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EFFECTS OF THE VENOM OF THE GREEN MAMBA, *Dendroaspis angusticeps* ON SKELETAL MUSCLE AND NEUROMUSCULAR TRANSMISSION

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1 The venom of the green mamba, *Dendroaspis angusticeps*, was tested for effects on neuromuscular transmission and skeletal muscle contractility in isolated phrenic nerve-hemidiaphragm preparations of the rat and mouse, chick biventer cervicis muscle preparations and in aneural cultures of embryonic chick skeletal muscle.

2 The venom (10 to 40 μg/ml) augmented the responses to indirect but not direct stimulation. As the venom did not have anticholinesterase activity and did not increase receptor sensitivity, it is likely that the venom enhanced release of acetylcholine.

3 Higher concentrations of venom (40 to 80 μg/ml) inhibited acetylcholine receptor sensitivity.

4 Prolonged exposure to the higher concentrations of venom produced a failure of muscle contractility. Signs of muscle degeneration were seen in skeletal muscle cultures.

Introduction

Investigation of venoms from elapid and hydrophid snakes has led to the discovery of several polypeptides that have specific pharmacological actions and are useful tools for study of neuromuscular processes. The toxins include α- and β-bungarotoxin from the Taiwan krait *Bungarus multicinctus*, cobratoxins from various species of *Naja*, and erabutoxins from the seasnake *Laticauda semifasciata* (for reviews, see Lee, 1972; Tu, 1977). Among the elapid family, cobra and krait venoms have been studied extensively but relatively little attention has been given to the pharmacological properties of venoms from the African mambas. Previously, neurotoxins have been found in the venoms of the western green mamba, *Dendroaspis viridis* (Banks, Miledi & Shipolini, 1974) and of the black mamba, *Dendroaspis polylepis* (Strydom, 1972). In the present study the actions of venom from the green mamba, *Dendroaspis angusticeps*, were examined on skeletal muscle and neuromuscular transmission. Some of these results were presented to the 3rd Symposium of the International Society on Toxinology European Section, London, September, 1978.

Methods

Rat phrenic nerve-hemidiaphragm preparation

The rat phrenic nerve-hemidiaphragm preparation (Bülbring, 1946) was mounted in Krebs-Henseleit solution of the following composition (mM); NaCl 118.4, KCl 4.7, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11.1; at 37°C and bubbled with O_2 containing 5% CO_2. The phrenic nerve was stimulated at a frequency of 0.1 Hz with rectangular pulses of 0.2 ms duration and of strength greater than that required to elicit maximal twitches of the hemidiaphragm. For direct muscle stimulation, neuromuscular transmission was abolished by the irreversible cholinoceptor blocking agent, erabutoxin b (5 μg/ml) (Tamiya & Arai, 1966). The hemidiaphragm was then stimulated directly through a hook electrode inserted into the rib tissue, with rectangular pulses of 2 ms duration at a frequency of 0.1 Hz and supramaximal strength. Paired preparations were set up from each rat, one hemidiaphragm for direct and the other for indirect stimulation.

Chick biventer cervicis nerve-muscle preparation

The chick biventer cervicis nerve-muscle preparation (Ginsborg & Warriner, 1960) was mounted and stimulated under identical conditions to those described for the rat hemidiaphragm. Indirect stimulation was via the motor nerve in the tendon while for direct stimulation of preparations irreversibly blocked with erabutoxin b (1 μg/ml), the stimulating electrode was moved into contact with the belly of the muscle. Periodically, in the absence of nerve stimulation, responses were obtained to acetylcholine or carbachol.

