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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—I

ENZYMATIC ACTIVITY ON FREE AND MEMBRANE BOUND SUBSTRATES*

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Abstract—The purified phospholipase A_2 of *Naja nigricollis* venom is a basic, relatively toxic protein, while the purified phospholipase A_2 of *Hemachatus haemachatus* is neutral and relatively non-toxic. In order to establish whether the difference in toxicity correlates with hydrolytic ability, we compared the two enzymes using substrates in various physical states such as mixed micelles, native soluble lipoprotein or organized in membranes. The purified phospholipids were used as mixed micelles with Triton X-100. When compared on purified egg L-α-phosphatidylcholine (PC), the two enzymes showed similar pH- and temperature-dependence and were equally affected by activators and inhibitors. *N. nigricollis* phospholipase A_2 had a V_{max} of 250 μ-equiv. per min per mg and a K_m of 4.2 mM, while *H. haemachatus* phospholipase A_2 had a V_{max} of 1052 μ-equiv. per min per mg and a K_m of 2.2 mM. Both enzymes favored the substrates in the liquid–crystalline state. With a buffered egg yolk dilution as substrate, a V_{max} of 356 μ-equiv. per min per mg and a K_m of 29 mM were found for *N. nigricollis*, while *H. haemachatus* had a V_{max} of 616 μ-equiv. per min per mg and a K_m of 25 mM. The hydrolysis of purified PC, L-α-phosphatidylethanolamine (PE), L-α-phosphatidylserine (PS) and L-α-phosphatidylinositol (PI) was followed with the substrates taken either singly or in various combinations. Significant differences in preference of the two enzymes were apparent on single substrates, such as the comparatively high hydrolysis of PC by *H. haemachatus* phospholipase A_2 and of PE by *N. nigricollis* phospholipase A_2. On mixtures of the four substrates, taken either in equal amounts or in proportions resembling the phospholipid distribution of human red cells, rat brain or electric eel Sachs organ, the sequence of substrate preference exhibited by the two enzymes was again strikingly different. A main feature of the *N. nigricollis* phospholipase A_2 was its high ability to hydrolyze PS. There was no essential difference between the actions of the two enzymes on fresh human red cells. However, erythrocytes from stored, outdated blood were hemolyzed and phospholipids were fully hydrolyzed by *N. nigricollis* phospholipase A_2, while the *H. haemachatus* enzyme was nonhemolytic and induced only limited hydrolysis. The same disparity in behavior could be demonstrated on fresh guinea pig erythrocytes. A comparison of hydrolysis, in permeable red cell ghosts and in Triton-solubilized membranes, by the phospholipases, revealed that the high preference of *N. nigricollis* enzyme for PS was masked by sequestration of this phospholipid within the ghosts.

Acidic, neutral and basic phospholipases A_2 (EC 3.1.1.4) have been isolated from snake venoms. In contrast to the acidic and neutral phospholipases which have relatively low toxicity, the basic phospholipases are highly toxic either as such or when in naturally occurring complexes with other subunits in venom [1, 2]. The relationship between toxicity and catalytic activity of phospholipase A_2 enzymes is as yet poorly understood. While there is evidence that catalytic activity is essential for toxicity [2], the relatively low toxicity of some venom phospholipases which have high catalytic activity suggests that the relationship is not straightforward. If enzymatic activity is to be implicated in toxicity, then one of the possibilities to be considered is that the toxic and non-toxic enzymes differ either in substrate preference and/or in ability to reach those substrates *in situ*. The present study compares the enzymatic activities of the toxic phospholipase A_2 from *Naja nigricollis* venom with the non-toxic enzyme from *Hemachatus haemachatus* venom, in an attempt to gain insight into the relationships between their enzymatic and pharmacological properties. These two phospholipases were chosen because, although they are both derived from venoms of elapid snakes and show a high degree of homology (their complete

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amino acid sequences being known), they differ greatly in toxicity [3, 4]. The basic phospholipase A_2 from *N. nigricollis* venom has an LD_{50} of 0.63 mg/kg when administered intravenously in mice, while the neutral phospholipase A_2 isolated from *H. haemachatus* venom has an LD_{50} of 8.6 mg/kg [1]. In future studies we plan to determine how structural modifications of these phospholipases alter their enzymatic and pharmacological activities.

