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Effects of *Naja nivea* venom on nerve, cardiac and skeletal muscle activity of the frog

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Summary

1. The effects of 1 mg/ml whole *Naja nivea* (Cape cobra) venom, and of three different toxins isolated from it, on spinal reflex excitability and cardiac and skeletal muscle activity of the frog (*Xenopus laevis*), were studied. Isolated spinal cords, sciatic nerve-gastrocnemius muscle units and isolated heart preparations were used.

2. Synaptic efficiency and/or motoneurone excitability ceased totally and irreversibly after the application of whole venom within 18·5 minutes. The application of α toxin caused polysynaptic reactivation of the motoneurones after the monosynaptic response elicited by electrical stimulation, i.e. it had an excitatory effect, which was retained after washing off the toxin. The effect of β toxin resembled that of α toxin, except that the effect was lost after the toxin was washed off. The δ toxin abolished all nervous reactivity irreversibly and was found to be 2·64 times more effective in this respect than whole venom.

3. Responses of the gastrocnemius muscle to electrical stimulation of its motor nerve ceased 10 to 20 min after application of the venom. The muscle usually still responded slightly to direct stimulation for a few minutes. The effect was irreversible. The venom did not only block neuromuscular transmission, but also affected the muscle fibre itself. Nerve conductivity was relatively resistant to the venom ; α and β toxins produced similar results but were less effective.

4. The application of whole venom (1 mg/ml) to the isolated heart resulted in complete spasm of the ventricle within 3 to 8 minutes. The atria continued contracting long after the ventricle ceased. When the venom was washed off, contractions of the atria were restored, while the ventricle remained irreversibly in a state of spasm. The α, β and δ toxins weakened the contractions of the ventricle, but did not produce spasm. The effect on the ventricle was reversible. Contractions of the atria remained apparently unaltered.

Introduction

Administration of whole venom from the Indian cobra (*Naja naja*) results in the death of small mammals, probably through a peripheral neuromuscular block leading to respiratory paralysis (Meldrum, 1965 ; Jimenez-Porras, 1968). According to Bhargava, Horton & Meldrum (1970), death following cobra poisoning is often preceded by convulsions, which, in the dog, are secondary to the anoxia produced by respiratory paralysis. However, Guyot & Boquet (1960) suggested that cobra venom (*Naja nigricollis*) might also have a direct convulsive action (i.e. it may affect neurones directly).

Application of either whole venom or a neurotoxic fraction of *Naja naja* to the exposed cerebral cortex of the rat, led to the appearance, in the somato-sensory evoked potential, of abnormal negative waves which persisted after the toxin was washed off (Bhargava *et al.*, 1970).

In the present paper, the effects of whole *Naja nivea* (Cape cobra) venom, and of three fractions isolated from it, on spinal reflex excitability and skeletal and cardiac muscle activity of the frog (*Xenopus laevis*) are reported.

Methods

A. *Cobra venom toxins*

When the effect of whole venom was tested, dried *Naja nivea* venom was dissolved in Ringer solution (1 mg/ml).

Three fractions of *Naja nivea* venom isolated by Botes, Strydom, Anderson & Christensen (1971) and Botes (1971) were also tested. These fractions were designated α, β, and δ toxins.

Toxin α differed from other snake venom toxins and contained 71 amino acid residues cross-linked by five intramolecular disulphide bridges, with none of the commonly occurring amino acids absent. Toxin β resembled other snake venom toxins and contained 61 amino acid residues cross-linked by four intra-molecular disulphide bridges and was devoid of methionine, alanine and phenylalanine. Toxin δ was shown to be identical in amino acid sequence with *Naja haje* toxin α.

Immunochemically, toxins β and δ were related, while toxin α was completely distinct.

These purified, dried toxins were also dissolved in Ringer solution for testing (1 mg/ml).

B. *Test preparations and recording techniques*

South African frogs (*Xenopus laevis*) were used.

