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AN ELECTROPHYSIOLOGICAL STUDY OF THE ACTION OF CRUDE *Enhydrina schistosa* VENOM

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1 Electrophysiological studies on the rat diaphragm revealed that crude *Enhydrina schistosa* venom has a blocking action on postjunctional acetylcholine (ACh) receptors.

2 This venom in concentrations of 0.125–1.0 μg/ml abolished the extracellular endplate potential (e.e.p.p.) without altering the presynaptic spike. When the phrenic nerve was stimulated at a frequency of 1 Hz the degree of depression of successive e.e.p.ps was approximately the same in records made before and after treatment with venom.

3 In concentrations of 0.25 and 0.5 μg/ml the venom reduced significantly the amplitude of miniature endplate potentials (m.e.p.ps). The frequency of m.e.p.ps did not increase but was decreased when the amplitude of the m.e.p.ps was much reduced.

4 The crude venom did not alter the resting membrane potential (RMP) of the muscle cell.

5 The venom inhibited the depolarization of the muscle membrane caused by carbachol.

6 It is concluded that an active component in the crude venom is responsible for a blocking action on postjunctional receptors.

Introduction

The crude venom of *Enhydrina schistosa*, a sea snake found in Malaysian waters, has been shown to paralyse skeletal muscle by blocking transmission across the neuromuscular junction (Carey & Wright, 1961; Cheymol, Barme, Bourillet & Roch-Arveiller, 1967; Geh, 1968). Attempts made to elucidate its site of action at the neuromuscular junction have revealed a presynaptic effect with relatively small doses (Geh & Chan, 1973; Toh, Geh & Chan, 1975); however, an action on postjunctional receptors by the crude venom has been suggested (Carey & Wright, 1961; Cheymol *et al.*, 1967; Geh, 1968). Since it is possible for crude snake venoms to have two main sites of action (Chang & Lee, 1963; Datyner & Gage, 1973a,b) the present electrophysiological study was designed to determine whether the action of crude *E. schistosa* venom is pre- or postjunctional or even both.

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Methods

The phrenic nerves and diaphragms were dissected from Wistar rats of either sex weighing 140–200 grams. The rats were killed by a blow on the head followed by section of the spinal cord in the neck. Both right and left hemidiaphragms were used and were bathed in a modified Liley's (1956a) solution of the following composition (mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ (aq) 1, NaH_2PO_4 (aq) 1, $NaHCO_3$ 12 and dextrose 25, aerated with 5% CO_2 in O_2.

The arrangements for extracellular and intracellular recording from the preparation have been described by Ferry & Marshall (1973). The diaphragm was pinned down in a perspex bath and illuminated from below. Medium flowed into the bath at approximately 1 ml/min and the amount in the bath was kept at 20 ml by a constant level device and at 37 ± 1°C. The phrenic nerve was stimulated through a pair of silver electrodes mounted in a perspex tube with pulses 5x threshold strength, with a pulse width of 0.05 ms and at 0.1 Hz. The bathing medium contained 1.3 μM (+)-tubocurarine chloride. Extracellular recordings (e.e.p.p.) were made with insulated silver wire electrodes (0.13 mm diam., diamel coated, Johnson,