MATERIALS AND METHODS

Materials. The neutral phospholipase A_2 (DE-I) was isolated from *H. haemachatus* venom following the procedure described by Joubert [5] and further purified on SP-Sephadex C-25 column by one of the authors (C. C. Y.). The basic phospholipase A_2 (CMS-9) was separated from *N. nigricollis* venom by chromatography on a column of CM-Sephadex C-25 and further purified on a DEAE–Sephacel column by the method of Yang and King [6]; the homogeneity was verified by disc electrophoresis.

L-α-Phosphatidylcholine from egg yolk, L-α-phosphatidylserine from brain, L-α-phosphatidylinositol from soybean, synthetic L-α-dimyristoyl and dipalmitoyl phosphatidylcholines, and Triton X-100 were purchased from the Sigma Chemical Co., St. Louis, MO. Bovine L-α-phosphatidylethanolamine was purchased from Supelco, Bellefonte, PA, and sphingomyelin from Applied Science Laboratories, Inc. State College, PA. Silica gel HR (Merck) was obtained from Brinkmann Instruments, Inc., Westbury, NY.

Phospholipid–Triton mixed micelles and egg yolk dilutions. Aliquots from the purified phospholipids in methanol–chloroform (2:1) were evaporated under a N_2 stream, and the dry material was solubilized in 0.1 M Tris–HCl buffer (pH 8.5) and supplemented with 10 mM Ca^{2+} and Triton X-100 in a 2:1 or 4:1 Triton to phospholipid molar ratio. The solutions were cleared by a few bursts of sonication in a Bronwill Biosonik III sonicator. The concentrations of the phospholipids were 6 to 10 mg/ml (~7.5 to 12.5 mM) when hydrolysis was assayed by titration of liberated fatty acids, and 0.6 to 1.2 mg/ml (~0.75 to 1.5 mM) when hydrolysis was determined by thin-layer chromatography. Egg yolk was diluted in isotonic saline buffered at pH 7.4 with 100 mM Tris–HCl buffer.

Red cell and red cell membrane preparations. Red cells were separated from whole citrated blood and washed three times in cold isotonic saline buffered at pH 7.4 with 10 mM Tris–HCl; the white blood cells were discarded at each step. The cells were suspended at 50% hematocrit in Tris-buffered saline supplemented with 1 mM Ca^{2+}. Permeable red cell membranes were obtained essentially according to the method of Dodge *et al.* [7] by hemolyzing washed red cells in 30 vol. of 10 mM Tris–HCl buffer, pH 7.4, at 4°. The membranes were sedimented at 50,000 *g* for 20 min in a refrigerated centrifuge, and repeatedly washed with the same buffer until creamy white. The membranes were resuspended, at the original cell volume, in the hemolysis buffer supplemented with 10 mM Ca^{2+}. Solubilized membranes were prepared by adding Triton X-100 to the membrane suspensions in an approximate molar ratio of 18:1 Triton to phospholipid, and the suspensions were cleared by a few bursts of sonication. The red cells, as well as the membrane suspensions, were adjusted to 1 to 1.2 mg phospholipid/ml.

