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Long-lasting convulsant effect on the cerebral cortex of *Naja naja* venom

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Summary

1. A neurotoxic fraction has been prepared from Indian cobra venom (*Naja naja*) by column chromatography on CM Sephadex.

2. When assayed for lethality in mice, or for neuromuscular blocking potency in the rat phrenic nerve-diaphragm preparation, this fraction was 2·4–2·9 times as potent as whole venom.

3. Application of either whole venom (0·5–1·0 mg/ml) or the neurotoxic fraction (0·25–1·0 mg/ml) to the exposed cerebral cortex of the rat led to the appearance in the somatosensory evoked potential of abnormal negative waves, resembling in amplitude and latency those produced by the cortical application of strychnine or curare.

4. This effect differed from that produced by strychnine or curare in that after washing off the toxin the abnormal responses persisted for as long as the experiment continued (8–9 hours).

Introduction

Venom from the Indian cobra (*Naja naja*), at intermediate doses, produces death in small mammals primarily by a peripheral neuromuscular block leading to respiratory paralysis (Meldrum, 1965b ; Jimenez-Porras, 1968). This peripheral neuromuscular block is produced by a basic polypeptide which acts post-synaptically to produce a non-depolarizing block of transmission (Meldrum, 1965a ; Chang & Lee, 1966).

Death following cobra envenomation is often preceded by convulsions. Brunton & Fayrer (1873, 1874) demonstrated that in the dog these were secondary to the anoxia produced by respiratory paralysis. Nevertheless the possibility remained that cobra venom has a direct convulsant action. Injection of *Naja nigricollis* venom directly into the hippocampus of the rabbit was shown by Guyot & Boquet (1960) to produce seizures which were clinically focal.

Characteristic changes in cerebral cortical potentials evoked by sensory stimulation follow the local application of strychnine, other convulsant drugs or curare (Bhargava & Meldrum, 1969). In the present paper we report the effects on somatosensory evoked potentials of the direct application to the rat cerebral cortex of whole cobra venom and of a neurotoxic fraction separated from it by column chromatography.

Methods

Venom fractionation. Freeze-dried *Naja naja* venom (250 mg dissolved in 10 ml water, adjusted to pH 4·5 with 0·1 N hydrochloric acid) was heated to 75° C for 15 min, cooled, centrifuged and the precipitate discarded. Three volumes of ice-cold acetone were added to the supernatant, and stood overnight in the cold. The precipitate was separated by centrifugation and dried in a vacuum. This partially purified venom (weight 164 mg) was placed on a CM Sephadex column (C 50, cation exchange resin) in 0·05 M ammonium acetate buffer, pH 5·7. Elution was performed with ammonium acetate buffer in four stages: (1) continuous gradient 0·05 M, pH 5·7–0·17 M, pH 6·6 (400 ml), (2) 0·17 M, pH 6·6 (200 ml), (3) continuous gradient 0·17 M, pH 6·6–0·7 M, pH 7·0 (400 ml), (4) 0·7 M, pH 7·0 (400 ml). An LKB Miniflow precision pump created a flow of 25 ml/h and 5 ml fractions were collected with an LKB Radirac Fraction Collector, with continuous monitoring of the effluent optical density at 254 nm and 280 nm. Fractions were freeze dried, weighed and stored at 4° C.

Toxicity. The toxicity of fractions relative to that of whole venom was assessed by intraperitoneal injection in mice (LAC Carshalton white, weight 25 g). The time to convulsions and to death was recorded.

Neuromuscular-blocking action. The rat phrenic nerve diaphragm preparation of Bülbring (1946) in a 5 ml bath of Tyrode's solution, bubbled with 5% carbon dioxide and 95% oxygen, was stimulated either directly or indirectly at 4 s intervals. The muscle twitch was recorded on a kymograph. Whole cobra venom or " neurotoxin " was dissolved in 5 ml of Tyrode's solution, and placed in the bath. The time to complete neuromuscular block was recorded.