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Anticholinesterase assay

The anticholinesterase activity of the venom was assessed by measurement of the cholinesterase activity of intact chick biventer cervicis muscles in the presence of the venom, by the colorimetric method of Ellman, Courtney, Andres & Featherstone (1961). Two biventer cervicis muscles from 20 day old chicks were used for each assay. The reaction flask contained 5.8 ml of 0.1 M phosphate buffer (pH 8.0) and was maintained at 32°C. The reaction was begun by addition of 0.2 ml acetylthiocholine iodide (0.075 M, freshly made up). At 15 min intervals for 1 h, 0.2 ml was removed and added to a cuvette containing 2.9 ml 0.1 M phosphate buffer (pH 8.0) and 0.1 ml 5,5-dithiobis-2-nitrobenzoic acid (0.01 M). A blank determination was performed on a flask containing tissue and venom (80 μg/ml) but no substrate. Cholinesterase activity of the venom itself was assessed in four flasks containing all the reagents but no tissue. The absorbance change at 412 nm, per min, in four control preparations was compared to that in the presence of the venom at two concentrations (20 and 80 μg/ml; four preparations each). All flasks were incubated for 30 min before addition of acetylthiocholine.

Isolated tissue electrophysiology

Mouse phrenic nerve-hemidiaphragm preparations were pinned to the base of a 10 ml bath. The bath was perfused at a rate of 2.5 to 5 ml/min with Krebs-Henseleit solution previously aerated with O_2 containing 5% CO_2. The muscle was maintained at a temperature of 28 to 30°C. Endplates were located by inspection under 400× magnification using a standard binocular microscope fitted with a Leitz UM 20/0.33 long-working-distance objective. Intracellular recordings of membrane potential and endplate potentials (e.p.ps) were made with conventional glass microelectrodes filled with 2 M potassium acetate (8 to 10 megohm resistance). Potentials were amplified with a WPI 701 electrometer (band width 0 to 30 kHz), monitored on one oscilloscope and also recorded on 35 mm film from a slave oscilloscope. Neuromuscular transmission was depressed by equilibration with physiological salt solution to which (+)-tubocurarine (3 to 5 μM) was added. To determine the action of the venom on the e.p.p., the nerve was stimulated at a frequency of 0.5 Hz with rectangular pulses of 0.2 ms duration and supramaximal voltage.

Effects of the venom were investigated by perfusion of the preparation with Krebs-Henseleit solution to which venom (2 to 20 μg/ml) had previously been added. This avoided disturbance of the bath, so that potentials could be measured from the same cell before and during exposure to the venom.

Cell cultures of embryonic chick skeletal muscle

Suspensions of myoblasts were obtained from the leg muscles of 10 to 11 day chick embryos by the method of Konigsberg, McElvain, Tootle & Herman (1960). Two ml of medium containing 5×10^5 cells/ml was added to a 35 mm plastic Petri dish that had previously been coated with collagen. The growth medium was Eagle's Minimum Essential Medium (M.E.M.; Eagle, 1959) containing 5% of a 50% v/v whole embryo extract and 15% horse serum. In order to prevent overgrowth of replicating fibroblast cells, the medium was replaced on the second day of culture, after the beginning of myoblast fusion, by one containing 10 μM cytosine arabinoside, a DNA synthesis inhibitor. Thereafter, the medium was replaced on every third day with fresh medium. Cultures were incubated at 37°C in a water-saturated atmosphere of 5% CO_2 in air. Myotube formation occurred at 2 to 3 days, and cultures were 7 to 10 days old when used (Harvey & Dryden, 1974).

Cultures were mounted on an inverted phase-contrast microscope and maintained at 37°C on a heated stage. Membrane potentials were recorded with an intracellular glass microelectrode filled with 3 M potassium chloride (electrode resistance 10 to 20 megohms) and mounted obliquely on a Leitz micromanipulator. Potential differences between the penetrating microelectrode and a chlorided silver wire bath electrode were monitored via a d.c.-preamplifier on an oscilloscope and on a pen recorder. Responses were obtained to iontophoretic application of acetylcholine. Microelectrodes for iontophoresis were filled with 0.5 M acetylcholine chloride and connected to a stimulator through one channel of a WPI 750 electrometer. The average resistance of the acetylcholine electrode was 100 megohms. Care was taken to select electrodes that did not leak drug spontaneously in the absence of a backing current. The dose of acetylcholine is expressed as nanoCoulombs (nC) of charge passed through the electrode. Responses, averaged from at least 10 cells, were calculated as depolarization as a percentage of the resting potential. All recordings were made in Eagle's M.E.M. which was maintained at pH 7.4 by bubbling with CO_2.