Incubations with enzyme and assays of enzymatic activity. Routinely, 1 ml aliquots of the phospholipid micelles, egg yolk dilutions, red cell or red cell membrane suspensions were preincubated at 37° in a shaking water bath for 2 min, following which enzyme was added in 0.1 ml of reaction media and incubation continued for the time indicated. The hydrolysis of single purified substrates and the overall hydrolysis of whole egg yolk phospholipids were estimated by titration of the liberated unesterified fatty acids, the reaction being stopped by the addition of an acidified extraction mixture; the fatty acids were titrated with dilute NaOH as described by Dole [8]. Enzyme-treated mixtures of the purified substrates were extracted with chloroform–methanol–acetic acid (1:2:0.5; by vol.), and the phases were separated, according to the method of Adamich and Dennis [9]. The phospholipids in the organic layer were then separated by thin-layer chromatography. With enzyme-treated red cells and cell membrane suspensions the reaction was stopped by addition of 10 mM EDTA, and the lipids were extracted with methanol–chloroform mixtures [10], washed [11] and separated by thin-layer chromatography.

Other assays. Thin-layer chromatography on silica-gel coated plates developed in two dimensions [12] was routinely used for phospholipid separation. Where a better separation of phosphatidylinositol from lysophosphatidylethanolamine was sought, we employed the solvent system described by Bowyer and King [13]. Lipid phosphorus was determined as described by Bartlett [14]. Hemolysis was estimated in the supernatant fractions obtained by centrifugation of the red cell suspensions. Readings were taken at 540 nm in a Beckman DU-2 spectrophotometer and corrected for the values of spontaneous hemolysis in control samples.

RESULTS

Enzymatic activity on purified substrates and egg yolk lipoprotein. Synthetic L-α-dipalmitoyl phosphatidylcholine or purified egg phosphatidylcholine were poorly hydrolyzed by the phopholipases A_2, in agreement with data published previously [15, 16]. On mixed micelles of the phospholipids, however, with the non-ionic detergent Triton X-100 added at a 2:1 or 4:1 molar ratio, linear hydrolysis-time curves were obtained, and the latter system was used for all further determinations on purified phospholipids. With purified egg phosphatidylcholine as substrate, the *N. nigricollis* phospholipase A_2 had an apparent K_m of 4.2 mM and a V_{max} of 250 μ-equiv. free fatty acids liberated/min per mg, while for *H. haemachatus* phospholipase A_2 the corresponding values for K_m and V_{max} were 2.2 and 1052 respectively. As seen in Fig. 1, both enzymes showed similar pH dependencies in Tris–HCl buffer, with two maxima at pH 6.0 and 9.0. Both enzymes also showed a similar steady rise in activity with increasing temperatures when tested between 10 and 70° (Fig. 2). Both

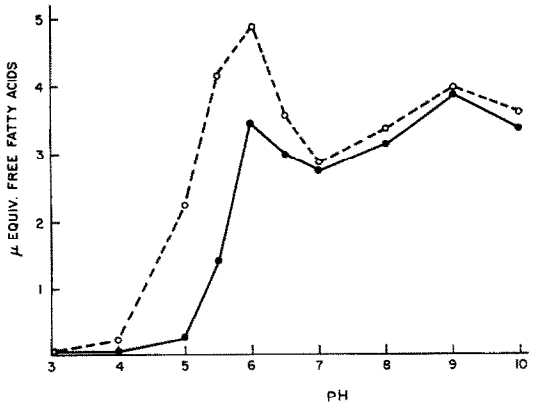

Fig. 1. pH Dependence of phospholipase A_2 activities toward egg phosphatidylcholine. Assay conditions: 1 ml of 7.5 mM phospholipid in 0.1 M Tris–HCl buffer containing 10 mM $CaCl_2$ and 15 mM Triton X-100 was incubated for 10 min at 37° with either 4 μg *N. nigricollis* or 1 μg *H. haemachatus* phospholipase. The pH of all incubation mixtures was checked before and after hydrolysis and was found to decrease by no more than 0.1–0.2 pH units, throughout the pH range of 3–10. Free fatty acids were assayed by titration. Symbols: (○– – –○) *N. nigricollis*; and (●——●) *H. haemachatus*.

