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COMPARISON OF A RELATIVELY TOXIC PHOSPHOLIPASE A_2 FROM *NAJA NIGRICOLLIS* SNAKE VENOM WITH THAT OF A RELATIVELY NON-TOXIC PHOSPHOLIPASE A_2 FROM *HEMACHATUS HAEMACHATUS* SNAKE VENOM—II

PHARMACOLOGICAL PROPERTIES IN RELATIONSHIP TO ENZYMATIC ACTIVITY*

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Abstract—Despite a remarkable degree of homology in amino acid sequence, the neutral phospholipase A_2 from *Hemachatus haemachatus* venom is much less toxic than the basic phospholipase A_2 from *Naja nigricollis* venom, the i.v. LD_{50} in mice for the two being, respectively, 8.6 and 0.63 mg/kg. Similarly following intraventricular injection into rats, the neutral phospholipase showed convulsant and lethal $dose_{50}$ values of about 7.5 and 15 μg per rat, respectively, whereas corresponding values for the basic phospholipase were 0.5 and 0.5 μg per rat. Death appears to be due to congestion, hemorrhage and edema in the lungs. Consideration of dosages required and times until onset of action suggests that, dependent upon the route of administration, the effect is either mediated via a central action or is due to a direct effect on the cardiac and/or respiratory system in the periphery. The pattern and extent of phospholipid hydrolysis in various brain regions was similar following intraventricular injection of the two phospholipases so that no relationship between phospholipid hydrolysis and lethal potency could be established. Concentrations of 5 and 10 μg/ml of the *N. nigricollis* and *H. haemachatus* phospholipases, respectively, were required to block electrical activity of the isolated single electroplax. The ultrastructural changes produced by both phospholipases were also similar. Parallel to the somewhat greater potency on the electroplax, *N. nigricollis* phospholipase produced slightly greater overall hydrolysis in the innervated and non-innervated membranes of the electroplax than did *H. haemachatus* phospholipase. The results suggest that these two phospholipases do not have a specific junctional effect and that the small difference in potency on the junction cannot be responsible for the large difference in lethality observed in mammalian species.

The main lethal components in many snake venoms are non-enzymatic toxins which have post-synaptic curare-like actions [1]. However, certain pre-synaptically acting toxins have phospholipase A_2 (EC 3.1.1.4) activity and, indeed, many venom phospholipases have potent and varied biological effects [2]. In general, basic phospholipase A_2 enzymes have been found to be more toxic than the acidic or neutral phospholipases. However, no direct relationship has been observed between biological potency and the level of *in vitro* enzymatic activity [2].

We chose for our studies two phospholipases, both derived from venoms of elapid snakes, whose complete amino acid sequences are known and show a high degree of homology. In spite of these similarities, they differ greatly in their toxicity. It is hoped that the use of these two phospholipases in our studies will allow us to determine if differences in toxicity can be related to differences in phospholipid hydrolysis. To define more clearly the role of phospholipase activity in toxicity, future studies will examine how structural modifications of the phospholipases alter their pharmacological and enzymatic effects.

In our preceding paper [3], we reported that a basic, toxic phospholipase A_2 from *Naja nigricollis* venom differs from a non-toxic, neutral phospholipase A_2 from *Hemachatus haemachatus* venom in substrate specificity. The *N. nigricollis* enzyme induced hemolysis and complete hydrolysis of membranal phospholipids in guinea pig red blood cells and stored human red blood cells, whereas the *H. haemachatus* enzyme was non-hemolytic and hydrolyzed only the externally oriented membranal phospholipids.

In this paper we have attempted to determine whether phospholipid hydrolysis induced by these two enzymes is correlated with their pharmacological

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activities, including i.v. LD_{50} values in mice, effects on the electrical activity and u/Tr_2 structure of the eel electroplax, potency following intraventricular injection into rats, and pathological effects in tissues of rats and mice.

MATERIALS AND METHODS

Pure phospholipases A_2 (EC 3.1.1.4) were isolated from *H. haemachatus* (Fraction DE-1) and *N. nigricollis* (CMS-9) crude venoms by one of the authors (C. C. Y.), using methods described in our preceding manuscript [3]. Triton X-100 was purchased from the Sigma Chemical Co., St. Louis, MO. Silica gel HR (Merck) and ninhydrin spray reagent were purchased from Brinkmann Instruments, Inc., Westbury, NY. Crystalline bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were obtained from the Sigma Chemical Co., and Fiske–Subba Row reagent and Folin–Ciocalteu reagent from the Fisher Scientific Co., Fair Lawn, NJ. β-Bungarotoxin was purchased from Miami Serpentarium Laboratories, Miami, FL.

LD_{50} *determinations*. Swiss–Webster mice (22–25 g) were injected intravenously via the tail vein with the phospholipases contained in 0.1 ml of mammalian Ringer's solution [4]. Times to death and signs associated with envenomation were recorded over an 8 hr period. Due to the limited supplies of pure phospholipases A_2, the LD_{50} value was estimated by injecting six animals at each of seven dosages (range 90–720 μg/kg) for *N. nigricollis* venom phospholipase A_2 and two animals at each of seven dosages (range 3800–11,000 μg/kg) for *H. haemachatus* venom phospholipase A_2. For both phospholipases the LD_{50} values were estimated as the midpoint between the highest dosage lethal to less than 50 per cent of the mice and the lowest dosage lethal to more than 50 per cent of the mice injected.

Extracellular recording from the eel electroplax. Electric eels *(Electrophorus electricus)*, about 1.5 m in length, were purchased from Worldwide Aquarium (Ardsley, NY) and maintained at 28° in an aerated and filtered aquarium. At room temperature, using extracellular recording electrodes, action potentials following direct and indirect (synaptic) membrane depolarization were recorded from single cells isolated from the Sachs organ [5], and incubated in eel Ringer's solution [6]. The preparation was stimulated every 30 sec with monophasic pulses of 0.1 msec duration.

