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ACTION OF *NAJA NAJA* AND *VIPERA PALESTINAE* VENOMS ON CAT BRAIN PHOSPHOLIPIDS *IN VITRO**

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Abstract—The action *in vitro* of *Naja naja* and *Vipera palestinae* venoms on phospholipids in cat brain slices, homogenates and mitochondria has been studied. Both venoms have no or little phospholipid splitting activity when applied to brain slices. *Naja naja* venom as well as its electrophoretically separated phopholipase A are able to hydrolyze lecithin, phosphatidyl ethanolamine, phosphatidyl serine and plasmalogen in cat brain homogenates and mitochondria, but *Vipera palestinae* venom is ineffective.

INTRODUCTION

THE role of phospholipase A in the neurotoxic action of snake venoms has been a subject of several studies.[1, 2, 3] Braganca and Quastel[4, 5] and Hudson, Quastel and Scholefield[6] ascribed the neurotoxicity of the Indian cobra (*Naja naja*) venom to its phospholipase A. It soon became clear, however, that the neurotoxic action of various venoms, including those of the Cobra is not related to this enzyme, since their neurotoxic and phospholipase A activities could be separated by different fractionation procedures.[7–13] On the other hand, a role has been ascribed to the venom phospholipase A in facilitating the penetration of neuropharmacologically active substances—curare and acetylcholine—into nervous tissue such as the squid giant axon.[14]

Previous studies in our laboratory[15] have revealed a marked difference in the action of phospholipases A from cobra (*Naja naja* and *Ringhals*) venoms and *Vipera palestinae* venom on the phospholipids in cell membranes. Whereas cobra phospholipase A is able to split the phospholipids of human and animal osmotic red cell ghosts[15] and of human platelets,[16] *Vipera palestinae* phospholipase A is ineffective on these systems. In view of the as yet unclear relationship between venom phospholipase A and neurotoxic activity it seemed of interest to study the action of these different venoms and their phospholipases on brain tissue.

MATERIALS AND METHODS

Pretreatment of animals

Domestic adult cats kept on a standard diet were used. The cats were killed by decapitation, the brains were removed immediately and thoroughly cleaned from blood vessels.

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Tissue preparations

Slices of whole fresh brain were cut free hand with a razor blade moistened with saline. White and grey matter was not separated, but it was tried to obtain samples containing about equal amounts of both. The average thickness of a slice was about 1·5 mm. Samples of 100 mg weight were used for each experiment.

Homogenates were prepared in a glass homogenizer with a Teflon pestle. The homogenation was carried out in saline or saline containing the appropriate venoms.

Mitochondria were isolated by gravitational separation from homogenates in 0·25 M sucrose solution, according to the procedure described by Biran and Bartley.[17] Protein content of mitochondria suspension was determined by the turbidimetric method.

Venoms

Vipera palestinae venom was obtained by the courtesy of Drs. H. Mendelsohn and E. Kochwa from the serpentarium of the Tel Aviv University, and freeze-dried in our laboratory. Cobra venom (*Naja naja*), freeze dried, was purchased from L. Light and Co. Ltd., Colnbrook, England. In some experiments boiled venom was used; the venoms were heated on a water bath at pH 5·5 for 15 min and then centrifuged.

Venom fractions

Phospholipase A from *Vipera palestinae* venom was obtained by fractionation on a diethylaminoethyl (DEAE) cellulose column.[18] The phospholipase containing fraction was boiled as above and then centrifuged in order to remove the precipitated hemorrhagin. Paper electrophoresis of the clear supernatant, performed at pH 7·0, using phosphate buffer M/15 ,showed one single protein band. Phospholipase A from cobra (*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. The appropriate protein bands were cut off from the unstained strips and eluted with distilled water at $+4^\circ$ overnight. Sodium chloride was added until isotonicity. Figure 1 shows 6 bands in the electropherogram. Only the fraction remaining at the application line and designated fraction 6, contained phospholipase A activity. Analytical ultracentrifugation of the electrophoretic phospholipase A fraction, dialysed against saline, was carried out with a model E, Spinco ultracentrifuge at 16·5°, using the standard 12-mm cell. A single peak was obtained corresponding to a sedimentation constant $S_{20} = 3{\cdot}65 \times 10^{-13}$.