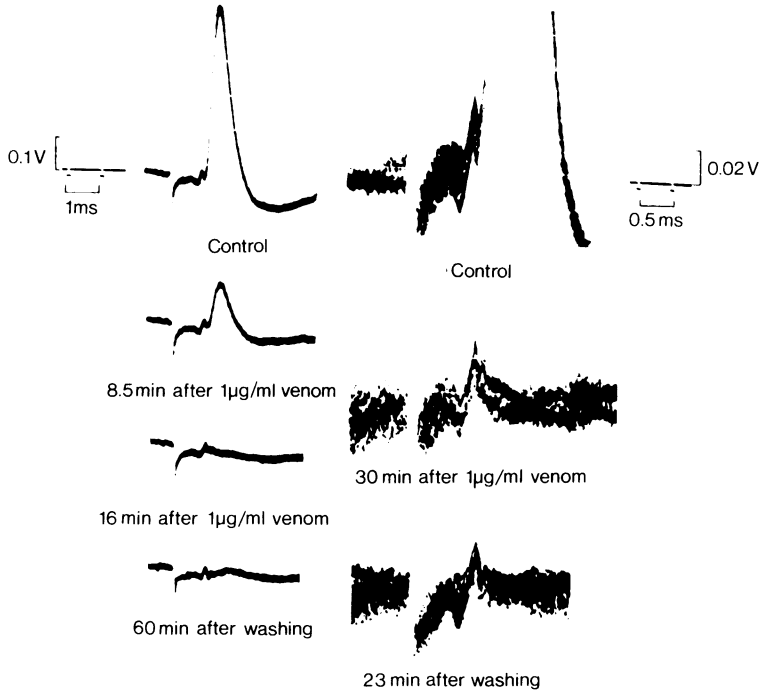

Figure 1 Extracellular records of activity at endplate region of rat phrenic nerve-diaphragm preparation 37°C (1.3 μM (+)-tubocurarine), showing (left) presynaptic spike followed by endplate potential, and (right) the similar records at 5x gain to show presynaptic spike, each trace being 10 superimposed responses.

Matthey & Co.) suitably supported in glass capillary tubings. One electrode was located at the endplate region, the indifferent electrode being placed in the medium. The endplate region was located in blocked preparations by positioning the electrode so that the amplitude of the endplate potential and its rate of rise were maximal and a presynaptic spike was seen. The preamplifier had a bandwidth of 0.2 Hz–10 kHz.

For intracellular recording of miniature endplate potentials (m.e.p.ps) and resting membrane potentials (RMP) the glass microelectrodes were filled with 3M KCl and had a tip resistance of 10–30 MΩ. The recording system had a bandwidth of 0.8 Hz–1 kHz for m.e.p.ps and was directly coupled for recording RMP.

Drugs

Crude *Enhydrina schistosa* venom (dried in a dessicator) was obtained from the Institute of Snake and Venom Research, Penang; (+)-tubocurarine chloride from Burroughs Wellcome and carbamylcholine chloride from BDH. All stock solutions of the drugs used were prepared in double distilled water, but final dilutions were in Liley's solution.

Results

Effect of E. schistosa *venom on extracellular endplate potentials*

In 10 experiments endplate potentials were recorded extracellularly in preparations which had been treated with (+)-tubocurarine 1.3 μM for at least 1 h to achieve a steady block of neuromuscular transmission.

After the addition of *E. schistosa* venom within the range 0.125–1 μg/ml, the amplitude of the extracellular endplate potential decreased. The extent of the decrease and its time course depended on the dose of venom, and was maximal within 15–40 minutes. The presynaptic spike remained despite considerable changes in the e.e.p.p. (Figure 1). Washing the preparation with fresh Liley's solution and/or stopping the stimulation did not restore the e.e.p.p. nor have any marked effect on the presynaptic spike. The persistence of the presynaptic spike affords an indication that the reduction of the e.e.p.p. is not artefactual; for instance, it is not due to the electrode moving off the original focus of activity.

It is concluded that *E. schistosa* venom does not affect the conduction of the presynaptic action

