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ISOLATION OF THREE DIFFERENT NEUROTOXINS FROM INDIAN COBRA (*Naja naja*) VENOM AND THE RELATION OF THEIR ACTION TO PHOSPHOLIPASE A

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Abstract—Six fractions of the venom of the Indian cobra (*Naja naja*) were separated by paper electrophoresis of which four (fractions 6, 3, 2 and 1) were toxic in anaesthetized cats or in mice, or in both species. Of the four toxic fractions only fraction 6 contained phospholipase; it was toxic to cats but not to mice. Fraction 3 was toxic to cats and mice and fractions 1 and 2 were toxic to mice but not to cats. Thus three different neurotoxic fractions were separated. In the anaesthetized cat the intravenous injection of fraction 6 caused the same effect as the injection of whole or boiled venom, i.e., a diphasic circulatory shock—an initial rapid fall in arterial blood pressure followed after partial recovery by a delayed gradual fall—with depression of cerebral cortical activity. The central vasoregulating mechanisms were apparently not affected during the shock since records taken from the cervical sympathetic showed increased activity. Fraction 3 did not produce the initial fall in arterial blood pressure but only the delayed phase of shock. In mice, the toxic effects of an intraperitoneal injection of whole venom resulted in convulsions, excitement, motor impairment and respiratory arrest; the injection of fractions 1 and 2 resulted in apathy, motor depression and convulsions before death, and the injection of fraction 3 in excitement, convulsions, repeated laboured respiration, and curling of the tail.

INTRODUCTION

SNAKE venoms are complex mixtures of toxins and enzymes, but the relations between the toxic and enzymatic activities have not been finally elucidated. Phospholipase A, protease, L-amino acid oxidase, cholinesterase, nucleases, phosphodiesterase, monoesterase, hyaluronidase have been found in most venoms and some of them, nucleases,[1] cholinesterase,[2] phospholipase A,[3] have been suspected to cause various envenomation syndromes. Above all, the phospholipase A of the Indian cobra (*Naja naja*) venom has been considered to possess neurotoxic activity,[3] although in recent publications this assertion is being dissented.[4, 5]

In the present study we have attempted to further clarify the problem of the toxicity of cobra venom phospholipase A by testing electrophoretically separated fractions on cats and mice. The contribution of the different venom fractions to the venom neurotoxicity and the causation of the diphasic shock, characteristic for the Indian cobra venom, was evaluated.

MATERIALS AND METHODS

Venom and venom fractions

Indian Cobra (*Naja naja*) freeze dried venom from L. Light and Co. Ltd., Colnbrook, England was used.

Phospholipase A from *Naja naja* venom was obtained by paper electrophoretic separation at pH 6·0, using phosphate buffer M/15. After electrophoresis a narrow segment (of about 1 cm width) was cut off from the middle of each strip (about 7 cm width) and stained with naphthalene-black. Figure 1 A shows 6 bands in the electropherogram. The appropriate protein bands were cut off from the unstained strips and eluted over night with distilled water at 4°. Sodium chloride was added until isotonicity and protein concentration was estimated by Lowry reaction.[6] Only the fraction remaining at the application line (fraction 6) contained the phospholipase A activity. The adjacent fraction 5 proved in most separations to be devoid of phospholipase A activity but in a few instances was contaminated with the enzyme. The phospholipase A containing fraction 6 was tested for the following activities: protease,[4] procoagulant,[6] L-amino acid oxidase[8] and hyaluronidase[9] and was found devoid of all of them. Analytical ultracentrifugation of fraction 6, dialyzed against saline, was carried out with a model E, Spinco ultracentrifuge at 16·5°, using the standard 12-mm cell, and a single peak was obtained corresponding to a sedimentation constant $S_{20}=3{\cdot}65\times10^{-13}$. Fractions 1 and 2 contained the direct lytic factor, a basic protein, capable to lyse washed red blood cells.[10]

For some experiments the venom was boiled and treated with heparin. For this purpose a saline–venom solution, 1 mg/1 ml, was heated for 15 min in a boiling waterbath at pH 5·5 and then centrifuged. The electrophoretic pattern of the supernatent was similar to that of the unboiled venom i.e. comprising 6 bands. 1 mg heparin (National Biochemicals Corporations, Heparin-sodium 100 U/mg) in 0·1 ml. saline was added to 1 ml. of the supernatant and the mixture was kept for 20 min at room temperature. A sediment appeared which is known to contain the direct lytic factor.[10] It was separated by centrifugation and electrophoresis of the clear supernatant showed that fractions 1 and 2 were absent (Fig. 1 B).

