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Presynaptic and postsynaptic sites of action of enhydrotoxin–a (EsNTx–a) isolated from *Enhydrina schistosa* venom

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Enhydrotoxin–a (EsNTx–a), one of the three toxins isolated from the venom of *Enhydrina schistosa* produced an irreversible blockade of indirect stimulation of rat phrenic nerve hemidiaphragm preparation in concentration of 0·5 to 1 μg ml^{-1}. The response to direct stimulation was not affected over a 4 h period. The neuromuscular blockade produced by EsNTx–a was antagonized in so far as twitches evoked by indirect stimulation are concerned, when the preparation was treated with approximately double the concentration of purified sea snake antivenin. The tension of tetanic contractions, although depressed in amplitude by the toxin, was sustained. However, it was converted to a twitch-like response in toxin–antivenin treated preparations. In the presence of eserine (1×10^{-6} g ml^{-1}), the twitch-like tetanus was dramatically restored in toxin–antivenin treated rat phrenic nerve hemidiaphragm preparations. Intracellular recordings showed that the frequency of spontaneous miniature end plate potentials (m.e.p.ps) recorded in EsNTx–a poisoned ($1{\cdot}24 \times 10^{-6}$ g ml) rat phrenic nerve hemidiaphragm preparations decreased to zero, while the m.e.p.p. amplitude was decreased in the later phase of block. The evoked end plate potentials (e.p.p.) in EsNTx–a poisoned ($1{\cdot}24 \times 10^{-6}$ g ml^{-1}) preparations progressively diminished to zero. The antivenin molecules may have an easy access to bind to the toxin receptor complexes at the postsynaptic site while the presynaptic binding site by the toxin may be relatively inaccessible due to their being intracellular. The presynaptic effect of the toxin was revealed only when the postsynaptic effect was abolished.

Short chain neurotoxins isolated from elapid and hydrophid venoms have strong affinity towards the postsynaptic cholinoceptive sites in mammalian skeletal neuromuscular junctions. Major toxins isolated from *Enhydrina schistosa* have been shown to have a postsynaptic site of action (Carey & Wright 1961; Tu & Toom 1971; Karlsson et al 1972; Gawade & Bhide 1978). Isolation and characterization of three neurotoxic components from the venom of *Enhydrina schistosa* collected from the coast of Bombay were described by Gawade & Gaitonde (1982). Enhydrotoxin–a (EsNTx–a), the principal lethal toxin, has an LD50 of 0·042 mg kg^{-1} in mice. It has a molecular weight 6885 and the molecule consists of 61 amino acid residues and 4 disulphide bridges. The amino acid composition of EsNTx–a is similar to that of other *Enhydrina* toxins reported earlier (Tu & Toom 1971; Karlsson et al 1972; Yu et al 1975), except for some differences in substitutions and additions in glutamic acid, proline and glycine residues. No attempt has been made to study in depth the mechanism of action of these toxins or their site of attachments to the receptors at the myoneural junction. In the present paper the application of toxin antibodies to help elucidate the mode of action of toxin is described.

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METHODS

Lyophilized *E. schistosa* venom was obtained by milking sea snakes in the laboratory with specially prepared glass tubes (Gawade et al 1981). The toxin EsNTx–a was isolated by a three-step purification procedure using Sephadex-G-75, CM-Cellulose and CM-Sephadex-C-25 chromatography (Gawade & Gaitonde 1982). Homogeneity of the toxin was examined by polyacrylamide gel electrophoresis, immunoelectrophoresis and gel diffusion.

Contractile responses

Experiments were carried out in vitro using albino rats 175–225 g, of either sex. The phrenic nerve–hemidiaphragm preparation (Bulbring 1946) was isolated and mounted in an organ bath containing 50 ml Krebs-Henseleit solution maintained at 37 °C and aerated with a mixture of oxygen (95%) and carbon

dioxide (5%). The nerve was stimulated at a frequency of 0·2 Hz by rectangular square wave pulses of supramaximal intensity and of 0·5 ms duraction. For direct stimulation, supramaximal square wave pulses of 1 ms duration were applied through a pair of electrodes placed directly on the muscle. Muscle contractions were recorded on a kymograph by means of a lightly loaded lever.