I. *Reflex excitability*

Isolated spinal cord preparations, as described by Loots & Meij (1972), were employed. The spinal cords were hemisected sagittally according to the technique used by Cerf & Carels (1966) and Davidoff (1972) to ensure rapid penetration of the toxins into the nervous tissue. The mean weight of the spinal cord preparations was 30 mg.

The isolated preparation was mounted in a recording chamber and allowed to stabilize in Ringer solution at 15° C ($\pm$1° C). The Ringer solution was then replaced by snake venom solutions at the same temperature. Work was carried out in a temperature-controlled room.

Stimulating and recording techniques employed were similar to those described in earlier studies (Holemans, Meij & Meyer, 1966 ; Holemans, Meij, Meyer & Loots, 1967). Reflex activity was elicited by square wave pulses which were supramaximal for reflex responses. These were applied to the tenth dorsal nerve root and motoneurone discharges were recorded on an oscilloscope screen from the corresponding ipsilateral ventral nerve root.

The effects of whole venom, and of the α, β and δ fractions were each studied in six preparations.

II. *Neuromuscular conduction and skeletal muscle activity*

Six sciatic nerve-gastrocnemius muscle preparations (mean weight 0·29 g) were employed to study the effects of whole venom and of the α and β toxins on neuromuscular transmission and muscle reactivity. These preparations were immersed in Ringer solution at room temperature (about 20° C), and after stabilization, the solution was replaced by Ringer solution containing venom (1 mg/ml).

The sciatic nerve was mounted on electrodes and stimulated continuously at 5 s intervals. The muscle twitch was recorded on a kymograph. Four additional gastrocnemius muscle preparations were stimulated directly; six sciatic nerves were used to study the effect of the venom on impulse conduction.

III. *Cardiac activity*

The effects of whole venom and of the three toxins were individually studied on six frog isolated hearts (mean weight 0·31 g). The hearts were isolated and perfused with Ringer solution and allowed to stabilize before the venom was added to the perfusion fluid (1 mg/ml). Contractions were recorded on a kymograph.

Results

I. *Effects on reflex excitability*

Spinal reflex excitability was tested by application of two consecutive stimuli to the tenth dorsal nerve root, and reflex spike responses were recorded from the corresponding ventral root. In previous studies on the spinal cord of the frog *in situ* from this laboratory (Holemans *et al.*, 1966; Meij & Holemans, 1968), a strongly facilitatory effect was obtained by the first of two consecutive stimuli (conditioning stimulus) on the monosynaptic response to the second (testing) stimulus. The facilitatory effect lasted several hundred ms, and the degree of facilitation was dependent on the interval between the two stimuli. Maximal facilitation was observed when the conditioning stimulus preceded the testing stimulus by about 30 milliseconds. The monosynaptic response to a single stimulus was usually very small. In the present study, isolated spinal cords were used and in these preparations, a single stimulus produced a strong monosynaptic spike response. When a second stimulus was applied after a 30 ms interval, the response to this stimulus was not facilitated but was smaller than the first response. This is probably due to recurrent inhibition of the motoneurones (Holemans & Meij, 1968). When the second stimulus was applied after longer intervals, the response to the second stimulus gradually improved, and after 50 to 70 ms intervals was approximately the same as the first response.

In the study reported here, two successive stimuli, separated by an interval of 50 to 60 ms were applied. At this interval the recurrent inhibitory effect of the first stimulus was no longer observed and this interval was also well within the limits for a facilitatory effect of the first stimulus on the response to the second stimulus, should a facilitatory effect have been present. In the preparations employed no such facilitatory effect was initially observed, apparently because the response to a single stimulus was already maximal. It was nevertheless decided to use two stimuli separated by this interval, in order to obtain a better idea of how reflex excitability is affected by the venom.

Preparations were not stimulated continuously, but tested approximately every minute, i.e. stimulated 4 or 5 times at 5 s intervals.

(a) *Whole venom*

The effect of whole venom is illustrated in Fig. 1.