Phospholipid and protein analysis in eel electroplax. Isolated single electroplax were incubated at room temperature for 30 min with the appropriate concentration of phospholipase A_2 in eel Ringer's solution. Incubation was terminated, and enzymatic activity was stopped by the addition of EDTA to a final concentration of 10 mM; the cells were frozen overnight. After thawing, the innervated and non-innervated membranes were separated by micro-dissection as described previously [7,8]. The membranes were blotted, lyophilized and weighed, and the lipid fraction was extracted with chloroform–methanol (2:1) [9]. Protein was determined on the residue using the method of Lowry *et al.* [10], as modified by Markwell *et al.* [11]. Phospholipids were separated by thin-layer chromatography on a micro scale using a modification of the method of Kleinig and Lempert [12] as described by Knickelbein and Rosenberg [13]. After visualization with iodine vapors and ninhydrin, the phosphorus contents of the separated spots were determined [13]. Total lipid phosphorus values were determined on aliquots of the lipid extract.

Electron microscopy. Isolated single electroplax cells were incubated at room temperature for 30 min with eel Ringer's solution containing phospholipase A_2. The reaction was terminated by placing the cells in a mixture of 3 per cent formaldehyde and 0.5 per cent glutaraldehyde in 0.005 M phosphate buffer, pH 7.2, containing 0.1 per cent magnesium chloride. Further steps of preparation, including postfixing in 2 per cent osmium tetroxide and photography in a Phillips EM 300 electron microscope were as described previously [14].

Intraventricular injection of phospholipase A_2. The phospholipases in 50 μl of artificial cerebrospinal fluid [15] were injected into the right lateral ventricle of male albino Sprague–Dawley rats (300–400 g) following the method of Noble *et al.* [16] as described previously [17].

Approximate values for convulsant $dose_{50}(CD_{50})$ and lethal $dose_{50}(LD_{50})$ were calculated using a minimum of four animals in each dosage group for each phospholipase A_2.

Phospholipid analysis in rat brain. For the *in vivo* studies, brains were removed after decapitation at a predetermined time interval (for rats injected with 2.5 μg of phospholipase A_2 from *H. haemachatus*) or at the time of death (all other animals). Each brain was placed immediately in cold mammalian Ringer's solution [4] which contained 10 mM EDTA, to block phospholipase activity, and while completely immersed in this solution was rapidly separated into the following regions: cerebellum, pons-medulla, cerebral cortex, and the rest of the brain. The brain regions were then homogenized in a Potter–Elvehjem homogenizer in mammalian Ringer's –EDTA solution.

For the *in vitro* studies, rats were decapitated and whole brain or brain regions were homogenized as described above except for the omission of EDTA. Phospholipase A_2 was added in a 50 μl vol. of mammalian Ringer's solution to a 1-ml aliquot of each homogenate, incubated in a shaker bath at 37° for 30 min and the lipids extracted. In a number of experiments, Triton X-100 was added prior to enzyme treatment in a 9:1 molar ratio of Triton–phospholipid.

Lipids were extracted using chloroform–methanol (1:3, v/v) according to the method of Folch *et al.* [18]. Thin-layer chromatography [19] was used to separate individual phospholipids which were visualized in iodine vapor and ninhydrin, scraped, and the phosphorus content determined [20].

Histologic preparation of tissues. Tissue sections (6 μm) were prepared from formalin-fixed paraffin-embedded tissues taken from the brain, heart, kidney, liver, lung, spleen, adrenal gland, and small and large intestine. These were stained with hematoxylin and eosin.

RESULTS

Lethality and convulsant effects in mice and rats.

Table 1. Convulsant and lethal effects of purified phospholipases A_2 (PLA_2) from *H. haemachatus* and *N. nigricollis* venoms following intraventricular injection into rats*

	Dose (μg/rat)	Total No. of rats	Convulsions		Death	
			No. of rats	Onset (min)	No. of rats	Min
H. haemachatus PLA_2	5.0	4	0		0	
	10.0	4	4	372 ± 40	0	
	20.0	8	8	255 ± 25	8	390 ± 20
N. nigricollis PLA_2	0.31	6	1	231	1	276
	0.62	12	7	196 ± 28	7	276 ± 20
	2.5	10	10	136 ± 13	10	209 ± 21

* Times are presented as means ± S.E. based upon the numbers of rats shown.

The approximate i.v. LD_{50} in mice was 0.63 mg/kg for *N. nigricollis* phospholipase A_2 and 8.6 mg/kg for *H. haemachatus* phospholipase A_2. The dose–response curves for both enzymes were very steep. Times to death ranged from 30 to 120 min with either phospholipase. Signs included decreased mobility, cyanosis, lacrimation and exophthalmos. Occasionally convulsions, most probably of the agonal type, were seen immediately prior to death.

The intraventricular LD_{50} in rats was about 15 μg/animal for *H. haemachatus* phospholipase A_2 and 0.5 μg/animal for *N. nigricollis* phospholipase A_2 (Table 1). Recurrent convulsive episodes were seen following intraventricular administration of both phospholipases, resembling those reported following administration of cobra (*Naja naja*) venom phospholipase [17]. The CD_{50} values for the *H. haemachatus* and *N. nigricollis* phospholipases were about 7.5 and 0.5 μg, respectively. Seizure episodes following administration of *H. haemachatus* phospholipase A_2 were, in comparison, more prolonged, whereas *N. nigricollis*-induced convulsions were more intense. In nearly all cases, rats injected with either phospholipase A_2 experienced dyspnea from about the time the animal began to convulse to the time of death, with death appearing to be due to respiratory failure.

Pathology. Two mice were injected intravenously with *N. nigricollis* phospholipase A_2 (1 mg/kg) and two with *H. haemachatus* phospholipase A_2 (10 mg/kg). Two rats were injected intraventricularly with the *N. nigricollis* enzyme (2.5 μg), while two others received the *H. haemachatus* enzyme (20 μg). All of the animals died from the injections, after which gross and histopathologic examinations at the light microscopic level were made. Gross examination showed visceral congestion including lung and liver congestion and subserosal petechiae in all animals. The kidneys of rats and mice injected with *N. nigricollis* phospholipase had renal tubular dilatation and albumin casts (Fig. 1). No glomerular lesions were apparent at the light microscopic level; however, the renal changes are probably due to toxin-induced glomerular damage since the casts are hyaline rather than the brown color seen with hemoglobin nephropathy. Lungs of all animals showed