Intracellular recordings

The left phrenic nerve hemidiaphragm preparation (Bülbring 1946) was used for intracellular recordings of end plate potentials. The muscle was pinned flat in a transparent sylgard dish. The nerve and muscle were freed from pericardial membrane and fatty tissue by microdissection, so that the point of entry of the microelectrode into the muscle was close to the selected myoneural junction. A silver wire hook acted as a reference electrode. Capillary microelectrodes of external tip diameter less than 0·5 μm filled with 3M KCl were used. The microelectrode was attached to a micromanipulator, and was placed near the end of the myelinated axon on the outside of the muscle fibre. The depth of the fluid in the bath above the preparation was about 3 mm and the bath fluid was grounded. The entire assembly was placed in a steel cage which was grounded to minimize electric noise. Recordings were carried out at room temperature (20 ° – 22 °C) using a WPI amplifier and a Tektronix oscilloscope. The magnesium concentration was increased to reduce the muscle twitches. In all experiments, solutions containing (in mM) NaCl 109, KCl 1·9, $CaCl_2$ 0·8, $MgCl_2$ 5·5 were used. The frequency and amplitude of miniature end plate potentials were recorded before and after exposing the preparation to the toxin. Evoked end plate potentials were recorded before and after exposing the preparation to the toxin action. All the values are expressed as mean ± s.e.

Monovalent E. schistosa *antivenin*

Antivenin against *E. schistosa* venom was prepared by hyperimmunizing a horse with adjuvant and purifying its serum by peptic digestion and ammonium sulphate precipitation (Gawade et al 1980). One ml of reconstituted purified sea snake antivenin neutralized 450 μg of *E. schistosa* venom, which corresponds to 304 LD50 in Kasauli mice. All the toxin–toxin antibody treatment experiments were carried out in a critical concentration of toxin ($1{\cdot}24 \times 10^{-6}$ g ml^{-1}) and antivenin (3×10^{-6} g ml^{-1}). The number of experiments (n) is stated in the results.

RESULTS

Effect of repetitive stimulation of EsNTx–a-paralysed phrenic nerve-diaphragm preparation in vitro *(n = 8)*

Repetitive stimulation (tetanus) was induced by indirect stimulation at a frequency of 50 Hz for 5 s. In the presence of EsNTx–a, a gradual reduction in the amplitude of tetanic responses occurred together with a similar diminution of the twitch response (Fig. 1). Even at the end of 40 min, although the amplitude was diminished to 80%, tetanus was well maintained. In the final stages of the block, tetanic concentrations were abolished. Post-tetanic potentiation (PTP) was evident until the contractions were abolished. When indirect stimulation no longer elicited a response, the response to direct muscle stimulation was unimpaired.

Antagonism with anticholinesterase (n = 5)

When eserine in a concentration of 2×10^{-6} g ml^{-1} was added during the neuromuscular block produced by EsNTx–a, only slight and transient recovery of the

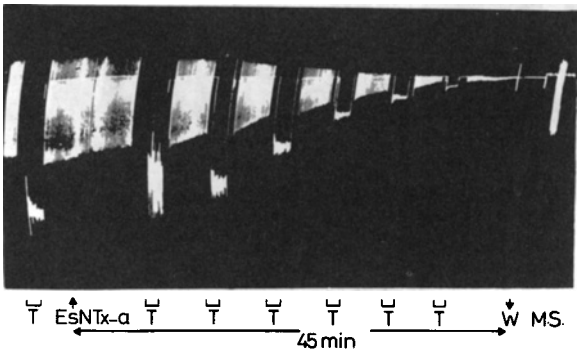

FIG. 1. Effect of repetitive stimulation on EsNTx–a paralysed phrenic nerve diaphragm preparation of the rat. Indirect stimulation (0·1 Hz; 0·5 ms). At arrow Enhydrotoxin–a (EsNTx–a) ($1{\cdot}24 \times 10^{-6}$ g ml^{-1}). At T repetitive stimulation 50 Hz per 5 s and M.S. direct muscle stimulation.