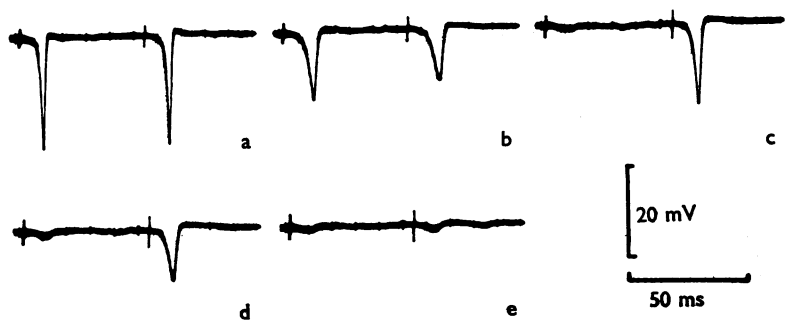

FIG. 1. The effect of whole venom of *Naja nivea* on spinal reflex excitability of the frog. (a) The stabilized monosynaptic spike responses to two consecutive stimuli. (b) The responses shortly after the addition of 1 mg/ml whole venom to the Ringer solution. (c), (d) and (e) The responses after progressively increasing time intervals. The first stimulus no longer elicited a spike response but still had a facilitatory effect in (c) and (d); in (e) this was also abolished.

Figure 1a shows two stabilized monosynaptic spike responses to two consecutive stimuli. Figure 1b shows the responses shortly after the Ringer solution was replaced by a 1 mg/ml whole venom in Ringer solution. Figures 1 c, d and e show the responses after progressively increasing time intervals. The monosynaptic reflex responses gradually declined, and the first stimulus (equivalent to a single stimulus) eventually failed to elicit a measurable monosynaptic response, but retained a facilitatory effect on the second stimulus for a while (Fig. 1 c and d). In some cases slight, apparently polysynaptic, responses were observed before excitability ceased.

Reflex excitability of motoneurones by electrical stimulation was abolished altogether after a mean time interval of 18·5 min in the six preparations studied. This effect of the whole venom was irreversible and no reflex responses were again elicited after perfusion with Ringer solution for as long as the experiments continued. Some were periodically tested for up to 12 hours. Measurable monosynaptic responses were obtained in control preparations stimulated and tested in the same way for more than 2 hours.

(b) *α Toxin*

The α toxin did not destroy reflex excitability, but the application of this toxin to six preparations caused, after a mean time interval of 6·1 min, the appearance of probably polysynaptic activity which followed both the electrically elicited monosynaptic responses (see Fig. 2).

The polysynaptic activity became more marked as time increased. When, after 20 min the α toxin was washed off with Ringer, the preparations continued to respond to stimulation in the same way; the polysynaptic activity increased further, eventually at the expense of the monosynaptic responses. The abnormal responses persisted for as long as the experiment continued (2 to 3 hours).

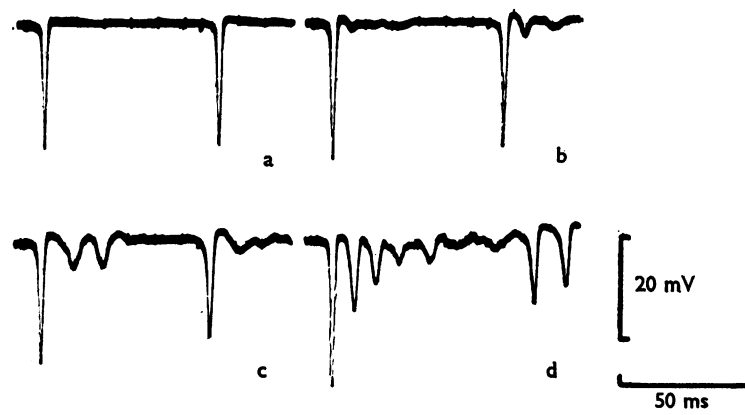

FIG. 2. The effect of *Naja nivea* α toxin on spinal reflex excitability in the frog. (a) Responses to two successive stimuli before the administration of α toxin. (b), (c) and (d) The responses at progressively increasing time intervals after the addition of α toxin. Polysynaptic activity appeared in (b) and increased at the expense of the second monosynaptic response (d).