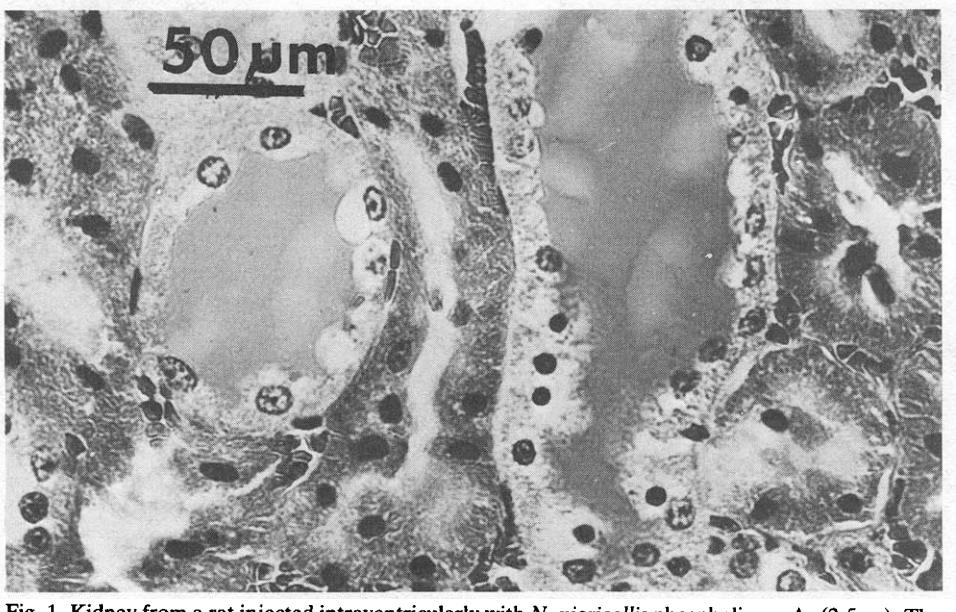


Fig. 1. Kidney from a rat injected intraventricularly with *N. nigricollis* phospholipase A_2 (2.5 μg). There are albumin casts in collecting ducts and distal convoluted tubules. Cytoplasmic vacuolization and nuclear pyknosis are also visible. Hematoxylin and eosin stain.

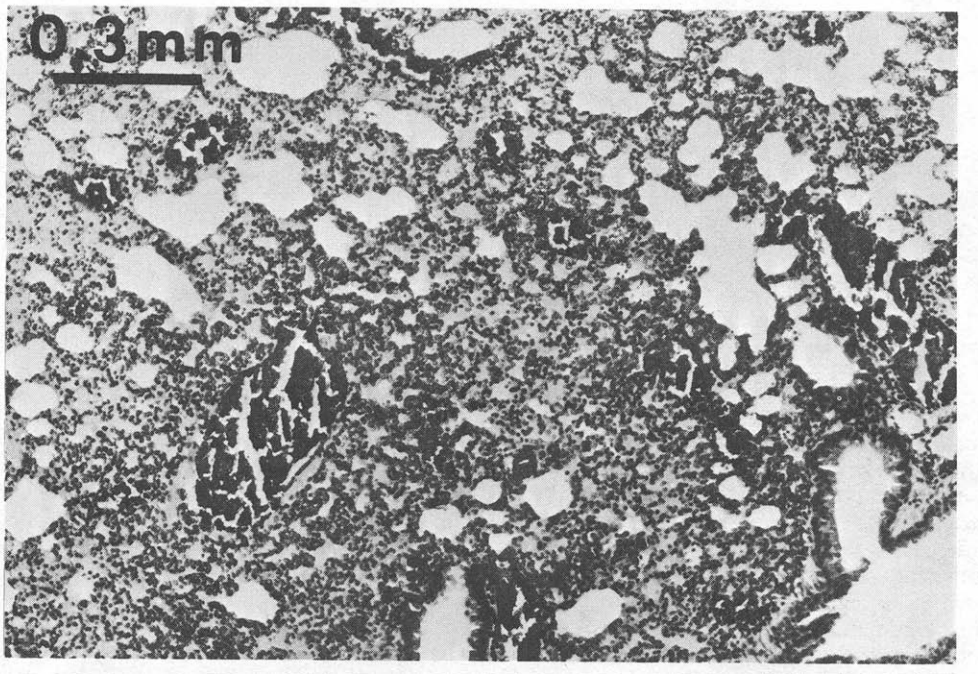


Fig. 2. Lung from a mouse injected intravenously with *N.nigricollis* phospholipase A_2 (1 mg/kg). Congestion, edema and hemorrhage are visible. Hematoxylin and eosin stain.

multifocal hemorrhages, congestion and alveolar edema (Fig. 2). The lesions could be a direct effect of the phospholipase or secondary to cardiac and respiratory failure, either centrally or peripherally mediated. These pulmonary changes were probably responsible for the deaths of the animals. The brains of the rats showed some mild suppurative chorioditis, probably associated with the trauma of intraventricular injections. The other organs appeared normal.

Hydrolysis of rat brain phospholipids in vivo. Phospholipid content in control whole brain and separate brain regions (results not shown) were in agreement with values in the literature [17,21].

Values of per cent hydrolysis are presented in Table 2 for intraventricular doses of 20 μg of each of the phospholipases A_2. A three-way analysis of variance indicated that the phospholipase A_2 from *H. haemachatus* produced a greater overall hydrolysis of phospholipids than the phospholipase A_2 from *N. nigricollis* ($P < 0.05$). Using the Newman–Keuls method [22,23], comparisons were made within classifications of phospholipase, region and phospholipid, and of interactions. Greater hydrolysis was seen in the cerebellum and cerebral cortex than in pons-medulla and rest of brain. Phosphatidylcholine and phosphatidylserine were hydrolyzed to a greater extent than phosphatidylethanolamine. There were no significant differences in the extent of phospholipid hydrolysis by the two phospholipases which could explain their marked differences in potency following intraventricular injection.