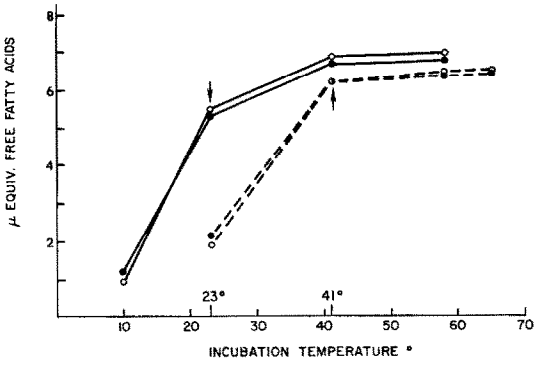

Fig. 3. Phospholipase A_2 activities toward dimyristoyl and dipalmitoyl phosphatidylcholines as a functional of transition temperatures. Assay conditions as in Fig. 1 except for the incubations being carried out at pH 8.5, with either 2 μg *N. nigricollis* or 0.5 μg *H. haemachatus* phospholipase. Symbols: (○) *N. nigricollis* phospholipase; (●) *H. haemachatus* phospholipase; (solid lines) dimyristoyl phosphatidylcholine; and (dotted lines) dipalmitoyl phosphatidylcholine. Transition temperatures at 23° and 41°, respectively, are indicated by arrows.

enzymes were equally activated by the addition of albumin and inhibited by palmitic acid and by the competitive inhibitor 2, 3-distearoyl-oxypropyl-β-hydroxyethylammonium acetate described by Rosenthal and Geyer [17]. Using synthetic L-α-dimyristoyl and L-α-dipalmitoyl phosphatidylcholines both below, at, and above their transition temperatures (23° and 41°, respectively), a strong preference for the liquid–crystalline form was evident for both the *N. nigricollis* and the *H. haemachatus* enzymes (Fig. 3).

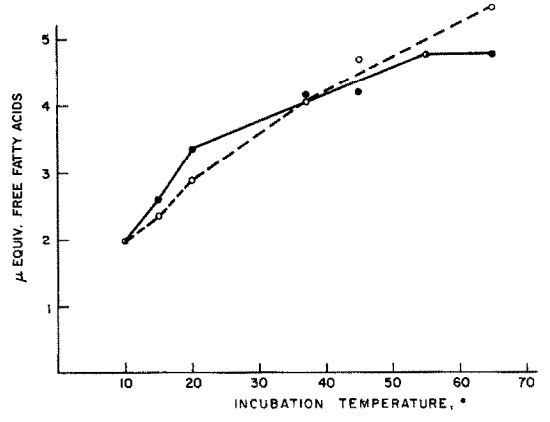

Fig. 2. Temperature dependence of phospholipase A_2 activities towards egg phosphatidylcholine. Assay conditions: 1 ml of 12.5 mM phospholipid in 0.1 M Tris–HCl buffer (pH 8.5) containing 10 mM $CaCl_2$ and 25 mM Triton X-100 was incubated for 20 min at the temperatures indicated with either 2 μg *N. nigricollis* enzyme on 0.5 μg *H. haemachatus* enzyme. Free fatty acids were assayed by titration. Symbols: (○– – –○) *N. nigricollis*; and (●——●) *H. haemachatus*.

The enzymatic activities of the two phospholipases were further compared on dilutions of egg yolk in Tris-buffered saline. Phospholipids in this native, soluble lipoprotein are readily hydrolyzed by both enzymes, without addition of detergent, the apparent K_m and V_{max} values being, respectively, 29 mM and 365 μ-equiv. free fatty acids liberated per min per mg for *N. nigricollis* and 25 mM and 616 μ-equiv. per min per mg for *H. haemachatus* phospholipase A_2. With egg yolk as substrate, the two enzymes showed largely similar pH dependencies, except for the optimal pH being 6.0 for *H. haemachatus* and 5.0 for *N. nigricollis*.