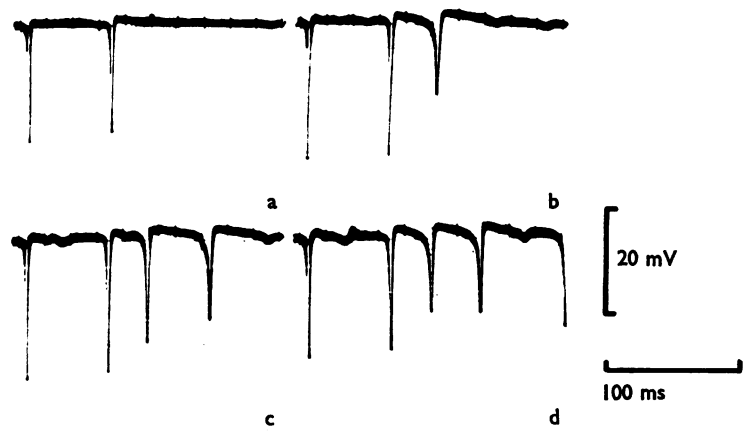

FIG. 3. The effect of *Naja nivea* β toxin on spinal reflex excitability in the frog. (a) Control responses. (b), (c) and (d) Massive synchronous motoneurone discharges follow the second of the two electrically elicited monosynaptic responses.

(c) β *Toxin*

β Toxin was also found not to destroy reflex activity. In the six preparations to which β toxin was applied, monosynaptic responses were elicited throughout the period of experimentation. After a mean time interval of 7·3 min massive synchronous motoneurone discharges followed the second of the monosynaptic responses after various intervals (see Fig. 3).

This excitatory effect of the β toxin was reversible, for when the toxin was washed off with Ringer after 20 min, the massive motoneurone discharges declined and eventually disappeared.

(d) δ *Toxin*

Synaptic excitability of motoneurones by electrical stimulation of the corresponding dorsal root was totally and irreversibly destroyed after application of this

toxin after a mean time interval of only 7 minutes. The δ toxin was found to be 2·64 times more potent in this respect than whole venom.

II. *Effects on neuromuscular conduction and skeletal muscle activity*

(a) *Whole venom*

It was found that muscle responses on stimulation of the motor nerve were depressed shortly after the Ringer solution was replaced by Ringer solution containing 1 mg/ml whole venom. The muscle twitch gradually became weaker and no response could be elicited after 6 to 10 min in the 6 preparations studied (see Fig. 4).

FIG. 4. Effect of whole venom of *Naja nivea* on neuromuscular conduction and skeletal muscle activity in frog. The muscle twitch rapidly became weaker and eventually no twitch was elicited by indirect stimulation. Note that the base line did not change, i.e. the muscle was in the relaxed state.

The muscle usually still responded slightly to direct stimulation for a few minutes, but was soon totally inexcitable and remained in the relaxed state.

The effect of whole venom was irreversible. The venom was washed off with Ringer solution and the preparations were tested at 10 min intervals, but no response was elicited through either indirect or direct stimulation for periods of 1 to 2 hours.

In control experiments with continuous direct stimulation at 5 s intervals for 1·5 hours, contractions decreased to one-third of the initial height. Rest for 30 min restored contractions to double the value at the beginning of the rest period.

Whole venom in Ringer solution (0·5 mg/ml) was applied to four additional gastrocnemius muscle preparations. These preparations were continuously stimulated at 5 s intervals until the responses decreased to one-third of the initial value. The venom was then washed off and the muscles were tested at 2 to 3 min intervals. The responses became weaker and disappeared irreversibly after 10 to 20 minutes.