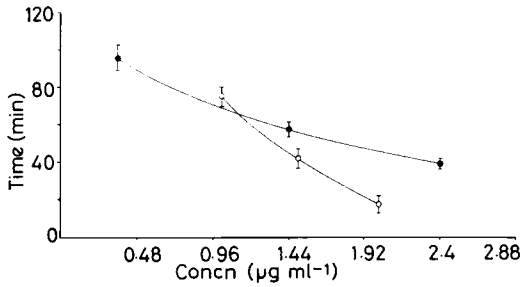

FIG. 2. Average time required for complete neuromuscular blockade of the rat phrenic nerve diaphragm preparation at various concentrations of tubocurarine (○——○) and Enhydrotoxin-a (●——●). Vertical bars indicate s.e. of 4–5 experiments.

contractions was obtained, and the block persisted after the transient recovery.

Comparison of neuromuscular blocking activity of EsNTx-a and tubocurarine (n = 8)

The neuromuscular blocking activities of EsNTx-a and tubocurarine were compared by noting the time required for complete neuromuscular blockade at various concentrations. As the concentration of tubocurarine was increased, there was a marked reduction in the time required for 100% neuromuscular blockade; the onset of action also became more rapid. On the other hand, there was a slow onset of action after addition of EsNTx-a. At a dose of $4{\cdot}8 \times 10^{-7}$ g ml^{-1} of toxin, the time required for 100% neuromuscular blockade was as long as $95{\cdot}8 \pm 6{\cdot}8$ min. With increasing concentration ($1{\cdot}49 \times 10^{-6}$ g ml^{-1}), however, the time was shortened significantly ($57{\cdot}3 \pm 3{\cdot}8$ min). Fig. 2 shows the relationship between the concentrations of EsNTx-a and tubocurarine, and the time (min) required for total neuromuscular block in the phrenic nerve hemidiaphragm preparation.

Effect on tetanus after toxin–antivenin treatment (n = 6)

Tetani were evoked by stimulation at 20 Hz for 5 s, and EsNTx-a, in a concentration of $1{\cdot}24 \times 10^{-6}$ g ml^{-1}, was added to the bath to abolish the responses. It took an average of $45 \pm 4{\cdot}8$ min for complete blockade. The preparation was then washed thoroughly with three successive washings and incubated with the sea snake antivenin for 45 min in approximately double the concentration of that of toxin. Antivenin was washed off with three consecutive washings and the preparation was indirectly stimulated to evoke twitches. Then at regular intervals, the muscle was stimulated indirectly with high frequency stimulation, i.e. 20 Hz for 5 s. The tetanus after such toxin–antivenin treatment was not maintained, being reduced to resemble a single twitch. However, the muscle responded normally to direct stimulation (Fig. 3).

Effect of monovalent E. schistosa *antivenin on phrenic nerve diaphragm preparation (n = 4)*

The effect of sea snake antivenin in a concentration $4{\cdot}5 \times 10^{-6}$ g ml^{-1} was studied. Tetanus was induced before the application of antivenin with stimulation at 20 Hz for 5 s and thereafter the preparation was exposed to antivenin. Antivenin treatment was without effect on the single twitch response to indirect stimulation or on tetanic contractions.