Cortical evoked potentials. The procedure for recording the responses to somatosensory stimulation from the exposed cerebral cortex of the rat anaesthetized with pentobarbitone has been described previously (Bhargava & Meldrum, 1969). Bilateral cortical cups were mounted so that the test solution could be applied to the pial surface of one cortex and a control solution of artificial cerebrospinal fluid (aCSF) (Bradbury & Davson, 1964) to the opposite cortex. For each somatosensory cortex computer averages were derived from consecutive responses to electrical stimulation of the contralateral forepaw. The whole venom, or " neurotoxin ", was dissolved in aCSF at 37° C, directly before application to the pial surface of the cortex.

Results

Column fractionation. The greater part of the heat-treated venom was totally excluded by the C 50 CM Sephadex and thus appeared in the effluent directly after the dead volume. Subsequently a nucleic acid fraction appeared and this was followed by a protein fraction. A fraction referred to as " neurotoxin " elutes when the buffer reaches a molarity of 0·17 M. The recovery of " neurotoxin ", after freeze-drying, was 21 mg. Two minor protein or polypeptide fractions were followed by a fraction eluting at 0·7 M, which weighed 11 mg.

Toxicity. Intraperitoneal injections in mice showed that the " neurotoxin " was the most toxic fraction. As happened after intermediate doses of whole venom, death was preceded by muscular weakness and convulsions. For the range of doses illustrated in Fig. 1A, there was an approximately linear relationship between the

$\log_{10}$ of the dose (of whole venom or " neurotoxin ") and the $\log_{10}$ of the time of death.

Death in 50 min was produced by 70 μg " neurotoxin "/100 g mouse or 170 μg whole venom/100 g mouse (giving a potency ratio of 2·4 : 1).

Neuromuscular block. Whole venom and " neurotoxin " both rapidly blocked neuromuscular conduction in the phrenic nerve diaphragm preparation. When the block was fully established the venom or toxin was washed off and the muscle continued to respond to direct stimulation for several hours but there was no recovery of neuromuscular conduction. The relationship between concentration of venom