Nerve trunks appeared to be relatively impermeable to cobra venom. Three sciatic nerve preparations were mounted on stimulating and recording electrodes and kept moist by applying drops of 1 mg/ml whole venom in Ringer solution, and stimulated at a frequency of 1 Hz for 2 hours. Conductivity remained virtually unchanged. Three more sciatic nerves were immersed in the same solution and tested every 30 minutes. Conduction was not affected for the first 30 min, but thereafter the action potentials became smaller. However, the ability to conduct was not altogether lost after 2 hours.

(b) *α and β Toxins*

Only the direct effect of α and β toxins on the muscle preparations was investigated. Neither α nor β toxin was as effective in destroying muscle responsiveness to electrical stimulation as was whole venom. Responses nevertheless ceased after 30 to 40 minutes. This effect was in both cases also irreversible.

III. *Effects on cardiac activity*

(a) *Whole venom*

The addition of whole venom (1 mg/ml) to the perfusion fluid resulted in spasm of the ventricle within 3 to 8 min in the six preparations studied (see Fig. 5).

The effect of the venom was irreversible when the perfusion was continued until the ventricle came to a complete standstill. A strong shock was ineffective in restoring ventricular activity.

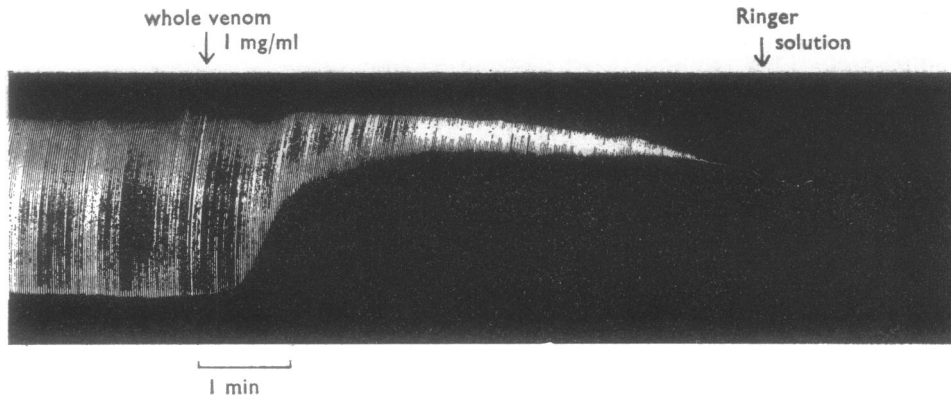

FIG. 5. The effect of whole venom of *Naja nivea* on the frog isolated heart. Within 5 min the ventricle was in a state of spasm. Contractions registered thereafter were entirely due to atrial activity. The subsequent slight drop of the base line was due to dilation of the atria.

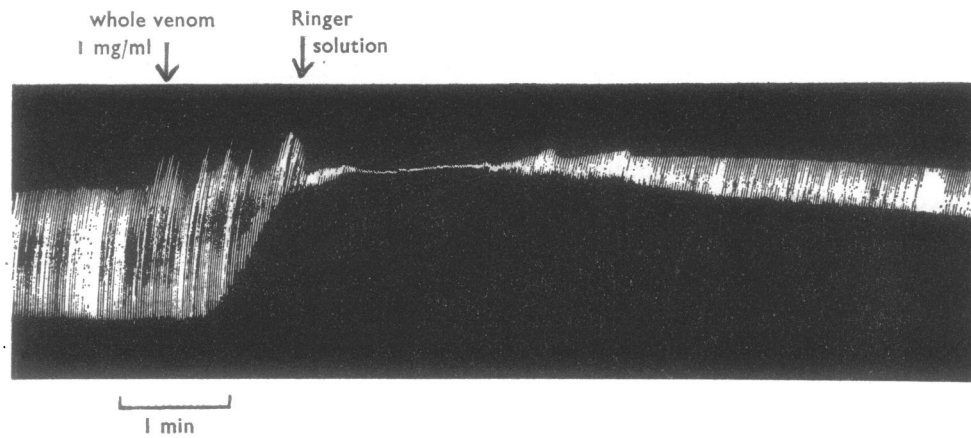

FIG. 6. Whole venom of *Naja nivea* was administered to the frog isolated heart and washed off within two minutes. The decline of ventricular contractions continued and the ventricle came to a standstill, but the atria continued contracting. After a while ventricular activity recovered to a small extent.