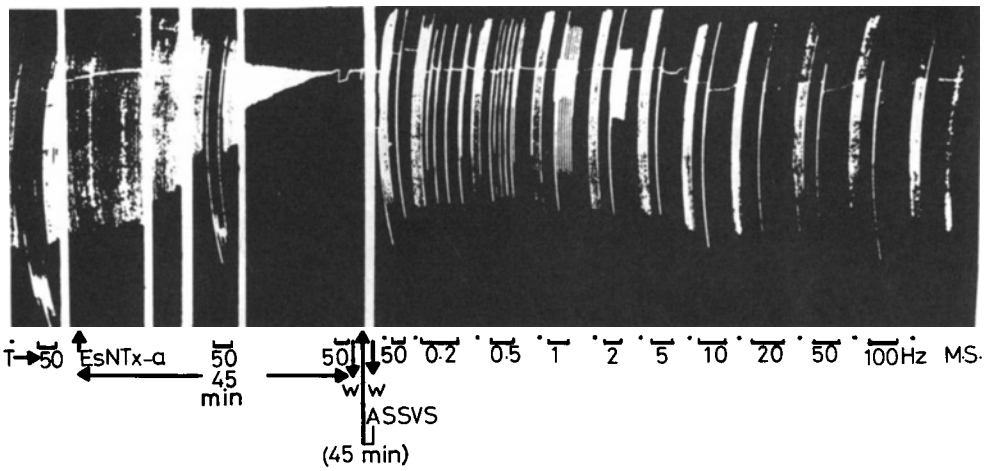

FIG. 3. Effect on tetanus at increasing frequencies after toxin–antivenin treatment in phrenic nerve diaphragm preparation of the rat. At dots indirect stimulation (0·1 Hz −0·5 ms). T repetitive stimulation for 5 s. At arrow EsNTx-a ($1{\cdot}24 \times 10^{-6}$ g ml^{-1}) and sea snake antivenin (ASSVS) 3×10^{-6} g ml^{-1}. M.S. direct muscle stimulation.

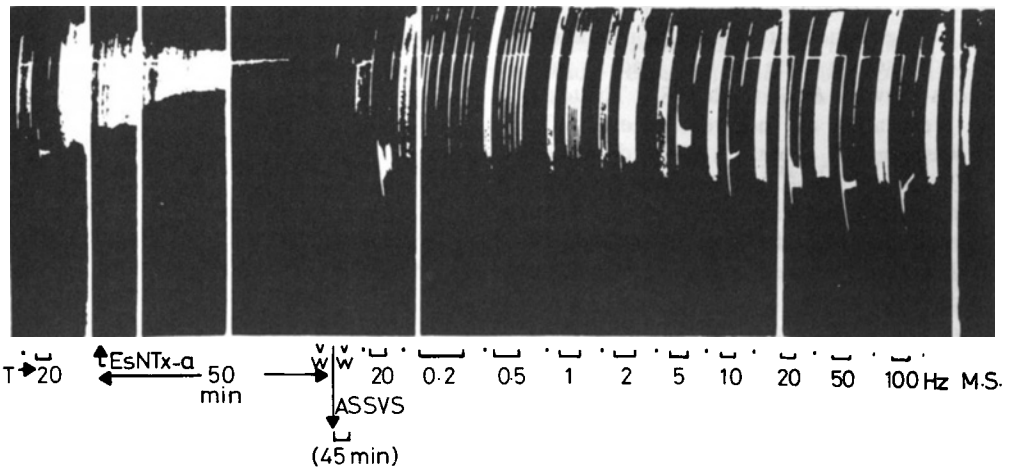

FIG. 4. Modification of tetanus by eserine after toxin–antivenin treatment in phrenic nerve diaphragm preparation of the rat. At dots indirect stimulation (0·1 Hz −0·5 ms). At arrow EsNTx-a ($1{\cdot}2 \times 10^{-6}$ g ml^{-1}) and sea snake antivenin (ASSVS) (3×10^{-6} g ml^{-1}). T repetitive stimulation (0·2 Hz to 100 Hz) for 5 s and M.S. direct muscle stimulation. Pre-treatment with eserine (1×10^{-6} g ml^{-1}).

Direct muscle stimulation was also not affected even after contact with the antivenin for up to 6 h.

Effect of eserine on toxin-antivenin treated preparation (n = 6)

The preparation was eserinized (1×10^{-6} g ml^{-1}) and incubated with toxin and thereafter with antivenin as described earlier. In such an eserinized preparation, the tetanus was well maintained indicating that eserinization restored the normal tetanic response of the nerve muscle preparation (Fig. 4).