The substrate preferences of the two enzymes were investigated on four purified phospholipids from natural sources, i.e, L-α-phosphatidylcholine from egg yolk (PC), bovine L-α-phosphatidylethanolamine (PE), L-α-phosphatidylserine from brains (PS) and soybean L-α-phosphatidylinositol (PI), with the substrates taken either singly, or in various combinations. Since we established previously that *H. haemachatus* phospholipase A_2 has a higher activity on egg phosphatidylcholine than the *N. nigricollis* enzyme, we adjusted the amount of the two enzymes so as to compare hydrolysis of the other substrates in conditions giving about equal PC hydrolysis. This was achieved by using 0.5 μg of *N. nigricollis* and 0.3 μg of *H. haemachatus* enzyme (Table 1). Additional differences in the substrate preference of the two enzymes were thus revealed, most obvious being the very poor activity of the *H. haemachatus* enzyme towards PE (Table 1). A comparison of the two phospholipases, both at concentrations of 1 μg/ml, showed about 80 per cent PE hydrolysis being induced by *N. nigricollis* and only 10 per cent by *H. haemachatus* phospholipase A_2 in a 5-min incubation (not in Table 1). About 80 per cent hydrolysis of PE in 5 min was reached only by increasing the amount of the *H. haemachatus* enzyme to 10 μg. Neither of

Table 1. Phospholipase A_2 activities toward substrates taken singly and in mixtures*

Substrates a	b	N	Per cent hydrolysis ($\bar{x}$ ± S.D.) *N. nigricollis* a	*N. nigricollis* b	*H. haemachatus* a	*H. haemachatus* b
PC		2	43 ± 4		44 ± 7	
PE		2	35 ± 7		1.5 ± 0.5	
PS		2	0 ± 0		0 ± 0	
PI		3	0 ± 0		0 ± 0	
PC + PE		2	37 ± 5	76 ± 8	45 ± 4	41 ± 9
PC + PS		2	54 ± 10	30 ± 3	56 ± 10	4 ± 1
PC + PI		3	58 ± 4	28 ± 3	53 ± 13	13 ± 8
PE + PS		3	29 ± 4	17 ± 6	4 ± 2	1 ± 1
PE + PI		3	30 ± 4	0 ± 0†	4 ± 1	0 ± 0†
PS + PI		3	5 ± 4	0 ± 0	2 ± 2	0 ± 0

* The reaction mixture (1 ml) contained: 0.6 mg phospholipid (approximately 0.75 mM), 0.1 M Tris–HCl buffer (pH 8.5), 10 mM $CaCl_2$, 3 mM Triton X-100 and either 0.5 μg *N. nigricollis* or 0.3 μg *H. haemachatus* phospholipase. In mixtures of two, the substrates were taken in equal amounts. Incubations were for 5 min at 37°. The phospholipids were separated by thin-layer chromatography [10]. Abbreviations: PC, phosphatidylcholine (egg yolk); PE, phosphatidylethanolamine (bovine); PS, phosphatidylserine (brain); and PI, phosphatidylinositol (soybean). N = number of experiments.

† Chromatography according to Ref. [13].

the enzymes was active on PS or on PI when these phospholipids were assayed as single substrates. However, with both enzymes, the hydrolysis of PE was increased by addition of PC. The addition of PC also induced the hydrolysis of PS and PI, while PE induced the hydrolysis of PS by the *N. nigricollis* enzyme. In all cases, when comparing the two enzymes at amounts which hydrolyze PC at about an equal extent, the *H. haemachatus* enzyme showed less activity on PE and PI and no significant activity on PS as contrasted to the phospholipase from *N. nigricollis* (Table 1).