When control measurements of membrane potential and dose-response curves to acetylcholine had been obtained, the culture medium was replaced with fresh medium containing the crude venom at different concentrations. In some experiments, measurements were made immediately following exposure of the culture to venom; in other experiments the culture was returned to the incubator before membrane potentials and dose-response curves were recorded. In those experiments where cultures were incubated with venom overnight, the venom-containing medium was sterilized by membrane filtration.

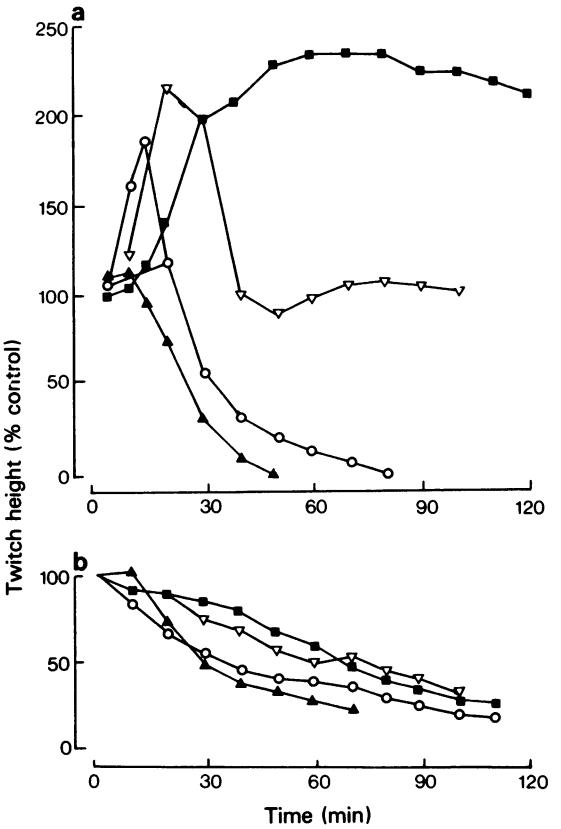

Figure 1 Effect of *Dendroaspis angusticeps* venom on the rat phrenic nerve-hemidiaphragm preparation. (a) Indirectly stimulated preparations; (b) directly stimulated preparations. (■) Venom 10 μg/ml; (▽) 20 μg/ml; (○) 40 μg/ml; (▲) 80 μg/ml.

Drugs

Dendroaspis angusticeps venom was obtained from Sigma Chemical Co. (Lot No. 102C-0730). Other drugs used were: acetylcholine chloride, acetylthiocholine iodide, carbachol chloride, cytosine arabinoside and (+)-tubocurarine chloride (all from Sigma Chemical Co.). Tissue culture media were purchased from Flow Laboratories, and erabutoxin b was generously supplied by Professor N. Tamiya, Tohoku University, Japan.

Results

Rat phrenic nerve-hemidiaphragm

At concentrations of 10 to 40 μg/ml of venom there was an immediate increase in the height of responses to indirect stimulation (Figure 1). With 10 μg/ml the twitch height increased to almost 250% of control, stabilizing at this augmented level after about 60 min. With 20 and 40 μg/ml the responses to indirect stimulation decreased after the initial augmentation. At a higher concentration of venom (80 μg/ml) there was a short-lived increase in the height of indirectly elicited contractions that was followed by progressive muscle paralysis (Figure 1).

In contrast, there was never any increase in twitch height in preparations that were stimulated directly. Concentrations of venom between 10 to 80 μg/ml caused a slow, progressive decrease in muscle contractions (Figure 1). The development of blockade was faster at the higher venom concentrations, although it was slower than that observed in indirectly stimulated preparations exposed to 40 or 80 μg/ml venom. The decline in twitch height could not be reversed by washing.

A second addition of venom to indirectly stimulated preparations during the augmentation produced by 10 μg/ml venom did not cause a further increase in twitch height, but rather hastened the onset of block.

Chick biventer cervicis nerve-muscle preparation

Qualitatively similar results were obtained on the chick biventer cervicis nerve-muscle preparation. The venom (10 to 40 μg/ml) markedly augmented the responses of indirectly but not directly stimulated preparations. At higher concentrations of venom (40 to 160 μg/ml) the augmentation was followed by a slowly developing, irreversible blockade of the twitch response. A similar blockade was observed on directly stimulated preparations but with a much longer time course. In some preparations a transient contracture occurred on addition of venom.