Effect of EsNTx–a on spontaneous miniature end plate potentials (m.e.p.ps) (n = 8)

In a bath solution containing high Mg^{2+} (5·5 mM) and low Ca^{2+} (0·8 mM) the frequency of spontaneous m.c.p.ps was decreased to zero in 40 to 70 min of exposure with EsNTx–a $1{\cdot}24 \times 10^{-6}$ g ml^{-1}) while m.e.p.p. size was decreased in the later phase of block. The results of one such observation are shown in Fig. 5. In two experiments, resting membrane potential was decreased by 5 mV (RP = mean 65 s.d. 5 mV).

Effect of EsNTx–a on evoked end plate response (n =4)

EsNTx–a ($1{\cdot}24 \times 10^{-6}$ g ml^{-1}) produced a complete block of neuromuscular transmission in 25 to 40 min i.e., complete elimination of postsynaptically recorded end plate potentials. The block was irreversible even after repeated washings for about 3 h (Fig. 6).

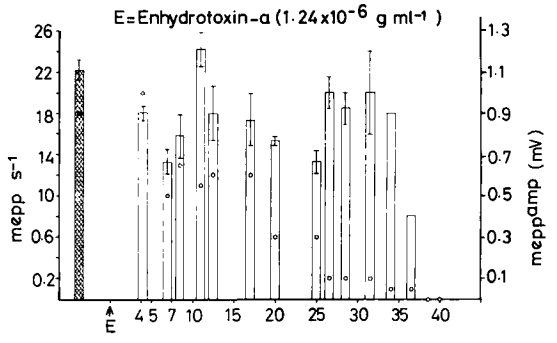

FIG. 5. Effect of Enhydrotoxin–a on frequency and amplitude of spontaneous m.e.p.ps in phrenic nerve diaphragm preparation of rat. Control frequency and amplitude are represented by (●) and crossed hatched column and by (○) and open columns after toxin application respectively.

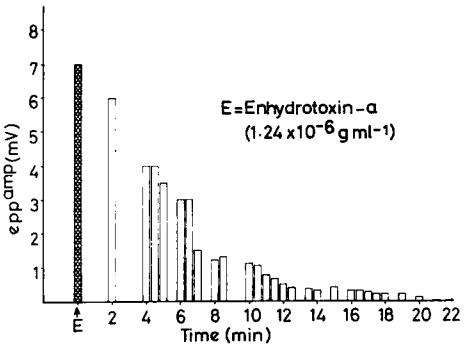

FIG. 6. Effect of Enhydrotoxin–a on the evoked and plate response in phrenic nerve diaphragm preparation of rat. Control amplitude is represented by cross hatched column and by open columns after toxin application.

DISCUSSION

The toxin has been shown to abolish acetylcholine-induced responses in both in vivo and in vitro preparations (Gawade & Gaitonde 1982). The toxin-poisoned preparation did not recover even after repeated washings for about 4 h (Gawade & Gaitonde 1982). This indicates that the toxin molecules have a high affinity for the cholinoceptive postsynaptic receptors. This powerful receptor binding resulted in almost irreversible blockade of ACh action on the postsynaptic site.

Although eserine failed to counteract the action of the toxin, antivenin brought about some reversal of block. Thus, after 45 min of incubation with antivenin, the responses to single shock indirect stimulation recovered completely. This is a specific action and is seen only when specific monovalent antivenin is used. Such a type of reversal has also been shown in the case of a block produced by *N. naja* venom when a specific monovalent antivenin is used (Chang 1960; Gaitonde & Joshi, unpublished data). Antivenin has thus a greater affinity for the toxin molecules.

Comparative studies of neuromuscular block produced by toxin and by tubocurarine revealed that there was always a delay in the onset of the toxin block. Although the latency was shortened with higher concentrations of toxin, it was never as short as with tubocurarine. The rapidity of action of the latter drug is suggestive of quick receptor saturation and is evident also by a steep dose response curve. In comparison, the dose response curve for the toxin was relatively flat. Once the toxin molecules become attached to the receptor, the binding becomes irreversible and can only be reversed by treatment with antivenin.