Phospholipase A activity

One ml. of tenfold saline diluted egg yolk was incubated at 37° for increasing periods of time with 0·05 ml venom or phospholipase A fraction containing 0·5 μg protein. The unesterified fatty acids (UFA) were determined by titration using the method of Dole.[11] The activity was calculated as m-equiv. of free acid released per enzyme fraction per minute. For experiments only those phospholipase A preparations were used which liberated at least 1·6 m-equiv. UFA/min per 1 μg protein.

Experiments on mice and cats

Intraperitoneal mouse—LD_{50} of whole venom and its separate fractions were determined on locally bred Swiss albino mice, using 5 animals per dose. Calculation of LD_{50} was made according to Reed and Muench.[12]

A second series of experiments was carried out in cats weighing 2–4 kg. Anaesthesia was started with ether and maintained throughout the experiment by repeated administration of thiopentone sodium, 10 mg/ml, injected into a cannulated femoral vein. Blood clotting was prevented by the intravenous administration of heparin (1,000 U/ml/kg). Physiological activities were recorded on a Grass Model 5 Polygraph by means of transducers. Blood pressure was registered from the cannulated femoral artery using a Statham transducer, and respiration by means of a thermocouple transducer from the cannulated trachea. The electrocardiogram (ECG) was

recorded from needle electrodes inserted into the extremities, and the electrocorticogram (ECoG) from stainless steel electrodes, insulated except for the tips, introduced through holes drilled into the cat's skull until reaching the brain surface in the right and left frontal, parietal and occipital areas.

RESULTS

Action of whole Cobra venom

Mice. The intraperitoneal LD_{50} in mice was 0·18 mg/kg. Mice injected with $2 \times LD_{50}$ showed convulsions, excitement and impaired movements, and died in respiratory arrest within 80 min.

Cats. The intravenous LD_{100} in anesthetized cats was 1 mg/kg, the animals dying within 2 hr from the moment of injection. In experiments on 12 cats, the injection of one LD_{100} caused a diphasic circulatory shock. Immediately after the injection a transient drop in blood pressure occurred, associated with apnea and bradycardia, high P waves and ST depression (Fig. 2B). Later, after the mean blood pressure had returned to the preinjection level, there occurred a temporary depression in the ECoG. In the period from about 10 min after injection till shortly before death, respiration was fast and pulse pressure decreased progressively, ECoG and ECG remaining normal (Fig. 2C). Near to death, 80–120 min after the injection (Fig. 2D), the ECoG slowly waned, the respiration became progressively bradypneic until respiratory arrest occurred. Thereafter the blood pressure dropped to zero and the animal died.

Recording of the action potentials from the cervical sympathetic chain (Fig. 3) showed increased firing starting shortly after venom injection, gradually increasing in frequency and amplitude, up to the time of death. Increased sympathetic firing together with maximally depressed ECoG 10 min before death are shown in Fig. 3B. At this time bradypneic respiration persisted, the sympathetic firing increasing at inspiration.

Action of Cobra venom fractions

Of the 6 paper electrophoretic Cobra venom fractions (Fig. 1A), three-combined fractions 1 and 2, fraction 3 and fraction 6, had neurotoxic activity, although having different species specificity and mechanism of action.

Mice. The LD_{50} for the combined eluates of fractions 1 and 2 was about 1·5 mg (protein) per kg body weight. Mice injected intraperitoneally with 2 LD_{50} of this combined eluate showed neurotoxic symptoms—apathy, motor depression, and close to death 3 hr after venom injection, gasping respiration, jumping and clonic convulsions.

Fraction 3 possessed stronger neurotoxic activity, the intraperitoneal LD_{50} being 0·5 mg/kg. Mice injected with this fraction showed excitement, jumping, convulsions, fast respiration with use of auxiliary respiration muscles, and curling of the tail. Death occurred early, not later than 30 min after the injection.

Animals receiving fraction 4 exhibited in some cases similar symptoms but the LD_{50} was much higher, above 2·5 mg/kg. Fraction 5 was not toxic at the doses injected, up to 2·5 mg/kg. Fraction 6, the only one which contained phospholipase A activity, was not toxic, even at doses of 5 mg/kg. This conforms to the findings of Master and Rao[4] with Indian cobra phospholipase A purified by starch gel electrophoresis.