We also determined per cent hydrolysis values with both enzymes at a dose of 2.5 μg. Brains were taken, at the time of death of those rats injected with the *N. nigricollis* phospholipase A_2, since this dose was lethal only for this enzyme. The overall level and individual values for hydrolysis were similar to those obtained at the 20 μg dose level (Table 2), which is lethal for both phospholipases A_2, indicating that their difference in toxicity is not correlated with

Table 2. Hydrolysis of phospholipids in rat brain regions after injection of 20 μg of phospholipase A_2 from *H. haemachatus* or *N. nigricollis* venoms*

	Per cent phospholipid hydrolysis					
	PC		PS		PE	
Brain region	*H.h.*	*N.n.*	*H.h.*	*N.n.*	*H.h.*	*N.n.*
Cerebellum	15 ± 6	10 ± 1	18 ± 6	29 ± 3	4 ± 4	2 ± 1
Pons-medulla	8 ± 1	12 ± 3	0	3 ± 2	4 ± 2	1 ± 1
Cerebral cortex	22 ± 4	16 ± 1	25 ± 6	8 ± 5	1 ± 1	1 ± 1
Rest of brain	22 ± 5	9 ± 1	10 ± 7	2 ± 2	2 ± 2	1 ± 1

* Rats were decapitated and brains were removed at the time of death. Values of per cent hydrolysis of individual phospholipids in each brain region are presented as means ± S.E. based on four experiments for each venom. Times until death are shown in Table 1. Hydrolysis of phosphatidylinositol was inconsistent. Abbreviations: *H.h.*, *H. haemachatus* *N.n.*, *N. nigricollis*; PC, phosphatidylcholine (lecithin); PE, phosphatidylethanolamine; and PS, phosphatidylserine.

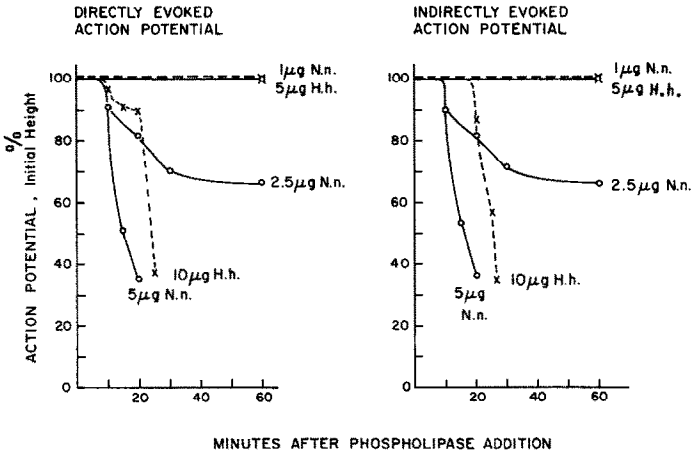


Fig. 3. Effect of phospholipases A_2 from *Naja nigricollis* (N.n.) and *Hemachatus haemachatus* (H.h.) venoms on electrical activity of the isolated single electroplax. Electroplax were mounted to expose the innervated membrane to phospholipase A_2 in 1 ml of eel Ringer's solution at room temperature, subsequent to a 30-min equilibration period. Zero time indicates the time of enzyme addition.

the extent of hydrolysis measured in brain regions.

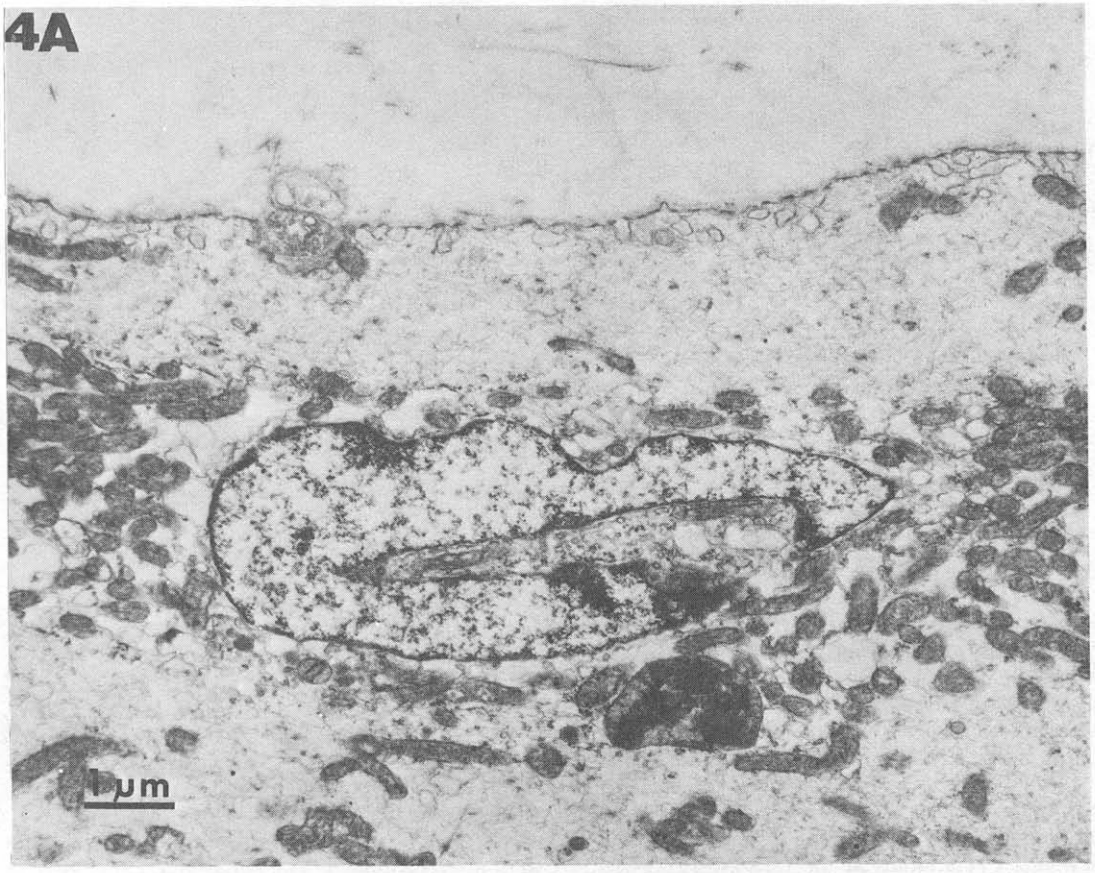
Hydrolysis of rat brain phospholipids in vitro. Phospholipase, in a range of concentrations (0.625–5.0 μg/ml), induced higher levels of hydrolysis in homogenized tissue *in vitro* (results not shown) than following intraventricular administration (Table 2). A comparison of hydrolysis levels of individual phospholipid species in the two conditions reveals that phosphatidylethanolamine is poorly exposed to enzymatic degradation in the intact tissue as contrasted to the homogenate. Homogenized tissue showed no consistent differences in hydrolysis among brain regions. The two phospholipases A_2 did not differ consistently in the extent of hydrolysis of phosphatidycholine or phosphatidylethanolamine; however, the phospholipase A_2 from *N. nigricollis* produced a greater hydrolysis of phosphatidylserine. The values of hydrolysis shown for both phospholipases A_2 in whole brains homogenized in mammalian Ringer's and Triton agree with values found for mixtures of purified phospholipids resembling the phospholipid composition of the rat brain [3].