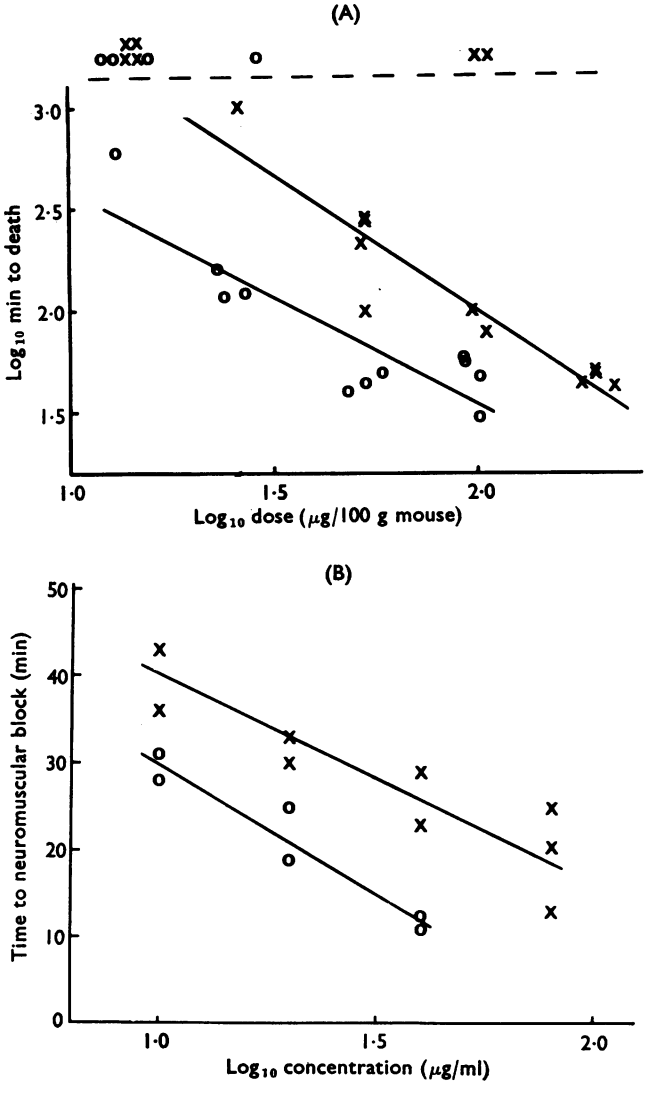

FIG. 1. A: Relationship between dose of whole venom (X) or the neurotoxic fraction (O) and time to death (or survival) in mice. Lines are calculated regression lines (ignoring survivors). B: Relationship between concentration of whole venom (X) or neurotoxic fraction (O) applied to the rat phrenic nerve diaphragm preparation and the time to complete neuromuscular block. Lines are calculated regression lines.

or " neurotoxin " and time to complete neuromuscular block is shown in Fig. 1B. Complete neuromuscular block can be produced in 25 min by either 15 μg " neurotoxin "/ml or by 43 μg whole venom/ml (giving a potency ratio of 2·9 : 1).

Cortical evoked potentials. Figure 2 illustrates the changes in somatosensory evoked cortical potentials that were seen after the topical application of whole venom. Characteristically after 10–20 min the response of the venom-treated cortex to stimulation of the contralateral forepaw showed a large negative wave with a latency to the peak of 40–75 ms. Initially this was intermittent, but in the following minutes it became more regular, of larger amplitude and shorter latency. When its amplitude was greatest the latency of the negative peak was 24–29 ms. Responses to ipsilateral stimulation remained insignificant. The primary evoked potential on the control hemisphere was unchanged (except for the occasional appearance of an early negative peak, latency 14–19 ms, associated with fluctuations in the depth of anaesthesia). At the same time as the abnormal negative wave appeared at the venom-treated cortex the control cortex showed a marked response to ipsilateral stimulation. This comprised a positive wave (peak latency 18–32 ms) followed by a negative wave (peak latency 36–45 ms). The relative amplitudes of the negative and positive waves varied greatly between experiments.

The venom was washed off after 30 min. In the next 5–30 min the abnormal negative wave usually stayed constant, but in some cases there were slight increases