The atria were not affected in the same way. The atria continued to contract long after the contractions of the ventricle had ceased. When the atria eventually came to a standstill, they were in the relaxed state. When the venom was washed off and perfusion with Ringer solution continued, contractions of the atria were restored, while the ventricle remained in a state of spasm.

If the venom was administered for only 1 to 2 min and then washed off, i.e. before the ventricular contractions were suspended, the ventricle recovered to some extent but eventually came to a standstill after about 30 minutes (see Fig. 6).

In control experiments the height of the contractions registered after 2 h of perfusion was still more than 75% of the initial value.

Whole venom in lower concentrations (0·5 mg/ml) was less effective and complete spasm of the ventricle was attained only after approximately 30 to 40 minutes.

(b) *α, β and δ toxins*

These toxins also affected contractions of the ventricle but to a smaller extent than did whole venom. The δ toxin was the most effective of the three. They did not produce ventricular spasm, but contractions became weaker. Furthermore, the effect was in all cases reversible (see Fig. 7).

Contractions of the atria were apparently not affected at all.

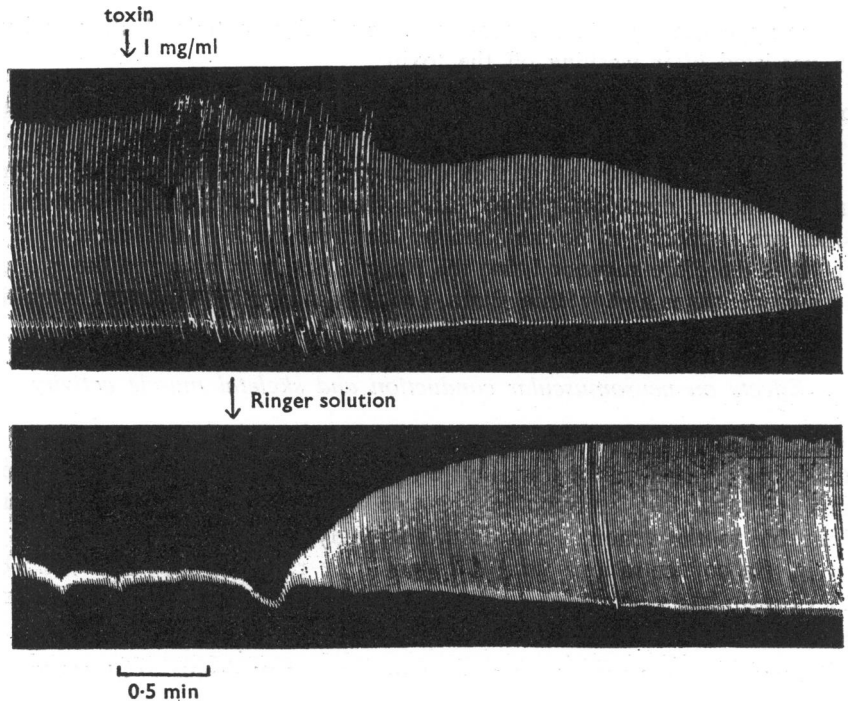

FIG. 7. The effect of *Naja nivea* δ toxin on contractions of the frog isolated heart. The top record demonstrates the effect when δ toxin was added to the perfusion fluid. It had an initial stimulating effect. Note that although the contractions of the ventricle weakened, the base line was altered very little. The lower record shows almost complete recovery of the ventricle when the toxin was washed off.