Cats. Combined fractions 1 and 2 were injected into 2 cats intravenously in the

amount of 1 mg (protein)/kg. One of the cats showed transient changes in the ECG—lowering of QRS voltage and ST elevation, lasting for about 10 min after injection. No other toxic effects were recorded.

Fraction 3 was given to 3 cats intravenously at doses of 0·25 mg/kg (1 LD_{100}). No changes were seen in blood pressure, ECG, ECoG and respiration at the moment of venom injection (Fig. 4A), but 120 min later ECoG slowly waned (Fig. 4B), respiration became sporadic and then stopped completely. Pulse pressure diminished progressively during the two hours following injection and fell to zero. During the period of apnea the ECG showed increasing bradycardia and anoxic changes (Fig. 4B).

Fraction 4 was as a rule non-toxic, but sometimes symptoms similar to those produced by fraction 3 could be elicited at higher doses (0·8 mg/kg), probably due to deficient separation from fraction 3.

Fraction 5 preparations were found non-toxic, even in high doses up to 1·5 mg/kg, when devoid of phospholipase A. In those cases in which fraction 5 possessed phospholipase A activity by contamination from fraction 6, the preparation did show neurotoxic activity.

Fraction 6, which had strong phospholipase A activity, was neurotoxic, the LD_{100} being approximately 1 mg/kg. The pharmacological effect was identical to that of the whole Cobra venom with the two typical shock phases described above.

The activity of phospholipase containing boiled—heparinized Cobra venom was indistinguishable from that of fraction 6 and of whole venom. The LD_{100} was similar, about 1 mg/kg.

DISCUSSION

Earlier studies by Houssay[13] suggested a correlation between the neurotoxic activity of Crotalidae venoms and their hemolytic action. Feldberg and Kellaway[14] attributed the steep fall in blood pressure after intravenous injection of Cobra venom to lecithinase which, by producing lysolecithin, liberates histamine from the tissues. Braganca and Quastel[3] reaffirmed the identity of neurotoxin and cobra venom phospholipase A. Cobra venom heated for 15 min at 100° retained both its neurotoxic and phospholipase A activities while other enzymes were inactivated. On the other hand, phospholipase A—containing fractions, separated from various snake venoms by electrophoresis or chromatography, were recently reported to be devoid of neurotoxic activity, the latter being recovered in other fractions.[15]

In the present study an animal specific action of different paper electrophoretically separated *Naja naja* venom neurotoxins, only one of them—fraction 6—having phospholipase A activity, was demonstrated. This was apparent not only from the observed resistance of mice to fraction 6 and of cats to fractions 1–2, but also from the different syndromes appearing in cats and mice. Fraction 6 produced in cats diphasic circulatory shock, depression of respiration, eventually leading to respiratory death, and early occurring ECoG depression and changes in sympathetic activity. Fraction 3 provoked early death and convulsions in mice, whereas in cats delayed respiratory arrest and death with late occurrence of similar electrophysiological response.

The diphasic circulatory shock ensuing in cats following intravenous administration of whole *Naja naja* venom, as well as of fraction 6 and of heated heparinized venom (phospholipase containing, devoid of direct lytic factor[10] and of the mice-neurotoxic activities related to fractions 1–2 and 3) resembles that described by Feldberg and

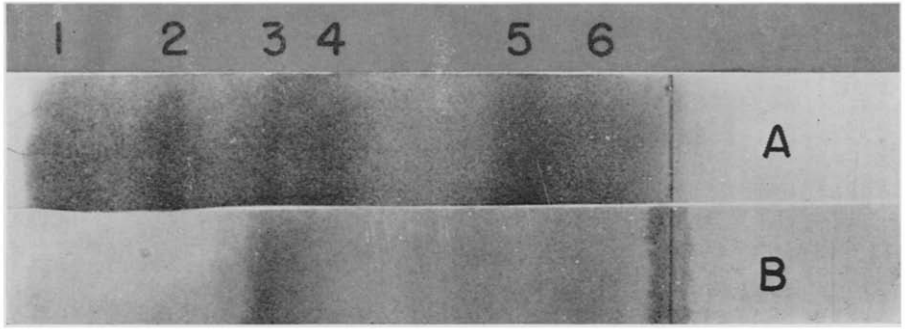

FIG. 1. Paper electropherogram of *Naja naja* venom—conditions as described in "Methods". A whole venom, B boiled, heparinized venom.