Our studies with tetanic stimulation tetanus revealed some interesting phenomena of toxin binding sites at the neuromuscular junction. Tetanus (20 Hz for 5 s) in a normal preparation can be shown for over a period of 3 h. Tetanus, although depressed in an amplitude, was well maintained in toxin-treated preparations until the terminal phases, when the indirect stimulation was almost blocked by toxin at $2{\cdot}8 \times 10^{-7}$ g ml^{-1}. In contrast, tubocurarine showed a well defined Wedensky inhibition. The effect of the toxin was evidently a failure of neuromuscular transmission, since the muscle responded to direct stimulation.

The sequence of events with respect to induced tetanus, in a toxin–antivenin treated preparation was as follows:

(i) Twitch responses and tetanus were blocked by toxin in the concentration ($1{\cdot}24 \times 10^{-6}$ g ml^{-1}) by the end of 50 min.

(ii) Antivenin treatment followed by a wash, restored the twitch response (stimulation (0·1 Hz, 2 ms) but not the tetanus (stimulus 20 Hz, 5 s).

(iii) At high frequency (10, 30, 40 Hz) instead of tetanus, the preparation responded with single high amplitude twitch response. Thus, high frequency repetitive stimulation failed to produce tetanus. The question arises, could this be evidence of a presynaptic action?

β-bungarotoxin, a presynaptically acting neurotoxin, has been shown to convert a tetanus into a twitch response (Chang & Lee 1963). This toxin may be reducing the quanta of ACh released by high frequency stimulation by a presynaptic action.

Addition of eserine after treatment with EsNTx–a and antivenin dramatically restored the sustained form of the tetanus. Eserine, by preventing its hydrolysis, builds up the concentration of ACh at the postsynaptic receptors sufficiently to enable the tetanus to be sustained.

Antivenin treament only partially restores the transmission as shown by a twitch response after tetanic stimulation. Antivenin molecules may have an easy access to the toxin receptor complexes at the postsynaptic sites but the presynaptic binding sites may be relatively inaccessible to antivenin. After antivenin treatment therefore, only the postsynaptic effect of the toxin is lost but presynaptic inhibition is still preserved.

Our data on intracellular recordings revealed that frequency of spontaneous m.e.p.ps was decreased to zero while the m.e.p.p. size was also affected in the later phase of block. Since a frequency change can reflect only presynaptic events (Del Castillo & Katz 1954; Liley 1956b), a decrease in spontaneous m.e.p.p. frequency further supports our contention of a presynaptic site of action of EsNTx–a, and this observation is in support of the 'twitch replacing tetanus' phenomenon seen in the preparation after treatment with antivenin. The progressive disappearance of evoked end plate potentials suggests a block either of presynaptic or postsynaptic sites. Both e.p.ps and m.e.p.ps were not restored inspite of repeated washings.

Snake neurotoxins of short chain having a molecular weight of about 7000 have been shown to produce neuromuscular paralysis by acting on the

postsynaptic membrane. Prejunctional and postjunctional sites of action have been attributed to the crude venom (Chang & Lee 1963; Datyner & Gage 1973a,b). This is the first time that we have been able to show that a toxin from *E. schistosa* venom having mol. weight 6900 possesses an action at both presynaptic and postsynaptic sites, although Schwab & Puffer (1978) did suggest such a possibility in the case of erabutoxin–a from the sea krait *Laticauda semifasciata*. The toxin with a molecular weight 6900, and having a presynaptic effect, is certainly different from the usual classes of snake neurotoxins, which are postsynaptic curarimimetic and phospholipase neurotoxins (Chang 1980, personal communication).

The snake neurotoxins may, in fact, fall into three broad categories: 1, postsynaptic neurotoxins; 2, presynaptic neurotoxins and 3, presynaptic and postsynaptic neurotoxins. The presynaptic site of predominantly postsynaptically acting neurotoxin has become evident because of the use of the specific antivenin. We, therefore, recommend the use of this type of 'model' for the elucidation of sites of action of venom-toxins at the myoneural junction.

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