Discussion

Effects on reflex excitability

Synaptic excitability of motoneurones by electrical stimulation of the relevant afferent nerve fibres was totally and irreversibly destroyed after application of whole venom and also the δ toxin, which was found to be 2·64 times more potent than whole venom. The nerve fibres were not easily affected by the venom, and blocking of the impulse probably took place at the synapse. The results reported above do not give an indication as to whether the presynaptic or postsynaptic membrane was affected. Inhibition or destruction of the chemical mediator at the synapse can be excluded because perfusion with Ringer solution did not restore reflex excitability. Two possibilities remain: (i) the formation or release of chemical mediator by the terminals might be permanently damaged; (ii) permanent structural changes in the postsynaptic membrane may take place which alter the permeability or the sensitivity to the effect of transmitter substance.

The application of α toxin caused the appearance of polysynaptic activity following the electrically elicited monosynaptic responses. This toxin therefore seemingly affected the excitability of interneurones which caused re-excitation of the motoneurones. The abnormal responses persisted after washing off the toxin with Ringer. This effect is comparable with the results reported by Bhargava *et al.*, 1970. They found that the application of either whole *Naja naja* venom or a neurotoxic fraction (0·5–1·0 mg/ml) to the exposed cerebral cortex of the rat also led to the appearance in the somato-sensory evoked potentials of abnormal waves, which persisted after washing off the toxin.

Application of β toxin also resulted in hyperexcitability of the motoneurones and/or interneurones, which was however, reversible. In these experiments the first massive synchronous additional discharge followed the second of the two electrically-elicited monosynaptic responses after about 30 ms, i.e. when the motoneurones are supposed to be inhibited by recurrent inhibition (Holemans & Meij, 1968). It is therefore suggested that the β toxin not only increased the excitability of the motoneurones and interneurones, but also suppressed recurrent inhibition.

Effects on neuromuscular conduction and skeletal muscle activity

Muscle responses were irreversibly depressed shortly after the application of whole venom. The α and β toxins were less effective in destroying muscle responsiveness to electrical stimulation. Nerve trunks appeared to be relatively impermeable to the venom. The results strongly indicate that whole *Naja nivea* venom in the concentrations used does not only result in neuromuscular block, but that it also has a direct toxic effect on skeletal muscle of the frog. The nature of this effect remains obscure.

The results reported here differ from the results of Bhargava *et al.* (1970) on the effect of *Naja naja* venom and a 'neurotoxin' on neuromuscular conduction in the phrenic nerve-diaphragm preparation of the rat. These authors found that when block was fully established and the venom or toxin washed off, the muscle continued to respond to direct stimulation for several hours. In agreement with the present result, however, there was no recovery of neuromuscular transmission.

Effects on cardiac activity

The addition of whole venom (1 mg/ml) to the perfusion fluid resulted in spasm of the ventricle which was irreversible when the perfusion was continued until the ventricle came to a complete standstill. Spasm of the atria did not occur and they continued contracting long after the contractions of the ventricle had stopped. When the atria eventually stopped, they were in the relaxed state. This might have been due to the fact that the wall of the atrium is very thin and contains only few muscle fibres. It could therefore have been dilated by the weight of the perfusion fluid which was continually supplied. Washing off the toxin with Ringer solution restored contractions of the atria.

The differences between the responses of the atria and ventricle show that the sinoatrial node was not easily affected by the venom and that the muscle fibres of the atria also seemed to be fairly resistant. The venom had, however, a severe toxic effect on the ventricle, which was also in a way the reverse of its effect on skeletal muscle, the latter remaining in the relaxed state.

α, β and δ toxins affected contractions of the ventricle to a much lesser extent than whole venom, and the effect was always reversible. It seems likely that these toxins interfered with the permeability of the membranes of the ventricle or the atrio-ventricular conductivity as the effect was readily reversed by washing off the toxin.

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