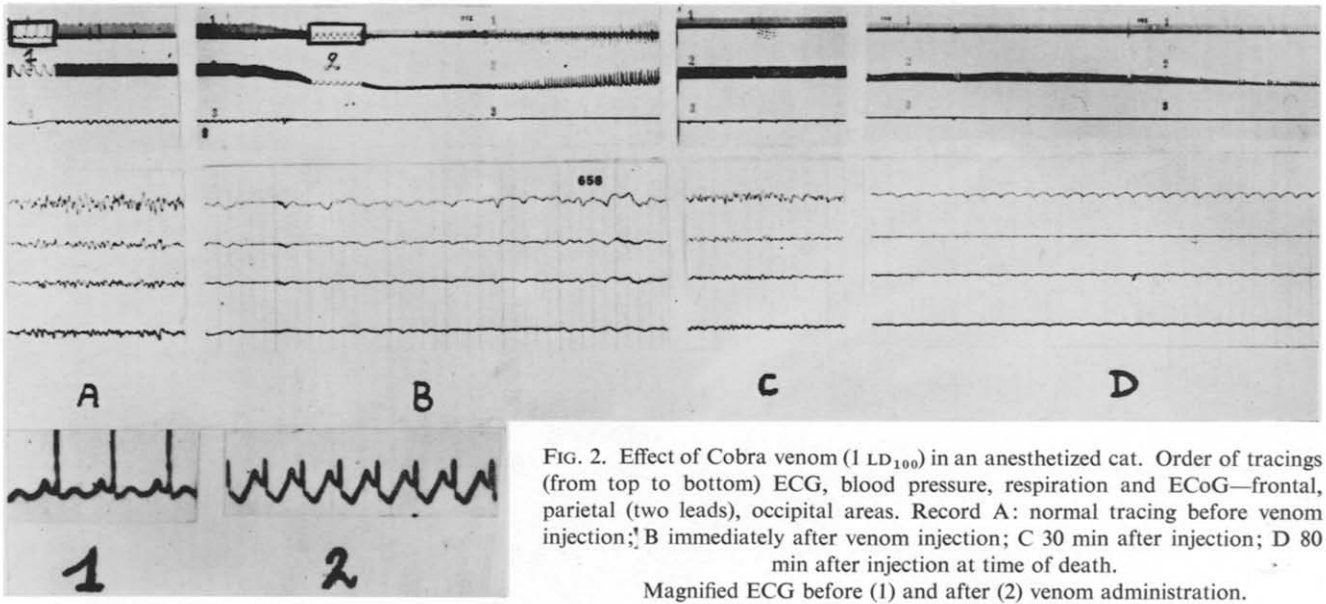

FIG. 2. Effect of Cobra venom (1 LD_{100}) in an anesthetized cat. Order of tracings (from top to bottom) ECG, blood pressure, respiration and ECoG—frontal, parietal (two leads), occipital areas. Record A: normal tracing before venom injection; B immediately after venom injection; C 30 min after injection; D 80 min after injection at time of death.
Magnified ECG before (1) and after (2) venom administration.

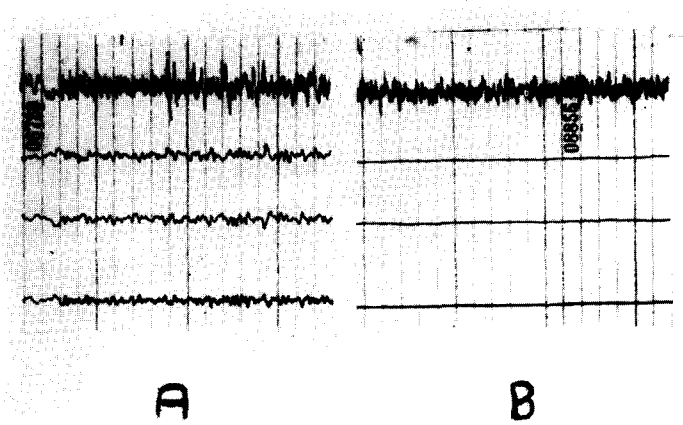

FIG. 3. Effect of Cobra venom (1 LD_{100}) in anesthetized cat on action potential from cervical sympathetic chain. A before venom injection; B 70 min after venom injection. Tracings from top to bottom sympathetic action potentials, ECoG—frontal, parietal and occipital.