Effects of phospholipases on electrical activity of the electroplax. The heights of the directly and indirectly evoked action potentials decreased over similar time courses after phospholipase A_2 addition (Fig. 3). The phospholipase A_2 from *N. nigricollis* venom appears to be about twice as potent as that from *H. haemachatus* venom. Concentrations of either phospholipase A_2 up to 400 μg/ml had no greater effect than that of 5 and 10 μg/ml concentrations (shown in Fig. 3) on latency or rate of action potential decrease. Complete block of the action potential was usually obtained in about 60 min. Attempts to reverse (30-min wash) the phospholipase A_2 effect after a 50 per cent decrease in action potential height were unsuccessful (results not shown). β-Bungarotoxin, a known potent pre-synaptic toxin [24,25], had no effect on electroplax electrical activity at a concentration of 200 μg/ml.

Electron microscopic findings. The effects of incubating the eel electroplax for 30 min in 200 μg of *H. haemachatus* phospholipase A_2/ml (Fig. 5) were similar to those reported for *Agkistrodon piscivorus* venom phospholipase A_2 [14] and should be compared to the control cell (Fig. 4). Mitochondrial swelling and vacuolization, disruption of membranal inpocketings, and the formation of clusters of small vesicles (approximately 0.1 μm) external to both the innervated and non-innervated membranes of the electroplax were observed (Fig. 5). The mitochondria and endoplasmic reticulum of the nerve endings were often distended. The extent of damage seen in cells treated with *N. nigricollis* venom phospholipase A_2 varied, for some unexplained reason, from little or no apparent damage to damage similar to that consistently observed with *H. haemachatus* venom phospholipase A_2 (Fig. 5). Due to this variation, no exact comparison can be made, between the two phospholipases A_2, on the extent of damage in the eel electroplax.

Phospholipid hydrolysis in the electroplax. The control values for phospholipid distribution (see footnote of Table 3) are in general agreement with previous reports [7,14]; however, we consistently observed that sphingomyelin and phosphatidylserine each constitute a greater percentage of total phospholipid content in the innervated than in the non-innervated membrane. Control total lipid phosphorus values ($\bar{x} \pm$ S.E.M.; three determinations) for the innervated and the non-innervated membranes were 1.8 ± 0.1 and 0.88 ± 0.06 μg/mg dry weight, respectively. The per cent hydrolysis of phospholipids is shown in Table 3. Since there is little difference in the amount of hydrolysis observed at concentrations of 200 μg/ml (whole cells) and 50 μg/ml (separated membranes), a plateau appears to have been reached for substrate availability in the time period studied.

A three-way analysis of variance for the data on the innervated and non-innervated membranes (Table 3) revealed ($P < 0.001$) that phospholipid hydrolysis by *N. nigricollis* phospholipase A_2 is greater than that by *H. haemachatus* phospholipase A_2. Phospholipid hydrolysis in the non-innervated membrane is greater than in the innervated mem-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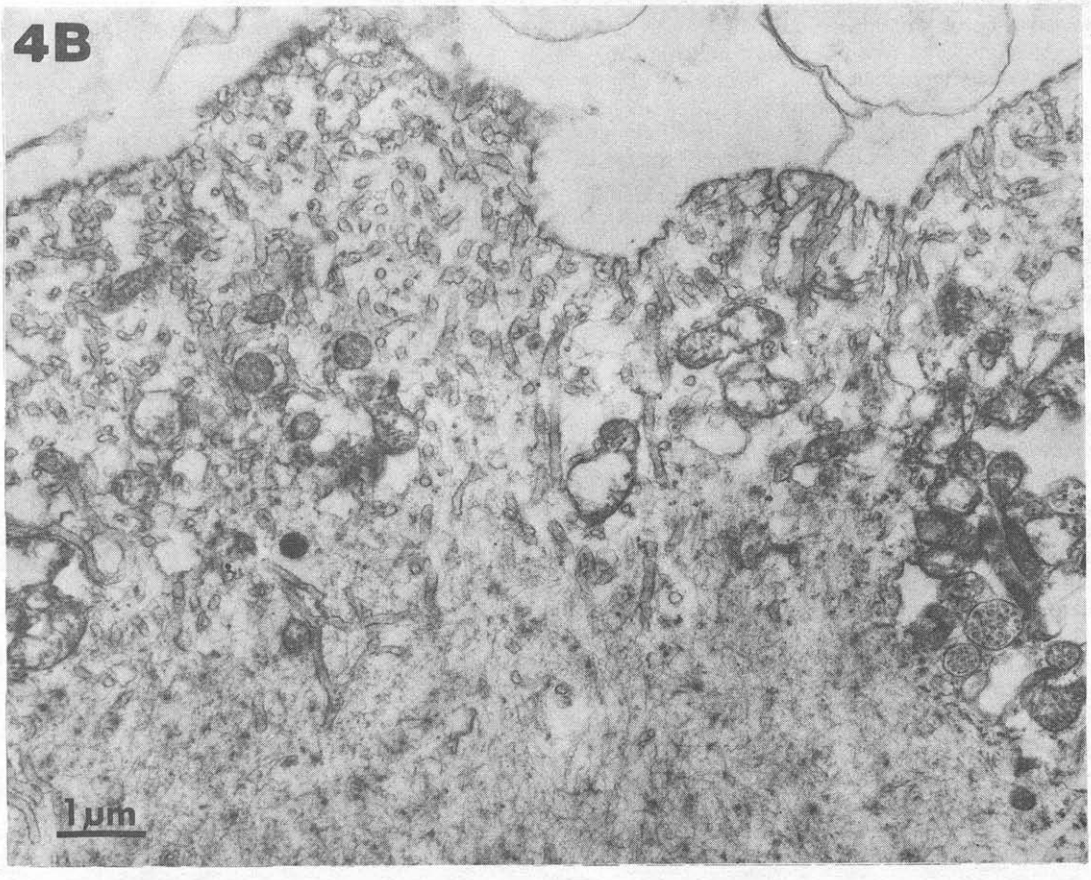
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Table 3. Hydrolysis of major phospholipids in the eel electroplax*