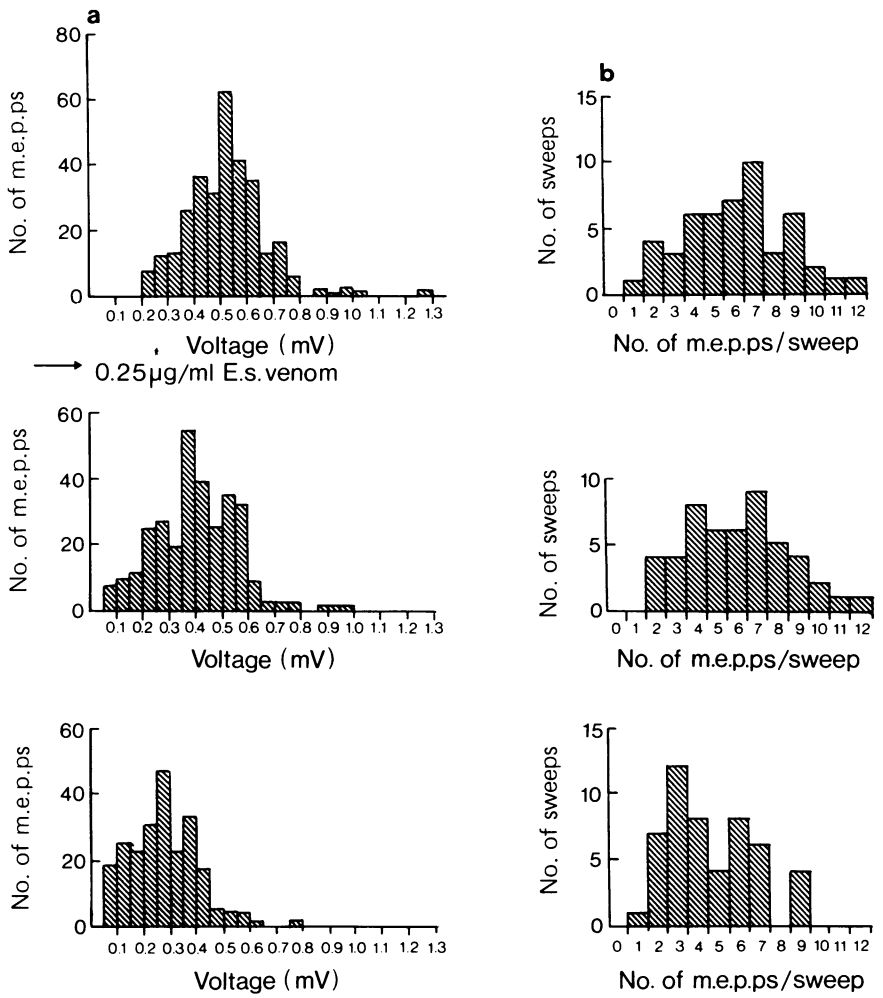

Figure 2 Miniature endplate potentials of rat phrenic nerve-diaphragm preparation (37°C) recorded intracellularly. (a), Histograms of m.e.p.p. amplitude; (b) histograms of number of m.e.p.p.s per 0.5 s sweep. All histograms constructed from 50 sweeps at 1 Hz. The preamplifier was set at a bandwidth of 0.8 Hz–1 KHz. Top: control conditions; Middle: 4 min after *Enhydrina schistosa* (E.s.) toxin 0.25 μg/ml; Lower: 9 min after E.s. toxin 0.25 μg/ml.

potential, but interferes with the release or action of acetylcholine.

Effect of E. schistosa *venom on tetanic rundown of extracellular endplate potentials*

In 10 preparations experiments were made on the effect of *E. schistosa* venom on the run-down at 1 Hz of the e.e.p.p. Before adding the venom, the 5th e.e.p.p. was 83% of the first (s.e. ± 1.47). After venom in concentrations of 0.125–1 μg/ml there was no significant change in the rate of tetanic run-down, although the amplitudes of the e.e.p.ps were reduced, as described above.

These experiments suggested there was no change in the fractional release of transmitter from the nerve terminals after *Enhydrina schistosa* venom.