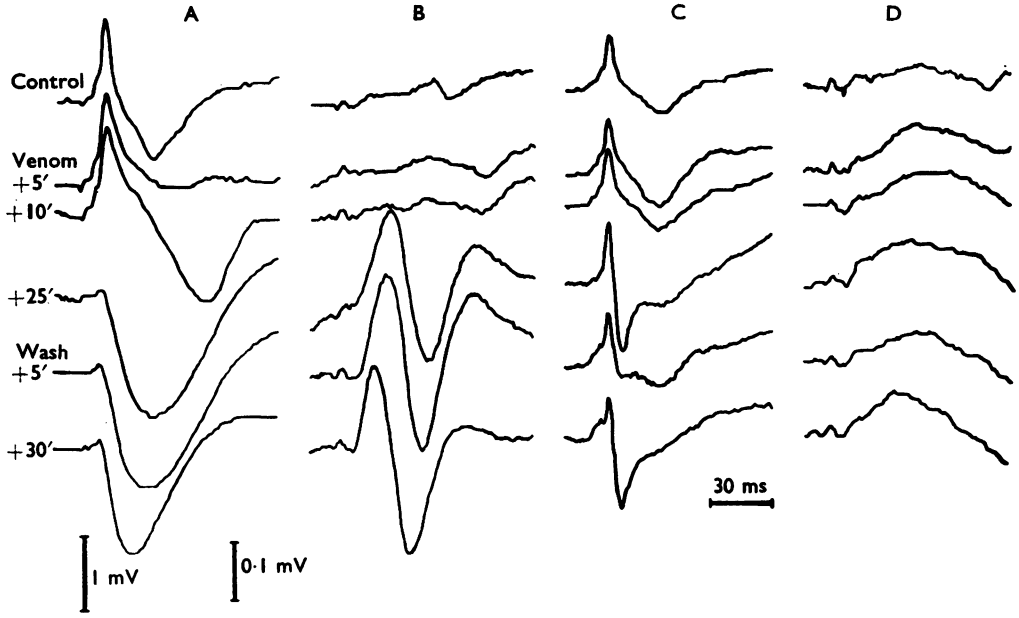

FIG. 2. Changes in cortical evoked potentials following the application of whole venom (0·75 mg/ml aCSF) to the left somatosensory cortex. Each trace is the average of thirty-two consecutive responses to peripheral stimulation at 2 s intervals. The stimulus was applied to the forepaw 10 ms after the commencement of each trace. The times relative to the venom application indicated against column A apply to all columns. A: Left cortex, response to stimulation of right forepaw. B: Right cortex, response to stimulation of right forepaw. C: Right cortex, response to stimulation of left forepaw. D: Left cortex, response to stimulation of left forepaw. 0·1 mV calibration applies to all traces except the lower three in column A (venom+25 min, wash+5 min, wash+30 min) to which the 1 mV calibration applies.

or decreases in amplitude. Responses were recorded up to 5 h after washing, and a slight decline in amplitude of the negative wave was seen, but a return to normal did not occur.

Table 1 shows that abnormal negative waves were not seen after 0·25 mg venom/ml, but a concentration of 0·5 mg/ml or more was effective.

Comparable changes were seen after the cortical application of the neurotoxic fraction (Fig. 3). The delay before the abnormal negative wave appeared in the

TABLE 1. *Production of abnormal negative waves in the somatosensory evoked potential after the cortical application of whole cobra venom or "neurotoxin"*

	Concentration (mg/ml)	Time of onset (min)	Time of max. amplitude (min)	Max. amplitude of neg. wave (mV)
Whole venom	0·1	—	—	0
	0·25	—	—	0
	0·5	16	30	1·3
	0·5	22	30+20W	1·7
	0·75	18	30	1·3
	0·75	18	30	1·3
	0·75	10	25	1·6
	1·0	18	30	4·3
	1·0	13	30	3·5
'Neurotoxin"	0·1	—	—	0
	0·25	15	30+5W	1·7
	0·5	14	30+5W	2·2
	0·75	3	20	1·9
	0·75	20	30+20W	2·1
	1·0	18	30	3·2

* When the maximum effect on the abnormal negative wave occurred after washing off the venom or "neurotoxin", the time interval is recorded as duration of application+interval after the washing (W) commenced.

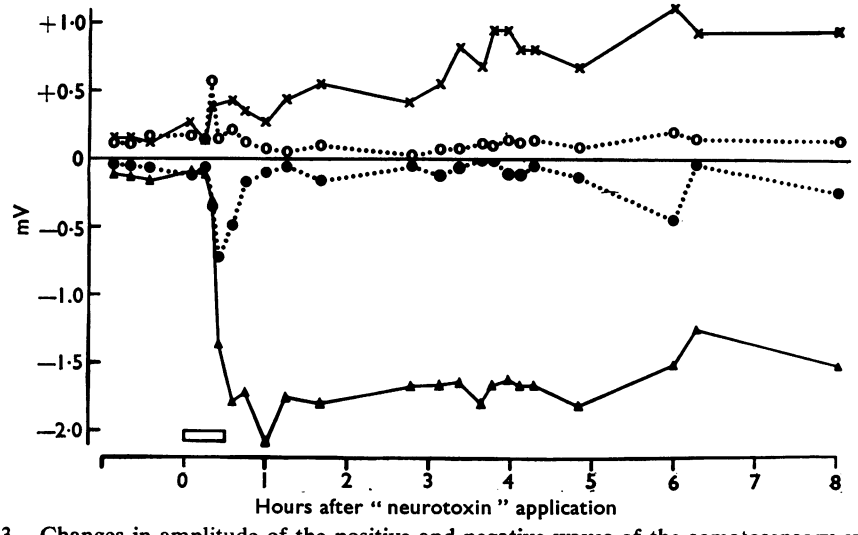

FIG. 3. Changes in amplitude of the positive and negative waves of the somatosensory evoked potential following application of "neurotoxin" (0·75 mg/ml) to the left somatosensory cortex for 30 min. ▲——▲, Surface negative wave, left cortex. ×——×, Surface positive wave, left cortex. ●····●, Surface negative wave, right cortex. ○····○, Surface positive wave, right cortex.