When all four substrates, supplemented with sphingomyelin as an internal standard, were combined either in equal proportions, or in proportions resembling the phospholipid distributions characteristic for the human red cell membrane [18], rat brain tissue [18] or electric eel Sachs organ [19], the two enzymes exhibited different sequences of substrate preference (Table 2). With the four substrates in equal proportions, the sequence for *N. nigricollis* phospholipase was PE ≫ PC = PS, while the *H. haemachatus* enzyme hydrolyzed PE and PC equally, with little or no hydrolysis of PS. No hydrolysis of PI occurred with either of the enzymes. With the three phospholipid model mixtures ('red cell', 'brain' and 'Sachs organ'), the sequences of substrate hydrolysis by the two phospholipases were strikingly different. The main feature was the ability of the *N. nigricollis* enzyme to hydrolyze PS, in contrast to the *H. haemachatus* enzyme which caused little or no hydrolysis. Because of the small amounts of PI in the model mixtures, it was not possible to measure accurately its hydrolysis.

Hydrolysis of substrates in red cell membranes. The red cell membrane, whose phospholipid composition and location within the membrane are well known [20], was used for comparing the hydrolytic ability of the two phospholipases on membrane-organized substrates. We exposed to enzymatic treatment red cell membranes in different stages of organization, such as intact cells in isotonic media, permeable red cell membranes prepared by osmotic hemolysis, as well as membranes solubilized by Triton X-100.

In intact human red cells obtained from freshly drawn blood, both phospholipases (120 μg/ml) induced a pattern of hydrolysis consistent with an attack on the externally located phospholipids, i.e. splitting of about 70 per cent of PC, 10–20 per cent of PE and little or no PS. The *H. haemachatus* enzyme was nonhemolytic in up to 2 hr of incubation, while *N. nigricollis* phospholipase induced 15–20 per cent hemolysis paralleled by a slight increase in PE hydrolysis (up to 25 per cent) in 2 hr.

While no essential difference between the actions of the two enzymes appeared where tested on red cells from freshly drawn blood, a striking disparity in behavior could be demonstrated on human red cells separated from stored, outdated blood obtained from the blood bank. These more fragile, ATP-depleted cells [21] were readily hemolyzed by *N.*

Table 2. Phospholipase A_2 activities toward mixtures of phospholipids taken in equal amounts and in 'model mixtures'*

Phospholipids mixture or model	N	Per cent hydrolysis ($\bar{x}$ ± S.D.) *N. nigricollis* PC	*N. nigricollis* PE	*N. nigricollis* PS	*H. haemachatus* PC	*H. haemachatus* PE	*H. haemachatus* PS
Equal amounts	4	45 ± 4	82 ± 3	46 ± 12	39 ± 6	39 ± 13	3 ± 4
Red cell	4–5	32 ± 12	77 ± 2	65 ± 9	47 ± 17	43 ± 11	13 ± 9
Rat brain	4	33 ± 11	78 ± 5	70 ± 4	49 ± 14	54 ± 9	17 ± 10
Eel Sachs organ	2	34 ± 1	75 ± 0	79 ± 12	37 ± 6	42 ± 10	0 ± 0

* Reaction mixture as in Table 1 except for the phospholipid concentrations being 1.2 mg/ml (approximately 1.5 mM). In the 'model mixtures' the phospholipids were taken in the following proportions: Red cell model: SM, 24; PC, 28; PI, 1; PE, 26; and PS, 13. Rat brain model: SM, 6; PC, 37; PI, 5; PE, 36; and PS, 12. Eel Sachs organ model: SM, 4; PC, 54; PI, 4; PE, 27; and PS, 5. Incubation and chromatography as in Table 1. In "Equal amounts" mixture, PI was not hydrolyzed; not determined in other mixtures. Abbreviations: PC, phosphatidylcholine (egg yolk); PE, phosphatidylethanolamine (bovine); PS, phosphatidylserine (brain); PI, phosphatidylinositol (soybean); and SM, sphingomyelin. N = number of experiments.