Effect of E. schistosa *venom on the resting membrane potential*

In each of 10 preparations the membrane potential of 30 cells was measured, the preparation was exposed to 2.5 μg/ml of venom and 30 min later the membrane

potentials of a further 30 cells was measured. At this time there was no response of the muscle to stimulation of the nerve. The overall mean and s.e. was calculated from the mean values in individual preparations. This overall mean and s.e. for control values was 70.61 mV (s.e. ± 1.45) and after the venom the overall mean was 71.60 mV (s.e. ± 1.05). There is no significant difference between these values ($P > 0.05$). It is concluded that *E. schistosa* venom has no significant effect on the membrane potential.

Effect of E. schistosa *venom on miniature endplate potentials*

Miniature endplate potentials were displayed on an oscilloscope with sweep time of 0.5 s and photographs were taken of successive sweeps. M.e.p.ps were recorded before and after the application of venom at 0.25 μg/ml or 0.5 μg/ml. The distribution of amplitude of m.e.p.ps was prepared from the photographs (Figure 2, left). The distribution of the number of m.e.p.ps in each sweep was also prepared as an index of m.e.p.p. frequency (Figure 2, right). The amplitude of m.e.p.ps began to decrease within 3–5 min, and in Figure 2 where sampling began 4 min after the administration of venom, there was a significant reduction of m.e.p.p. amplitude compared to control ($P \leqslant 0.001$) without any marked change in their frequency distribution. When sampling began 9 min after the application of venom, there was a further reduction in m.e.p.p. amplitude with an apparent reduction in m.e.p.p. frequency. It is considered that the apparent reduction of m.e.p.p. frequency is a reflection of the reduction of some m.e.p.p. amplitudes to within the noise of the recording system. It is concluded that *E. schistosa* venom reduces m.e.p.p. amplitude and is likely to have little or no effect on the frequency of release of quanta of transmitter.

Effect of E. schistosa *venom on depolarizing effect of carbachol*

Experiments were done on 11 preparations in which the effect of venom on the carbachol-induced depolarization of muscle cells was investigated. In control preparations, membrane potentials of about 25–30 cells per diaphragm were sampled. The preparations were exposed to carbachol for 30 min and the membrane potentials were sampled again. The effects of 1 μM to 1 mM concentrations of carbachol were measured.

In the treated preparations the membrane potentials of 30 cells per diaphragm were sampled before and after 2.5 μg/ml venom, there being no significant change, as mentioned previously. *E. schistosa* toxin at 2.5 μg/ml was used to give a rapid large and irreversible effect (Geh, 1968). The toxin-treated preparations were then exposed to carbachol for 30 min before sampling of the membrane potentials was carried out.

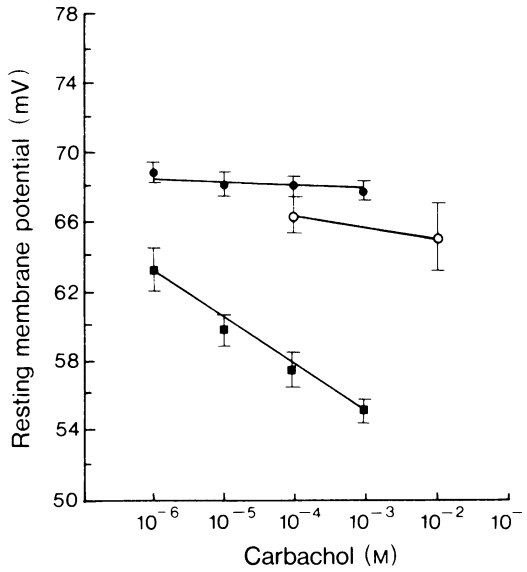

Figure 3 Membrane potential at the endplate region of rat diaphragm (37°C). (●) Control values of membrane potential measured throughout the experiment and related to membrane potential in presence of carbachol. (■) Membrane potential recorded for each concentration of carbachol in bathing fluid. (○) Membrane potential recorded for each concentration of carbachol added to bathing fluid already containing *Enhydrina schistosa* venom 2.5 μg/ml. Each point represents mean of membrane potentials sampled from 150–180 cells from a total of 5–6 diaphragms. Vertical lines show s.e. mean.