During the development of the augmentation of responses to indirect stimulation, there was no marked increase in the responses to acetylcholine and carbachol (see Figure 2 for a typical experiment with acetylcholine contractures and 20 μg/ml venom). When tested at the time of maximum augmentation of indirectly elicited contractions, responses to both acetylcholine and carbachol were depressed (Figure 3).

Cholinesterase activity

Venom (80 μg/ml) had no ability to hydrolyse acetylthiocholine within a 60 min incubation at 32°C, indicating that the venom itself did not have significant cholinesterase activity.

With intact chick biventer cervicis muscles, control cholinesterase activity per muscle was 0.66 ($\pm$0.04) $\times$ 10^{-3} absorbance units/min (mean $\pm$ s.e. mean of 4 determinations), while in the presence of 20 and 80

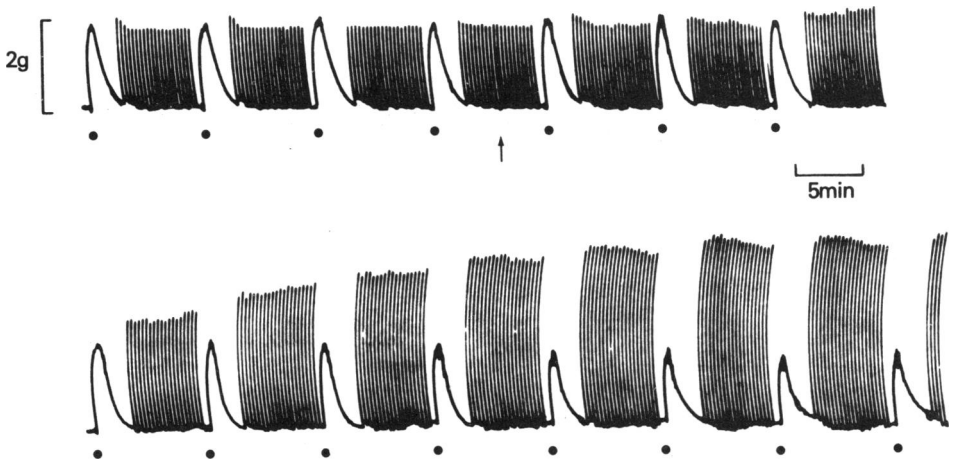

Figure 2 Effect of *Dendroaspis angusticeps* venom (20 μg/ml) on responses of the chick biventer cervicis nerve-muscle preparation to indirect stimulation (0.1 Hz) and acetylcholine (2 mM). Acetylcholine was added at the dots and washed out after 30 s. Venom was added at the arrow, and again after each acetylcholine washout.

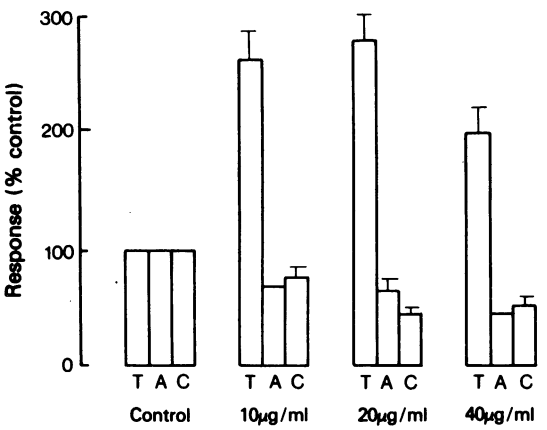

Figure 3 Effects of *Dendroaspis angusticeps* venom on responses of the chick biventer cervicis preparation to indirect stimulation (T), acetylcholine (A, 2mM) and carbachol (C, 20 μM). Responses to acetylcholine and carbachol were determined at peak twitch augmentation. Each column represents the mean of at least 6 preparations; vertical lines show s.e. mean.

μg/ml venom the activity per muscle was 0.55 ($\pm$0.05) $\times$ 10^{-3} and 0.80 ($\pm$0.06) $\times$ 10^{-3} absorbance units/min, respectively.

