentration had been too low in the undialysed antivenene to produce significant effects.

Overall, the antivenene, when added after TSV had developed its effects, counteracted the postsynaptic action of the venom and this was indicated by the return of m.e.p.ps. However the presynaptic effect on e.p.ps was reversed at very few endplates. Furthermore the frequency of m.e.p.ps, when they returned, was often high, an indication again of a persistent presynaptic lesion.

Discussion

The crude venom of the tiger snake clearly has two major sites of action at the neuromuscular junction, the nerve terminal and the postsynaptic receptors. In this respect, TSV is similar to the venom from *Bungarus multicinctus*, which also has presynaptic and postsynaptic effects (Lee & Chang, 1966). The earlier potentiation and later depression of evoked transmitter release in TSV (Fig. 4A) may have been caused by two different fractions of the venom. Potentiation was seen most clearly with a concentration of TSV of 0·1 μg/ml, whereas only depression occurred with a concentration of 10 μg/ml; at the intermediate concentration of 1 μg/ml, a mixture of the two effects was seen (Figure 4A). The increase in m.e.p.p. frequency caused by the venom was calcium-dependent, suggesting that the venom increases the permeability of the presynaptic membrane to calcium ions At concentrations of 1 μg/ml and above, TSV eventually inhibited evoked transmitter release.

The presynaptic effect of TSV was not secondary to changes in presynaptic action potentials because the quantal content of e.p.ps fell even before small changes in nerve terminal spikes could be detected. When endplate currents had become very small in TSV, the amplitude and time course of presynaptic spikes were sometimes changed, but not greatly. These later changes in the nerve terminal spike suggest that same other fraction of the venom might affect action potentials, but this is not a prominent effect compared with the inhibition of transmitter release which must be due to interference with the transmitter-release mechanism. This conclusion is supported by the observation of increases in the latency of e.p.cs and the frequency of m.e.p.ps. It is interesting that the venom appears to change the normal Poisson statistics of the release process, and also causes clumping and a reduction of the number of vesicles in nerve terminals (N. Lane, personal communication). It is possible that the two phenomena are related, but clearly, further experimental validation is needed before more inferences can be drawn from the two sets of observations. It seems very unlikely that the fall in the quantal content of e.p.ps is caused by a reduction in the number of quanta available for release (so-called 'depletion'). If depletion occurred to a significant extent, Poisson statistics would probably not apply (the probability of the release of a quantum would have increased) unless the number of quanta available for release fluctuated according to a Poisson distribution (Vere Jones,

1966). Poisson statistics were in fact obeyed by e.p.ps (Fig. 4A) until their amplitude could no longer be measured accurately.

The presynaptic effects of TSV appear similar, in many respects, to those of β-bungarotoxin (Lee & Chang, 1966). At certain dose levels both cause an initial rise in the frequency of m.e.p.ps, followed by a fall in frequency, although in the case of TSV, the postsynaptic effect of the venom makes it difficult to say with complete confidence that such a fall had taken place. Lee & Chang (1966) showed that β-bungarotoxin reduced the size of e.p.ps, probably through an interference with excitation-secretion coupling: nerve terminal spikes were still present when β-bungarotoxin had abolished e.p.ps. Tiger snake venom appears to have comparable effects on the evoked release of acetylcholine from nerve terminals. β-Bungarotoxin caused marked structural changes in the nerve terminals, including a depletion of synaptic vesicles (Chen & Lee, 1970) and tiger snake venom has been found to have significant effects on the nerve terminal structure also (N. Lane, personal communication).

The postsynaptic effect of TSV was expected, but its reversibility was not. It is fundamental to the use of snake neurotoxins as quantitative markers of acetylcholine receptors that they bind to these receptors irreversibly. The fraction of TSV which had postsynaptic effects binds reversibly to receptors: the postsynaptic effects of TSV could be reversed either by washing out the venom or by adding antivenene to the bath. It was noticed that the presynaptic effects could not be so readily reversed, and this may be a result of the structural changes (N. Lane, personal communication) in presynaptic terminals produced by TSV.

There is no information available about the structure of the fraction of TSV which binds to acetylcholine receptors. It would be interesting to compare the structure of this fraction with that of α-bungarotoxin and other snake neurotoxins that bind to receptors. Such studies may yield more information about the structure and affinities of acetylcholine receptors.

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