Phospholipase A activity

One ml egg yolk diluted ten times with saline was incubated at 37° for increasing periods of time with venoms or phospholipase A fractions (containing 0·5 μg protein as estimated by the Lowry reaction.[19]) The unesterified fatty acids liberated were determined by titration using the method of Dole.[20]

Incubation procedure

Incubation of tissue was generally carried out with shaking at 37° for 60 min. Suitable amounts of venoms or venom fractions dissolved in 2 ml saline were used for each 100 mg tissue. The respective amounts were chosen so as to have equal

phospholipase activity when tested on egg yolk. When different times of incubation or different amounts of venoms were used, this will be indicated in the experiments. In some experiments brain slices were incubated with venom in the presence of purified lysolecithin or cat blood or plasma in which lysolecithin had evolved by pretreatment with whole *Naja naja* venom. Two ml of whole blood or plasma were preincubated at 37° for 20 min. with 2·5 mg venom and then added to a brain slice. The system was incubated for 1 hr more and the slice washed once with 10 ml saline before phospholipid extraction. When lysolecithin was used, 3 mg of purified preparation were added to the brain slice in 2 ml saline containing 2·5 mg *Naja naja* venom.

Phospholipid extraction procedure

The tissue slices, homogenates or mitochondria were extracted following removal of the venom-containing supernatant. The mitochondria were precipitated in 0·25 M sucrose in a Spinco centrifuge at 11,000 rev/min. Extracts of the supernatants, evaporated to dryness, were shown not to contain even traces of phospholipid as determined by chromatographic analysis. Following separation the tissues were kept in 2 ml methanol for 30 min at +4° in order to inactivate traces of remaining venom. The tissue slices were then homogenized in the methanol solution. Subsequently 4 ml chloroform were added to the tissue in methanol solution and the extraction proceeded at room temperature for 30 min. After centrifugation of filtration the residue with the filter paper was extracted twice more with methanol chloroform (1 : 2, v/v). The three extracts were combined, filtered and evaporated to dryness in a nitrogen stream. The residue was re-extracted with the above solvents in two portions and made up to 1 ml in a volumetric flask.

Silicic acid—paper chromatography. Separation of the various phospholipids from the extracts and their quantitative estimation were performed as described previously.[22] Usually 40 μl of the extract were used for each strip and spots of two strips combined for combustion. All analyses were made in duplicate.

RESULTS

Both *Naja naja* and *Vipera palestinae* venoms had no or little phospholipid splitting activity when applied to brain slices in large amounts—per 100 mg tissue 5 mg *Vipera palestinae* venom or 2·5 mg *Naja naja* venom, in this proportion having equal phospholipase activity when tested on egg yolk. Prolongation of incubation time, raising venom concentration, or addition of calcium ions were not effective in procuring splitting of the phospholipids in brain slices. On the other hand, the phospholipids: phosphatidyl ethanolamine, phosphatidyl serine, lecithin and plasmalogen in brain homogenates and mitochondria were readily split by *Naja naja* venom but not by *Vipera palestinae* venom (Figs. 2 and 3 and Table 1, the latter giving data only for splitting of lecithin.) Calcium ions increased the rate of phospholipid splitting in brain homogenates in the case of *Naja naja* venom, but did not affect the result obtained with *Vipera palestinae* venom. The enhancement of brain homogenate splitting by *Naja naja* venom in the presence of calcium ions is in line with the observation of Dawson[23] on the requirement of calcium ions for the hydrolysis of lecithin and phosphatidyl ethanolamine by purified *Naja naja* phospholipase A.