Effect on endplate potentials

Mouse phrenic nerve-hemidiaphragm preparations were partially paralysed by perfusion with physiological saline containing (+)-tubocurarine (3 to 5 μM). After the preparation stopped contracting in response to indirect stimulation, subthreshold endplate potentials could be recorded.

Subsequent exposure to venom (at 2 and 10 μg/ml) caused such a dramatic increase in the amplitude of the endplate potentials that the preparations began to twitch in response to stimulation, making intracellular recording impossible. Typical records of endplate potentials before and 5 min after application of 2 μg/ml venom are shown in Figure 4. The time course of the endplate potentials did not change appreciably in the presence of the venom and there was no change in muscle resting membrane potential throughout the experiment.

Effect on aneural muscle cultures

Postjunctional blocking activity of the venom was assessed on aneural cultures of chick embryonic skeletal muscle. Effects on resting membrane potential and cell morphology were also tested.

At concentrations of 5 to 40 μg/ml the venom depressed responses to iontophoretic application of acetylcholine (Figure 5). Lower concentrations (0.1 to 2 μg/ml) had no effect, even after overnight incubation.

Three hours incubation of cultures with 5 or 10 μg/ml of venom did not significantly depress the average resting membrane potential, but a 25 to 50% reduction was found following 2 h in 20 or 40 μg/ml. The depolarization was general accompanied by signs of cellular damage, such as extensive vacuole formation, extrusion of cytoplasm and peeling of myotubes from the surface of the culture dish (Figure 6). It

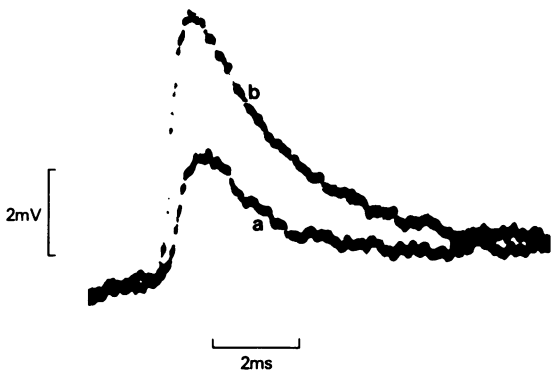

Figure 4 Typical endplate potentials (e.p.p.) recorded in a mouse phrenic nerve-hemidiaphragm preparation paralysed by (+)-tubocurarine (5 μM). (a) Control e.p.p.; (b) e.p.p. after 5 min exposure to 2 μg/ml *Dendroaspis angusticeps* venom.

should be noted that reduction or abolition of acetylcholine responses was found with concentrations of the venom that caused neither depolarization nor obvious cellular damage, although it is not known if fibre input resistance was altered by venom. Additionally, in cultures in which large areas had been destroyed, cells could be found with membrane potentials more negative than −20 mV but the acetylcholine responses of these cells were almost completely blocked.

Discussion

The venom of *Dendroaspis angusticeps* had three concentration-dependent actions on skeletal muscle: at low concentrations it augmented the contractions of indirectly stimulated preparations; at higher concentrations it inhibited responses to acetylcholine; and at still higher concentrations it caused a failure of muscle contractility.

Perhaps the most interesting effect of the venom was the marked augmentation of twitch responses of indirectly stimulated rat phrenic nerve-hemidiaphragm and chick biventer cervicis preparations. In an earlier report that concentrated on the cardiovascular actions of green mamba venom, Osman, Ismail & El Asmar (1973) described a similar effect on the rat hemidiaphragm. These authors suggested that the augmentation might be due to a supersensitization of the cholinoceptors, although no evidence was presented to support such a hypothesis. Since the venom did not augment the contractions of directly stimulated preparations, the site of action would appear to be at the neuromuscular junction rather than on the muscle fibres themselves. It was, thus, possible that the venom had anticholinesterase activity, increased postsynaptic receptor sensitivity or increased acetylcholine release.