Tissue	Source and concn of phospholipase	Per cent Hydrolysis PC	PS	PE
Whole cells	*H. haemachatus* (200 μg/ml)	43, 36		40, 49
	N. nigricollis (200 μg/ml)	53, 40	71, 55	60, 35
Innervated	*H. haemachatus* (50 μg/ml)	25 ± 7	2 ± 10	9 ± 5
membrane	*N. nigricollis* (50 μg/ml)	37 ± 2	36 ± 5	20 ± 8
Non-innervated	*H. haemachatus* (50 μg/ml)	33 ± 6	21 ± 7	36 ± 9
membrane	*N. nigricollis* (50 μg/ml)	47 ± 3	70 ± 8	63 ± 6

* Measurements were made after incubation of whole single cells for 30 min with indicated source and concentration of phospholipase A_2. Values represent duplicate determinations for single cells and ± S.E.M. of four determinations for the separated membranes. Phosphatidylinositol was not hydrolyzed. For statistical analysis see Results section. Control per cent distribution: Whole cells: SM, 6.2 ± 0.5(5); PC, 57 ± 3(7); PI, 3.0 ± 0.6(7); PS. 7.0 ± 1.6(7); and PE, 26 ± 0.5(7). Innervated membranes: SM, 8.5 ± 1.1(8); PC, 50 ± 2(10); PI, 3.9 ± 0.4(9); PS, 11 ± 1(9); and PE, 29 ± 1(10). Non-innervated membrane: SM, 3.2 ± 0.3(11); PC, 55 ± 1(11); PI, 4.6 ± 0.9(9); PS, 7.6 ± 0.5(11); and PE, 28 ± 1(11). Abbreviations: PC, phosphatidylcholine (lecithin); PS, phosphatidylserine; PE, phosphatidylethanolamine; SM, sphingomyelin; and PI, phosphatidylinositol.

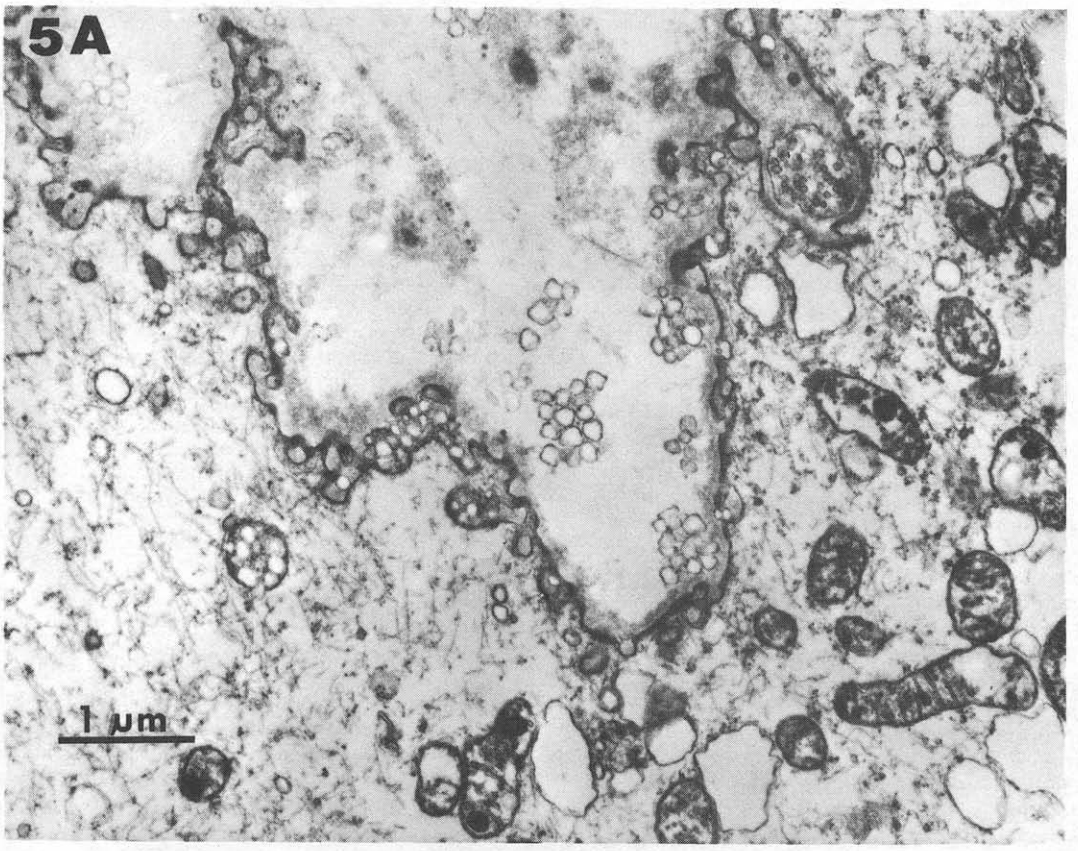
brane. The Newman–Keul's test [22,23] showed ($P < 0.05$) that *N. nigricollis* phospholipase A_2 hydrolyzes phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine at levels higher than *H. haemachatus* phospholipase A_2 hydrolyzes phosphatidylserine or phosphatidylethanolamine. In addition, *N. nigricollis* phospholipase A_2 hydrolyzes phosphatidylserine at a level higher than *H. haemachatus* phospholipase A_2 hydrolyzes phosphatidylcholine. The overall amount of phosphatidycholine hydrolysis is not significantly different between the two phospholipases. *H. haemachatus* phospholipase A_2 demonstrated a substrate preference for phosphatidylcholine over phosphatidylserine. Phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine in the non-innervated membrane are more readily hydrolyzed than phosphatidylserine and phosphatidylethanolamine in the innervated membrane. The amount of phosphatidylcholine hydrolysis is not significantly different between the two membranes. In the innervated membrane, phosphatidylcholine is more readily hydrolyzed than phosphatidylethanolamine.