As may be seen from Figs. 4 and 5 the isolated phospholipase fraction of the *Naja naja* venom was able to split the phospholipids in brain homogenate and mitochondria equally well as whole *Naja naja* venom. Apparently no other venom components, as for instance the direct lytic factor (DLF, capable of hemolyzing washed red blood cells[15]), are required for the action of the *Naja naja* phospholipids in brain homogenate and mitochondria. This is exemplified also by the observation that boiled or heparinized venom, the latter being devoid of DLF activity, were effective in brain homogenate splitting, equally to the untreated venom (Fig. 4).

TABLE 1. ACTION OF VENOMS ON BRAIN TISSUE

		Lecithin P (μg/100 mg tissue)					
		Naja naja			*Vipera palestinae*		
	Incubation time (min)	Control	Venom	Venom Ca^{2+}	Control	Venom	Venom Ca^{2+}
Slices	60	39·0	35·0		39·0	37·0	
	180	48·4	42·8		48·4	42·8	
	60	37·4	38·4	36·0	37·4	39·8	36·5
Homogenates	60	32·0	11·9		32·5	35·7	
	60	42·5	20·5	6·25	41·0	43·0	37·5
Mitochondria	60	23·5	7·65		23·5	27·4	
	60	21·7	11·2		21·7	19·2	

Incubation system: per 100 mg tissue 2 ml saline containing 2·5 mg *Naja naja* venom or 5 mg *Vipera palestinae* venom, at 37°. Calcium was added to a final concentration of 2 mM.

The protein content of the mitochondria suspension was 6·8 mg, as compared with 8·5 mg present in 100 mg brain tissue.

In order to investigate whether products, evolving by the action of *Naja naja* venom on blood, such as lysolecithin, might enable the *Naja naja* phospholipase to split the phospholipids in brain slices, experiments were carried out in which *Naja naja* venom was acted upon brain slices in the presence of purified lysolecithin or cat blood or cat plasma preincubated with the venom. It was found that also in the presence of venom-treated blood or plasma (preincubated for 20 min at 37°), the *Naja naja* venom did not induce phospholipid splitting in the brain slices (with venom treated blood: lecithin phosphorus in control slice 46·8 μg/100 mg tissue venom-treated 46·6 μ g/100 mg tissue; with venom-treated plasma 51 and 51·5 μg/100 mg tissue, respectively. Furthermore, addition of purified lysolecithin (3 mg/100 mg tissue) did not enable *Naja naja* venom to attack the phospholipids in the slices (lecithin phosphorus in control slice 51 μg/100 mg tissue, after action of the venom 49·5 μg/100 mg tissue).

DISCUSSION

The observations made in the present study on the action of the venoms of *Vipera palestinae* and *Naja naja* on cat brain phospholipids add further evidence for a specific difference between their phospholipases A, which had been previously demonstrated by applying the enzymes to other biological structures. Similarly to the action of these venoms and their phospholipases on osmotic red cell ghosts[15] and blood platelets,[16] *Naja naja* venom as well as its isolated phospholipase readily splits the phospholipids: phosphatidyl ethanolamine, phosphatidyl serine, lecithin and plasmalogen in cat

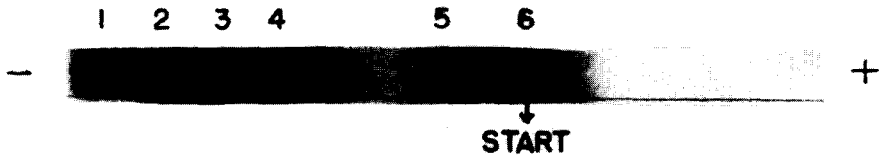

FIG. 1. Paper electropherogram of *Naja naja* venom. Phospholidase A activity is contained in fraction 6 only.