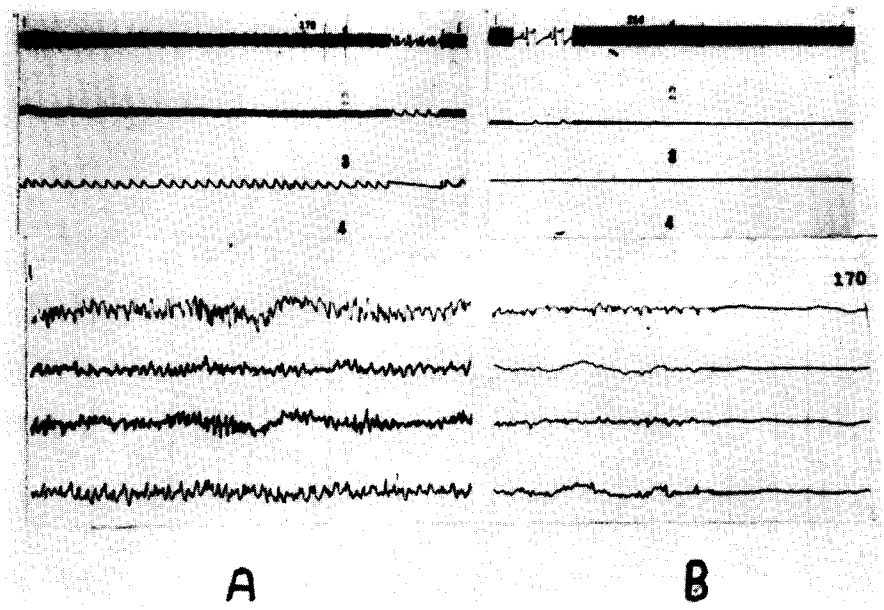

FIG. 4. Effect of Cobra venom fraction 3 (1 LD_{100}) in anesthetized cat on functions as in FIG. 2. A at moment of injection; B shortly before death, about 2 hr after venom injection.

Kellaway[14] for whole *Naja naja* venom. After recovery from the immediate steep drop in blood pressure, there was a late occurrence of a secondary gradual descent until respiratory death supervened. Fraction 3 did not produce diphasic shock but only a late lethal effect appearing synchronously with the second part of the diphasic shock caused by whole venom or fraction 6. Thus, whereas in whole venom-induced intoxication the phospholipase A-containing fraction 6 plays a role in the causation of both shock phases, fraction 3, which is devoid of phospholipase A, contributes to the delayed shock phase only.

Although the primary shock has been attributed by Feldberg and Kellaway to the histamine liberating action of the venom, a direct action of the venom phospholipase A on the central nervous system, manifested in our experiments by transient ECoG depression and changes of respiratory rhythm, cannot be excluded.

It was established in the present study that *Naja naja* venom provokes increased cervical sympathetic action potentials with primary depression of ECoG waves. In contradistinction, Vipera palestinae venom neurotoxin has been shown in a previous study to reduce cervical sympathetic firing immediately upon injection without simultaneously affecting the cortical activity.[16] It appears therefore that neurotoxins from different snakes act on different sites in the central nervous system.

Phospholipase A from different snake venoms have been shown to have different substrate specificity. The phospholipase A of both cobra and *Vipera palestinae* venoms hydrolyse phospholipids in soluble state, such as in egg yolk and plasma. However, whereas the cobra phospholipase A readily attacks the phospholipids in human osmotic red blood cell ghosts[10] and blood platelets,[17] cat brain homogenates and cat brain mitochondria,[18] the *Vipera palestinae* phospholipase A has no such activity. This may reflect a possible biochemical correlation with the difference in neurotoxicity of the separated phospholipase-containing fractions of the *Naja naja* and *Vipera palestinae* venoms, the former being toxic to cats, the latter non-toxic to both mice and cats.[15]

A specific action of cobra venom phospholipase A on nervous membranes was demonstrated by Tobias,[19] who used the isolated lobster giant axon. The inactivation of the action potentials was not accompanied by electron microscopically demonstrable membrane changes. The assumption of a direct central action of *Naja naja* phospholipase A, however, as yet lacks an in vitro corrolary, since the enzyme, which is able to split phospholipids in brain homogenates, does not act on intact brain slices.[18]

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