DISCUSSION

The cause of death in mice injected with the phospholipases intravenously, as well as in rats receiving the phospholipases by the intraventricular route, appears to be due to the marked congestion, hemorrhage and edema in the lungs which may be a direct effect of the phospholipases or secondary to cardiac or respiratory failure. The other major pathological finding was kidney damage which was associated with the action of *N. nigricollis* phospholipase administered either intraventricularly or intravenously. It was mentioned previously [26], although data were not provided, that *N. nigricollis* phospholipase A_2 induces kidney and lung damage.

Since the pathological findings were similar following peripheral and central injection of the phospholipases, the question arises as to whether this is a centrally or peripherally mediated effect. Our finding that *N. nigricollis* phospholipase induces kidney damage, even when administered centrally, strongly suggests that this phospholipase, at least, is able to penetrate through the blood–brain barrier, contrary to previous observations [27–30]. The total amount of each phospholipase required to cause death following intraventricular injection into rats (approximately 0.5 and 15 μg, respectively, per rat) is much less than is required after intravenous injection into mice (approximately 13 and 170 μg, respectively, per 20 g mouse). Other reports have also shown that phospholipase A_2 from *Naja naja* venom [17] and from *Vipera ammodytes* venom [31] is more potent following the intraventricular route of administration as compared to peripheral routes of injection. This suggests that sites in the brain are more sensitive to, and may mediate the lethal effects of, the phospholipases, whether administered centrally or peripherally. If this is the case, however, it is difficult to explain why the time to death is 3 or more hours following intraventricular injection. These findings suggest that the ultimate cause of death (lung disturbances) following intraventricular injection may be a centrally mediated indirect effect acting on sensitive structures in the brain, with relatively long periods of time being required for the effect to become manifest in the lungs (perhaps secondary to a centrally induced cardiac failure). Following intravenous administration, the time to death is shorter because the phospholipase acts directly on the cardio-respiratory system, although the sensitivity of the structures is less than that of the brain structures. Further support for this hypothesis is a difference in effects on the electrocardiogram (ECG) between the two routes of administration (unpublished obser-

Fig. 4. Electron micrograph of control single electroplax. (A) Innervated surface. (B) Non-innervated surface. Note lack of vesicles external to the membrane and relatively normal appearance of mitochondria. Large nucleus in panel A shows some precipitated chromatin material. Note junctional region in panel A.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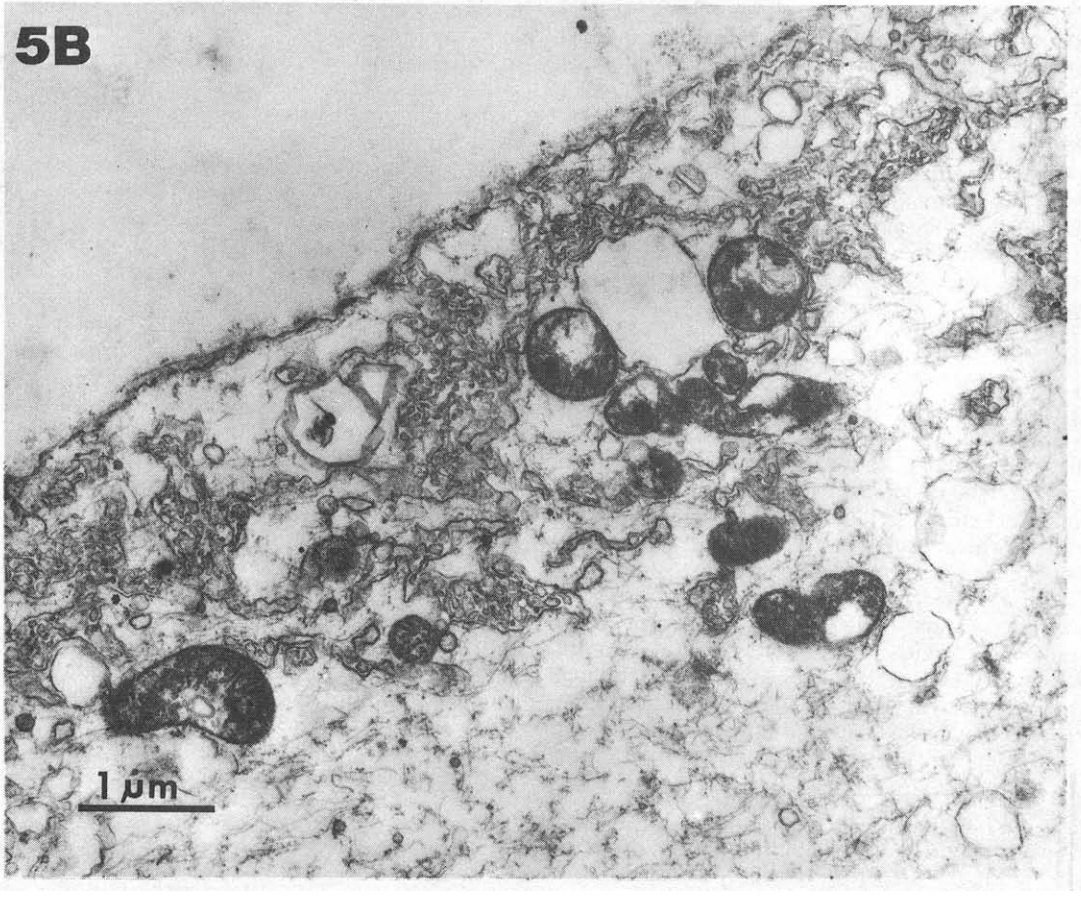
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1 µm

vations). Intravenous injection of *N. nigricollis* phospholipase A_2 in mice produced ventricular tachycardia, progressing rapidly to ventricular fibrillation which occurred approximately 1 min before cessation of respiration. Intravenous administration of *H. haemachatus* phospholipase, however, had no effect on the ECG. In contrast, after central administration into rats, the primary effect of both phospholipases involved a pattern of atrial tachyarrhythmias and decrease in atrioventricular conduction which increased in severity until cessation of respiration; ventricular fibrillation was not observed. Preliminary results in our laboratory also showed that an i.v. lethal dose of *N. nigricollis* phospholipase A_2 is associated with much higher levels of phospholipid hydrolysis in heart than are produced by an equal but non-lethal dose of the *H. haemachatus* enzyme. In contrast, the levels of hydrolysis in lung tissue were very similar for both enzymes. These results suggest that the toxicity of *N. nigricollis* phospholipase A_2, when administered intravenously, is due to a direct effect on the heart, while the less toxic enzyme has a peripheral action on some other tissue.