primary evoked response was similar (except for an unusually rapid effect in one experiment). The latency of the negative peak and its maximum amplitude were also similar. Two animals showed a significant enhancement of the amplitude of the primary positive wave (peak latency 10–12 ms).

As with whole venom, the large negative wave at the treated cortex was accompanied by a response at the control cortex.

Evoked responses were recorded in two animals for 7·5–8·5 h after washing off the neurotoxin. In both cases the abnormal negative wave was still present at the end of this period. It was abolished by spreading depression or transient asphyxia, but with recovery of the cortex it reappeared.

The venom fraction eluted by 0·7 M buffer was also tested on the rat cortex in four experiments but did not reproduce the effect of whole venom or neurotoxin.

Discussion

The principal action of whole cobra venom and of the neurotoxic fraction on the somatosensory evoked potential was to produce an abnormal negative wave with latency to the peak of about 26 ms. This effect is closely similar to that produced by the cortical application of curare or strychnine (Bhargava & Meldrum, 1969). The latter substances when applied topically to the cerebral cortex are both convulsant agents. It is not known whether they cause direct excitation or whether they block an intracortical inhibitory process. The venom had a slower onset of action than strychnine or curare. However the effect of strychnine of curare is readily reversed (in 15–90 min) by washing, but the effect of the venom persists after washing for at least 8 h. This sustained effect is similar to the action of neurotoxin at the neuromuscular junction where the block in transmission is also "irreversible". At the neuromuscular junction a non-depolarizing blockade of cholinergic transmission is produced (Meldrum, 1965a ; Chang & Lee, 1966). This suggests the possibility that blockade of a cortical cholinergic synapse could be responsible for the effect observed. This possibility is supported by the observation that treatment of the rat cortex with eserine (10^{-3}M) for 60 min blocks the usual effect on the evoked potential of strychnine 4×10^{-4}M but not that of strychnine 2×10^{-3}M (Bhargava, 1969).

Compared with whole cobra venom, the neurotoxic fraction was nearly three times as lethal or as effective at producing neuromuscular block. The evoked potential changes indicated that the neurotoxic fraction was also more potent than the whole venom at the cortex, but the dose-response measurements were not sufficiently quantitative to allow calculation of an exact ratio. Larsen & Wolff (1968) stated that CM cellulose chromatography of cobra venom yielded fractions with 6–9 times the lethality of whole venom. This is similar to the potency ratio of 6·7 determined by Yang (1965) for "cobrotoxin" from Formosan cobra venom (*Naja naja atra*).

The fraction eluted by 0·7 M buffer which did not produce the convulsant effect on the cortex was probably the "cobramines A and B" of Larsen & Wolff (1968), which is the "cardiotoxin" of earlier authors (Meldrum, 1965b).

Whole venom of *Naja nigricollis* was shown by Guyot & Boquet (1960) to produce convulsions in rabbits when injected into the hippocampus in very small quantities (3·5 μg/kg). Seizure activity in the hippocampus is readily provoked by curare (Feldberg & Fleischhauer, 1963), suggesting that similar pharmacological mechanisms

are responsible for the convulsant effects on the cortex and the hippocampus of curare or cobra venom.

Whether neurotoxins or other venom components can produce convulsions by direct action on the brain after systemic administration remains uncertain. Experiments with ^{131}I-labelled cobra venom (Tseng, Chiu & Lee, 1968) have shown a very slow but measurable penetration of label from plasma to cerebrospinal fluid after administration of either whole venom or neurotoxin.

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