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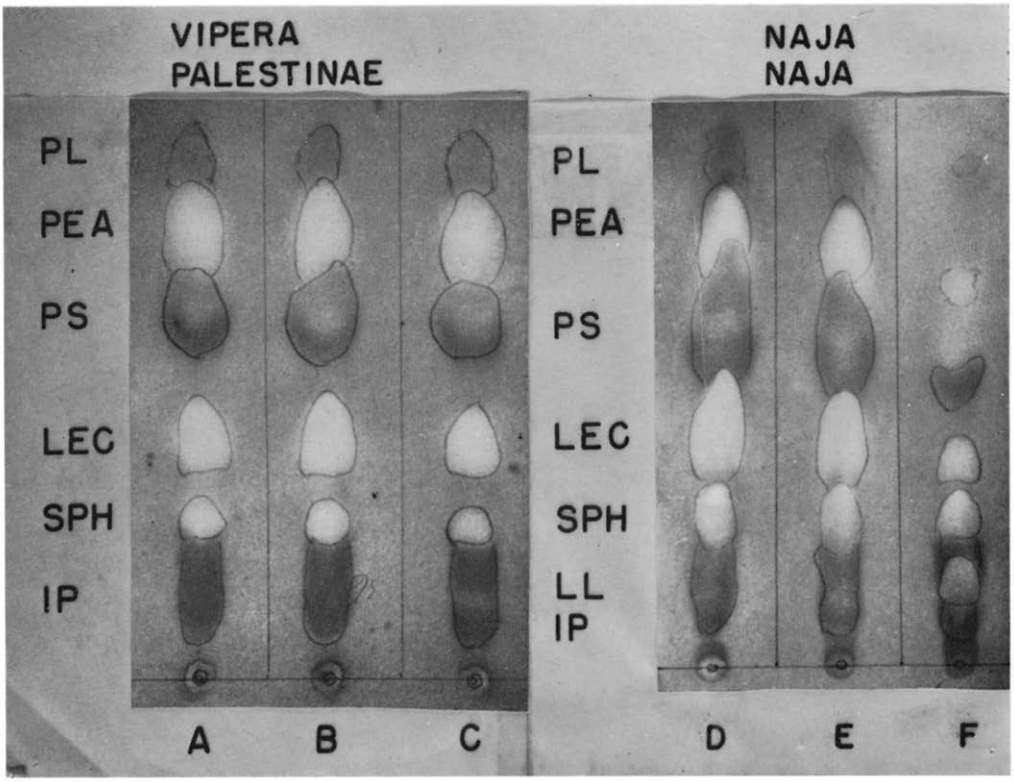

FIG. 2. Action of venoms on brain slices and homogenates. *Vipera palestinae* venom: A control, B slice, C homogenate. *Naja naja* venom: D control, E slice, F homogenate. Concentrations as in legend of Table 1. Incubation at 37° for 60 min. Abbreviations: PL plasmalogen; PEA phosphatidyl ethanolamine; PS phosphatidyl serine; LEC lecithin; SPH sphingomyelin; IP inositol phosphatide; LL lysolecithin.

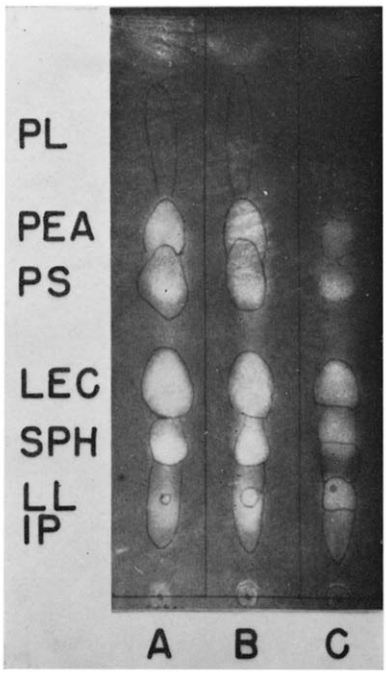

FIG. 3. Action of venoms on mitochondria. A control, B treated with *Vipera palestinae* venom, C treated with *Naja naja* venom. Concentrations as in legend of Table 1. Incubation at 37° for 60 min.