We confirmed previous observations [32–36] that *N. nigricollis* phospholipase A_2 is more toxic than *H. haemachatus* phospholipase as judged by our results in mice and rats following intravenous and intraventricular administration. Can this difference be related to differences in the types of phospholipid hydrolyzed? In our accompanying paper [3] we showed that these two phospholipases do differ in their substrate specificities. Our measurements of phospholipid hydrolysis in brain are of relevance to this problem since, as noted above, the central nervous system seems to be sensitive to the intraventricular injection of small amounts of these phospholipases. We found, however, that no correlation can be drawn between phospholipid hydrolysis and toxicity. We have, of course, made our analyses on large areas of the brain, and it is possible that selective differences might have been observed had we used more discrete areas. Our results resemble, in some ways, those of Gubensek *et al.* [31] who were unable to find a direct relationship between enzymatic activity and toxicity of two phospholipase A_2 isoenzymes isolated from *V. ammodytes* venom.

Following intraventricular injection, the pattern of phospholipid hydrolysis induced by the phospholipases does not agree with the specificity patterns observed *in vitro* on mixtures of pure phospholipids [3] or on brain homogenates. The *in vitro* results suggest that phosphatidylethanolamine is inaccessible to the intraventricularly administered phospholipases. The addition of Triton to the whole brain homogenate further increases the hydrolysis of phosphatidylethanolamine and makes the selectivity patterns of hydrolysis by the two phospholipases similar to those observed on pure phospholipids in mixed micelles with Triton [3].

We observed differing extents of hydrolysis in the different brain regions. This may be due partially, at least, to the fact that not all regions of the brain are equally exposed to the phospholipases when they are administered into the right lateral ventricle. In addition, the relative amounts of neuronal cell bodies (gray matter) and axonal fibres (myelin; white matter) vary in the different regions. Regardless of regional differences in phospholipid hydrolysis, the survival of animals, under conditions where 10–15 per cent of brain phosphatidylcholine and phosphatidylserine is hydrolyzed, was unexpected.

On a cellular level the electroplax functioned with even greater phospholipid hydrolysis, in agreement with results on the squid axon [37] and previous studies with electroplax [7]. *N. nigricollis* phospholipase was only about twice as potent as *H. haemachatus* phospholipase in blocking electrical activity (Fig. 3), a much smaller difference than the 15 to 30-fold difference we observed in CD_{50} and LD_{50} in rats or LD_{50} in mice. The effects of these two phospholipases on the electrical activity of the electroplax are similar to those obtained using *A. piscivorus* venom [7] which is a rich source of a relatively non-toxic phospholipase. The ultrastructural changes produced by equal doses of the two phospholipases, that is, vesiculation and mitochondrial disruption, were also similar. The relatively low concentrations of these two phospholipases required to block electrical activity of the electroplax, as compared to the pre-synaptically acting β-bungarotoxin, also suggest that they do not have a specific presynaptic action. Furthermore, both phospholipases (5 μg/ml) decrease directly and indirectly elicited muscle twitches simultaneously (unpublished observations) on the rat phrenic nerve diaphragm, a preparation known to be sensitive to β-bungarotoxin [24,25; unpublished observations], as well as to postsynaptically acting toxins.

It is of interest to compare the extent of hydrolysis and substrate specificity of these two phospholipases on electroplax phospholipids to determine whether the phospholipid substrates are accessible to the enzymes and whether the effects on electrical activity are due to phospholipid hydrolysis. In addition, because we were able to measure separately hydrolysis on the innervated and non-innervated surfaces of the cell, we could determine whether these phospholipases had any selective action on the conductive innervated membrane. Phosphatidylinositol was not hydrolyzed by either phospholipase, in agreement with previous reports using phospholipase A_2 [7,19,38,39]. We found overall phospholipid hydrolysis to be greater in the non-innervated than in the innervated membrane (Table 3). This may be due to a greater difficulty in access of the enzymes to the innervated membrane. An observation not supporting this suggestion is that phosphatidylcholine hydrolysis is about equal in the two membranes, whereas phosphatidylserine and phosphatidylethanolamine hydrolysis is much greater in the non-inner-

Fig. 5. Electron micrograph of *H. haemachatus* phospholipase A_2 treated single electroplax. Cells were exposed to the enzyme (200 μg/ml) in eel Ringer's for 30 min. (A) Innervated surface. Note the formation of numerous vesicles external to the innervated membrane and some mitochondrial disruption. (B) Non-innervated surface. Disruption of some of the mitochondria and of the branched inpocketings of the membrane is visible.

vated membrane. The differential hydrolysis in the two membranes seems to reflect structural differences determining specific phospholipid exposure. Moreover, the phospholipid composition of the two membranes varies, and so does their function.

Hydrolysis of phospholipids in the eel electroplax by *N. nigricollis* phospholipase is greater than that by *H. haemachatus* phospholipase (Table 3). In addition, *N. nigricollis* phospholipase A_2 hydrolyzed the three phospholipids to about the same extent, whereas *H. haemachatus* phospholipase A_2 demonstrated a substrate preference for phosphatidylcholine over phosphatidylserine. This agrees with the results obtained on purified substrates in a mixture simulating the eel electroplax [3]. The 2-fold difference in potency on the eel electroplax might be explained by the greater hydrolysis induced by *N. nigricollis* phospholipase and the substrate specificity differences between the two phospholipases.

In conclusion, the great difference in lethalities of the two phospholipases does not correlate with either pattern of phospholipid hydrolysis observed in brain or the small difference in effectiveness on electrical activity and phospholipid splitting in the eel electroplax. It is possible that the differences in potency of these two phospholipases following intravenous injection result from a direct membrane effect of *N. nigricollis* phospholipase on the heart. The lethality following central administration may not necessarily be associated with phospholipid splitting, but does appear to be centrally mediated.

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