In the chick biventer cervicis nerve-muscle preparation responses to acetylcholine and carbachol were unaffected, or depressed, at times when responses to indirect stimulation were increased three fold. This supports the earlier conclusion that there is no increase in muscle contractility and also makes it unlikely that there has been a supersensitization of cholinoceptors, as suggested by Osman *et al.* (1973). That there was little difference in the effect of the venom on responses to carbachol and exogenous acetylcholine suggests that the venom does not act as an anticholinesterase. This conclusion was confirmed by the cholinesterase assay, where biochemical tests indicated that the venom at concentrations up to 80 μg/ml has no significant anticholinesterase activity. By a process of elimination, it may be concluded that the augmentation of twitch height produced by venom is a result of a presynaptic action to increase transmitter release. More direct evidence in favour of this conclusion was obtained from electrophysiological experiments on the mouse phrenic nerve-hemidiaphragm preparation where the venom increased the amplitude of the endplate potential without the concomitant prolongation that would be associated with an anticholinesterase effect.

At concentrations of venom higher than those causing augmentation, there was a slow irreversible blockade of the twitch responses. Although a similar effect was observed on directly stimulated preparations, it was faster in indirectly stimulated preparations, suggesting that the antagonism is largely at the neuromuscular junction. Since responses of the chick biventer cervicis preparation to acetylcholine, carbachol and indirect stimulation were depressed simultaneously, the antagonism would appear to be postjunctional rather than by a failure of transmitter release. The presence of neurotoxins that cause nondepolarizing blockade by binding to postjunctional cholinoceptors is a common feature among elapid snake venoms (see Lee, 1972); such toxins have already been reported in *Dendroaspis viridis* venom (Banks *et al.*, 1974) and in *Dendroaspis polylepis* venom (Strydom, 1972).

Electrophysiological studies on aneural cultures of chick embryo skeletal muscle allowed investigation of effects of the venom on the postjunctional cholinoceptors in the absence of interference due to effects of the venom on the presynaptic nerve terminal. Receptors on cultured muscle have previously been shown to be similar pharmacologically to receptors on more conventional skeletal muscle preparations (for review, see Harvey & Dryden, 1977). At concentrations which had no effect on the average resting