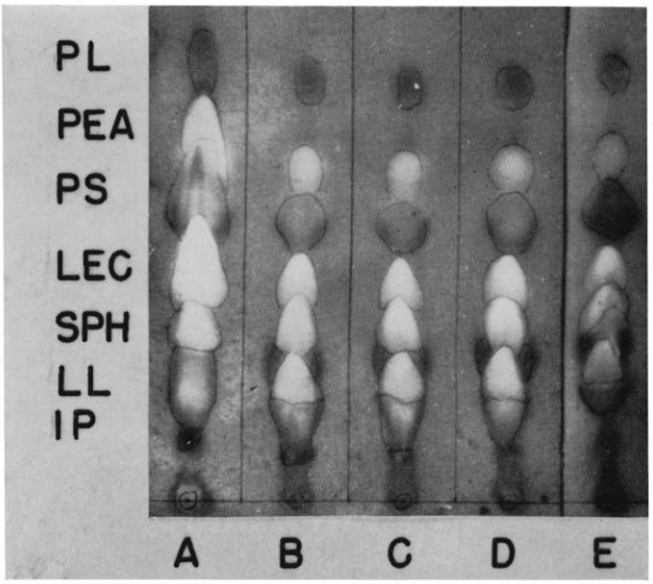

FIG. 4. Action of boiled and boiled-heparinized *Naja naja* venom and of *Naja naja* phospholipase electrophoretic fraction on brain homogenate. A control, B venom, C boiled (15 min on waterbath at pH 5·5) venom, D heparinized-boiled venom, E phospholipase fraction. Systems: per 100 mg homogenate 1·5 ml saline containing 0·75 mg venom or in C, D and E amounts having equivalent activity on egg yolk. Heparin (National Biochemicals Corporations, Heparin–sodium 100 U/mg was added in an amount of 0·75 mg in 0·1 ml saline. Lecithin phosphorus values expressed in μg/100 mg homogenate, after incubation at 37° for 60 min: A 47·6; B 22·6; C 21·1; D 25·2, E 23·2.

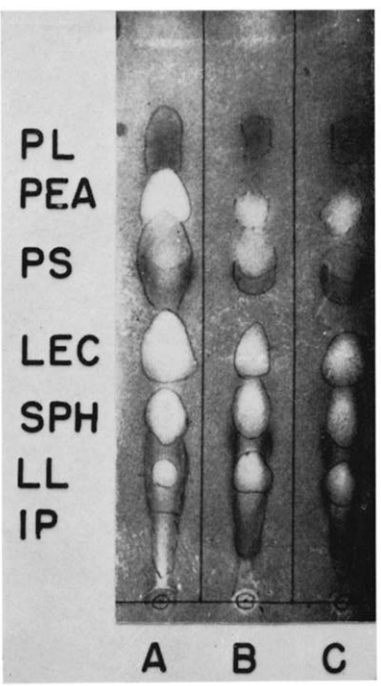

FIG. 5. Action of *Naja naja* phospholipase fraction on mitochondria. System: 1·5 ml mitochondria suspension in saline (protein content 8·4 mg) containing 1·44 mg venom or 0·85 mg phospholipase fraction, in this proportion having equal activity on egg yolk. A control, B venom, C, phospholipase fraction. Lecithin phosphorus values in suspension, expressed in μg ,after incubation at 37° for 60 min: A, 23·7; B, 12·5; C, 15·6.

brain homogenates and mitochondria, whereas *Vipera palestinae* venom is unable to do so, although applied in quantities, having equal enzymatic activity on egg yolk phospholipids.