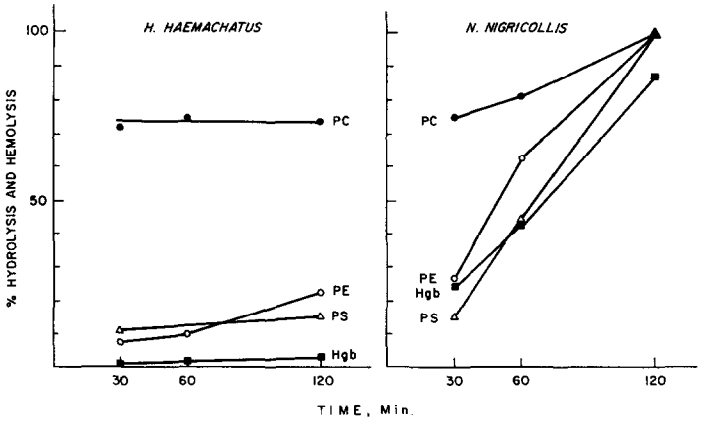

Fig. 4. Phospholipid hydrolysis and hemolysis induced by phospholipases A_2 in human erythrocytes separated from stored blood. Assay conditions: 1-ml red cells suspensions in isotonic saline, containing 10 mM Tris–HCl (pH 7.4) and 1 mM $CaCl_2$, were incubated at 37° with 120 μg of either *H. haemachatus* or *N. nigricollis* phospholipase A_2, for the times indicated. Phospholipids were extracted and separated by thin-layer chromatography. Symbols: (●) phosphatidylcholine; (○) phosphatidylethanolamine; (△) phosphatidylserine; and (■) hemoglobin.

nigricollis phospholipase, complete hemolysis with hydrolysis of all phospholipid substrates being reached by the end of a 2–hr incubation. In contrast, these ATP-depleted red cells were not hemolyzed by the *H. haemachatus* enzyme, and phospholipid hydrolysis did not advance beyond that of the exposed outer lipid layer, as observed with the fresh red cells (Fig. 4). In experimental conditions as shown in Fig. 4, addition of EDTA (50 mM), which inhibits the venom phospholipase A_2 activity [22], abolished the hemolysis caused by the *N. nigricollis* enzyme, while addition of heparin, in amounts per weight equal to those of enzyme, had no effect. Heparin is known to prevent hemolysis induced by venom direct lytic factor [23].

The difference in hemolytic and subsequent phospholipid splitting ability of the two phospholipases was further documented on fresh guinea pig red cells which are naturally more susceptible to venom-induced hemolysis [23]. Indeed, the *N. nigricollis* enzyme induced marked hemolysis and complete hydrolysis of the membrane phospholipids, while *H. haemachatus* phospholipase was non-lytic and phospholipid hydrolysis was only slight (Fig. 5). In contrast to the intact red cell on which the enzymatic attack by the phospholipases is limited to the outside of the cell, the permeable membrane prepared by osmotic hemolysis is considered as freely accessible to the enzyme from both sides. Both enzyme preparations hydrolyzed isolated red cell membranes readily, the *H. haemachatus* phospholipase exhibiting an overall higher activity than the *N. nigricollis* enzyme when compared at equal concentrations (Fig. 6). The order of substrate preference was similar for both enzymes, i.e. PE > PC > PS, except for the two lower concentrations of *N. nigricollis* enzyme where PS was hydrolyzed slightly more than PC. While, for the *H. haemachatus* enzyme,