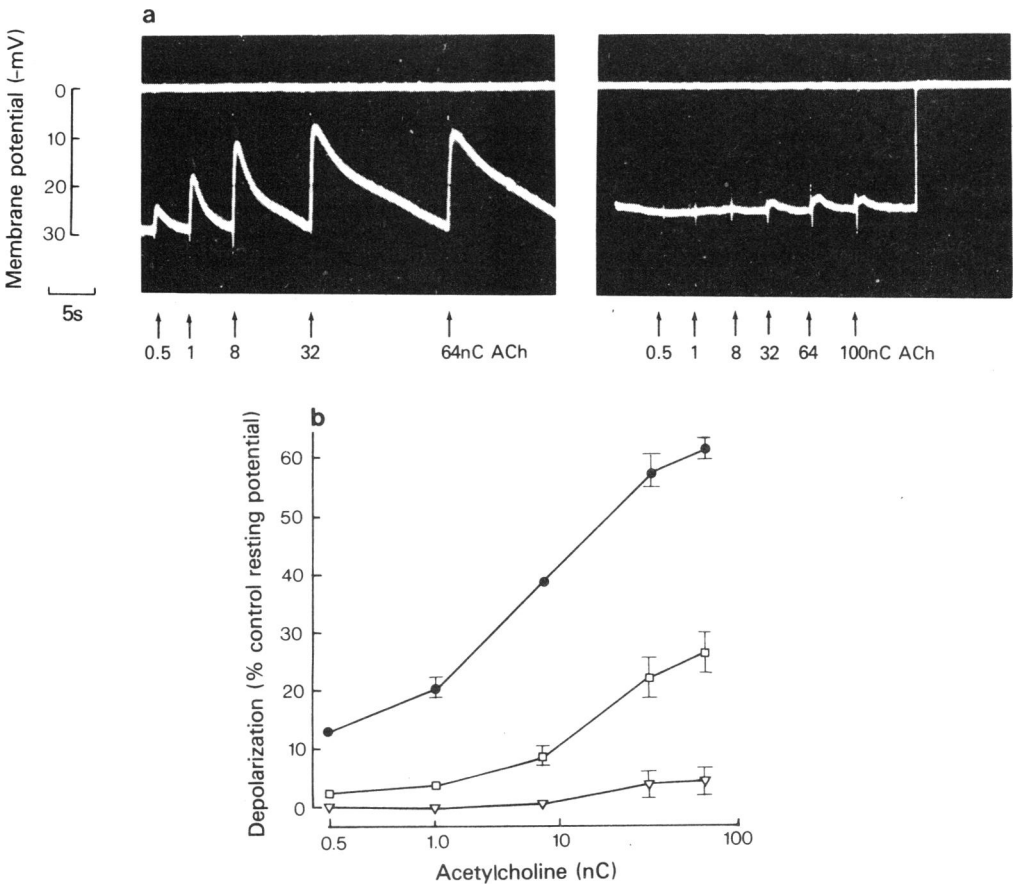

Figure 5 (a) Effects of *Dendroaspis angusticeps* venom on responses of cultured chick embryonic muscle fibres to iontophoretic application of acetylcholine. Left panel: a typical control dose-response curve; right panel: dose-response curve in a myotube after 80 min exposure to 20 μg/ml venom. (b) Mean dose-response curves to acetylcholine before and after venom: (●) control responses; (□) responses after 80 min exposure to 5 μg/ml venom; (▽) responses after 80 min exposure to 20 μg/ml venom. Each point represents the mean of at least 10 determinations; vertical lines show s.e. mean.

membrane potential, the venom markedly depressed the depolarization elicited by iontophoretically applied acetylcholine, suggesting that one or more components in the venom have postjunctional receptor blocking actions. At higher concentrations the venom had a cytotoxic effect on the muscle fibres in tissue culture which resulted in cell destruction. This direct action of the venom on skeletal muscle may be the cause of the slow decrease in twitch height of directly stimulated rat hemidiaphragm and chick biventer cervicis preparations that was observed at higher concentrations of venom.

Thus, the venom of *Dendroaspis angusticeps* has several actions on skeletal muscle and on neuromuscular transmission, including an unusual facilitatory effect. Further studies on isolated venom components are in progress in order to elucidate more precisely the mechanisms of action of the venom. Previous studies on purification of polypeptides from green mamba venom failed to isolate a single component that approached the activity of crude venom when injected into mice (Viljoen & Botes, 1973; 1974), but it is hoped that more extensive pharmacological testing will be successful.

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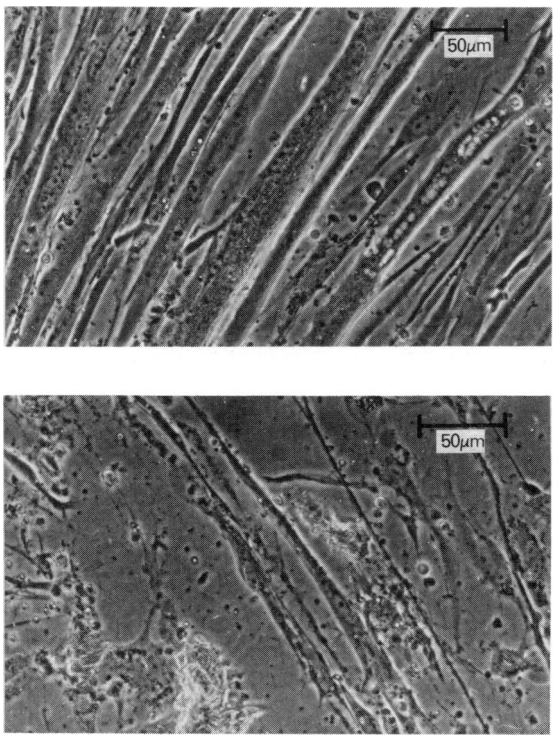

Figure 6 Cytotoxic action of *Dendroaspis angusticeps* venom on cultured chick embryonic muscle fibres. Upper panel: phase-contrast photomicrograph of a culture before addition of venom. Lower panel: photomicrograph of the same culture after 60 min exposure to 20 μg/ml venom. Bar indicates 50 μm.

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