There is a marked analogy between the actions of *Naja naja* phospholipase A on the red blood cell and cat brain phospholipids in that the enzyme is not or only little able to attack the phospholipids in the intact cells (washed erythrocytes and brain slice), but readily splits their phospholipids when the cell membrane has been altered or disrupted (osmotic ghosts, brain homogenate and mitochondria). Seemingly, the analogy does not hold when comparing the action of whole *Naja naja* venom on brain slices with that on intact red blood cells, since the venom does produce complete phospholipid splitting in the latter but not in the former. This discrepancy, however, is only apparent since the phospholipid splitting in the erythrocytes is conditioned by another *Naja naja* venom component, a basic protein designated direct lytic factor (DLF), which itself having no phospholipase activity, alters the erythrocyte membrane, so as to render its phospholipids susceptible to the venom phospholipase. In contradistinction, the direct lytic factor is not able to render the phospholipids in the intact brain cells available to the action of *Naja naja* phospholipase. The difference in the response of intact erythrocytes and brain cells to *Naja naja* venom, therefore, does not reflect a different susceptibility of the membranes to the *Naja naja* phospholipase but to the DLF. Interestingly, DLF is not required for the phospholipid splitting action of the *Naja naja* venom on brain homogenate and mitochondria. These relationships have been schematically presented in Table 2.

TABLE 2. DIFFERENCE IN ACTION OF VENOMS AND THEIR PHOSPHOLIPASES ON VARIOUS BIOLOGICAL SYSTEMS

	Phospholipid splitting*			
	Vipera palestinae		*Naja naja*	
	Whole venom	Isolated phospholipase A	Whole venom	Isolated phospholipase A
Cat brain slice	—	—	—	—
Cat brain homogenate	—	—	+	+
Cat brain mitochondria	—	—	+	+
Erythrocyte	—	—	+†	—
Erythrocyte osmotic ghost	—	—	+	+
Platelet	—	—	+	+
Egg yolk	+	+	+	+
Plasma	+	+	+	+

* Using amounts of venoms or phospholipase fractions, equally effective on egg yolk.

† *Naja naja* DLF alters erythrocyte membrane enabling phospholipase to act.

It would seem an attractive hypothesis to relate possible differences in neurotoxicity or neurotoxicity-facilitating action of phospholipases A from various snake venoms to the specific ability of these enzymes to attack phospholipids in brain tissue. However, the inability of the allegedly neurotoxic *Naja naja* phospholipase to split the phospholipids in 'intact cells' in brain slices, as contrasted to its ability to split phospholipids in brain homogenates and mitochondria, raises the questions of how to explain penetration of cobra phospholipase into brain cells or whether such penetration is at all necessary for neurotoxic venom action. Evidence has been put forward for

the ability of snake venom phospholipase to affect whole cell preparations of brain or spinal cord resulting in inhibition of respiratory activity and uncoupling of oxidative phosphorylation.[6] Such effects would presuppose access of the enzyme to the mitochondria. As indicated by the experiments *in vitro* it is improbable that the *Naja naja* DLF would be responsible for facilitation of phospholipase penetration *in vivo*. Furthermore, in the light of the experiments in which *Naja naja* phospholipase was applied to brain slices in the presence of venom-treated cat blood or plasma, or of purified lysolecithin, there is no evidence for facilitation of phospholipase penetration by factors evolving in blood due to venom action. Whereas, therefore the present observation that *Naja naja* phospholipase produces no or only little splitting of the phospholipids in intact brain cells does not clarify the problem of its possible neurotoxicity or neurotoxicity-facilitating action, the possibility must be considered that venom phospholipases induce 'minor chemical reactions' in the cell membrane, as suggested by Rosenberg and Podleski,[24] and evidenced by leakage of enzymes.[25] Also in that case, however, the specific difference in venom phospholipases, demonstrated by using various biological systems, could be determinative in venom neurotoxicity, since even such 'minor changes' induced in the membrane phospholipids may presuppose a specific ability of the enzyme to reach its substrate.

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