The results of these experiments are summarized in Figure 3. The application of *E. schistosa* toxin resulted in a smaller effect of carbachol, the dose-ratio being greater than 10^4. It is concluded that *E. schistosa* toxin reduces the effect of carbachol on the endplate.

Discussion

Effective blocking doses (Geh, 1968) of crude *Enhydrina schistosa* venom abolished the e.e.p.p. progressively without having much effect on its presynaptic spike potential (Figure 1). Since this spike reflects activity in the motor nerve terminal (Eccles, Katz & Kuffler, 1941; Hubbard & Schmidt, 1963; Katz & Miledi, 1965a), its integrity suggests that the terminal depolarization resulting in activated sodium conductance is probably not affected by the venom. However, the depolarization-secretion coupling that is responsible for the release of the transmitter (Katz & Miledi, 1965b) may or may not have been changed. A

block of postjunctional receptors may be responsible for the progressive disappearance of the e.e.p.p. The tetanic rundown experiments do not suggest an effect of *E. schistosa* venom on transmitter release and therefore tend to support the hypothesis of a postjunctional action.

The action of the venom on m.e.p.ps lends more support to its postjunctional action on the receptors. Here the amplitude of the m.e.p.ps progressively declined but their frequency showed little change even when there was already a significant fall in potential. Since frequency change can reflect only presynaptic events (del Castillo & Katz, 1954; Liley, 1956b), the absence of changes indicates that the venom probably does not have presynaptic action on transmitter release. The fall in frequency that occurred late in the experiment may be due to the loss of potentials below the noise level as the potential amplitude fell. The fall in m.e.p.p. amplitude itself may reflect both pre- and postsynaptic events; however, more likely, postsynaptic ones (Hubbard, Llinas & Quastel, 1969). In our experiments rapid decline of potential amplitude with time (Figure 2a) indicates an action of the venom blocking the postjunctional receptors, thereby preventing acetylcholine action, rather than a decrease in the quantal content of acetylcholine (Elmqvist & Quastel, 1965), for if the venom interfered with the synthesis of acetylcholine resulting in depletion of acetylcholine stores, one would expect the change in amplitude to occur only after prolonged stimulation.

The shift to the right of the dose-response curve with carbachol (Figure 3) supports the possibility of a postsynaptic action. The progressive and irreversible nature of *E. schistosa* venom block was demonstrated in our present experiments and has often been shown previously (Carey & Wright, 1961; Cheymol *et al.*, 1967; Geh, 1968; Geh & Chan, 1973).

From electrophysiological studies, it appears therefore that effective paralysing doses of crude *E. schistosa* venom probably exert an effect by combining irreversibly with the postjunctional receptors without altering significantly the resting membrane potential of the muscle cells. Nevertheless, an effect of the crude venom on the motor nerve terminal, whether prior to or together with, its action on the postjunctional membrane cannot be ruled out entirely. Geh & Chan (1973) have suggested that in the cat, relatively small non-paralysing doses of the venom could possibly have an initial prejunctional effect whereas effective blocking doses have been thought to combine irreversibly with postjunctional receptors. Moreover Toh *et al.* (1975) have shown that after chronic treatment in guinea-pig, sublethal doses of crude *E. schistosa* venom caused destruction of mitochondria in the motor nerve terminal. However, no gross evacuation of synaptic vesicles or changes in the axonal membrane were seen. Since all these studies have been carried out with the crude venom it is not surprising that manifestations of various effects have been indicated depending on dose, experimental animals and techniques. Pre- and postjunctional sites of action have been attributed to other crude snake venoms as well (Chang & Lee, 1963; Datyner & Gage, 1973a,b).

It is not possible at this stage to identify which component(s) or combination of active constituents in the crude *E. schistosa* venom that can be responsible for the effects seen now and in previous investigations. The only solution is to study the mode of action of purified active fractions; at present, experiments are being carried out towards this end.

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