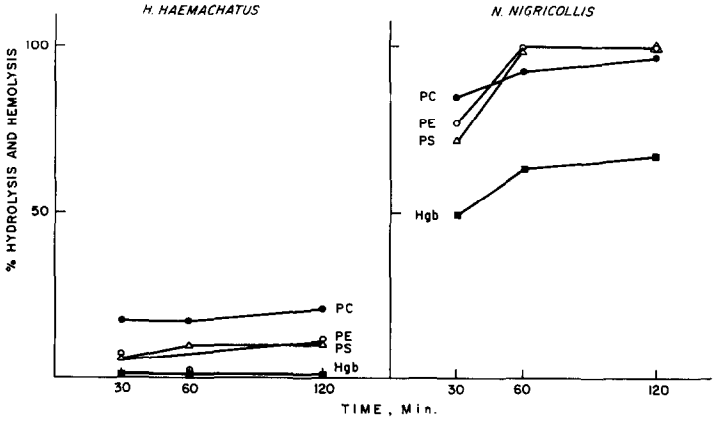

Fig. 5. Phospholipid hydrolysis and hemolysis induced by phospholipase A_2 in guinea pig erythrocytes. Assay conditions as in Fig. 4 except for the enzymes being in a concentration of 30 μg/ml. Symbols: (●) phosphatidylcholine; (○) phosphatidylethanolamine; (△) phosphatidylserine; and (■) hemoglobin.

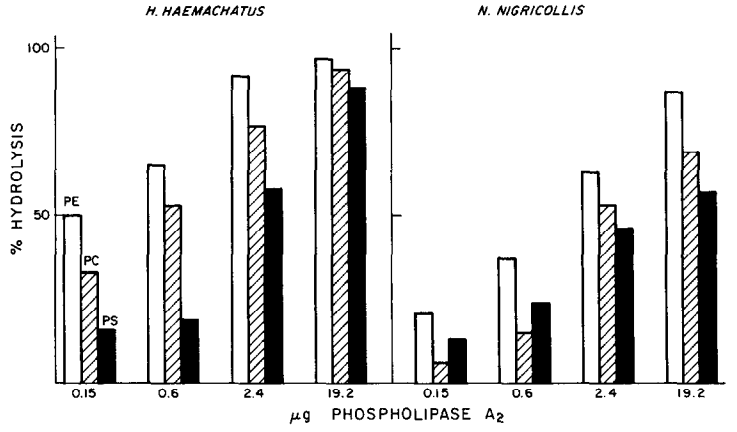

Fig. 6. Phospholipid hydrolysis induced by phospholipases A_2 in isolated human red cell membranes. Assay conditions: 1-ml membrane suspensions in 10 mM Tris–HCl buffer (pH 7.4) and 10 mM $CaCl_2$ were incubated for 5 min at 37° with the amounts of enzyme indicated. Phospholipids were extracted and separated by thin-layer chromatography. Symbols: (open bars) phosphatidylethanolamine (PE); (hatched bars) phosphatidylcholine (PC); and (solid bars) phosphatidylserine (PS).

this sequence agrees well with the findings on the "red cell" model phospholipid mixture, it is at variance with the sequence established for *N. nigricollis* on the same model (Table 2), which was characterized by a high preference for PS over PC. Since this finding suggested that a significant degree of PS sequestration within the membrane still prevails in the permeable ghosts, we checked this assumption by adding the two enzymes to membrane preparations which had been completely solubilized by addition of Triton X-100 at a high Triton/phospholipid molar ratio (~18:1). Indeed, on the Triton-solubilized membranes, the *N. nigricollis* enzyme recovered the characteristic substrate preference, as evidenced by the hydrolysis sequence PE = PS > PC (Fig. 7). A high degree of PS hydrolysis by *N. nigricollis* enzyme could also be promoted by storage of permeable membrane preparations at 4° for about 3 days. At the high Triton/phospholipid ratios used in the solubilized membrane experiments, Triton X-100 had opposite effects on the two phospholipases, enhancing the activity of *N. nigricollis* phospholipase and partially inhibiting that of *H. haemachatus* (compare columns D with C in Fig. 7 and with hydrolysis at 2.4 μg enzyme in Fig. 6).

DISCUSSION

Studies of enzyme kinetics and substrate specificities of phospholipase are hindered by the very low activity rates obtained with purified phospholipases. In the present study, therefore, we assayed *N. nigricollis* and *H. haemachatus* phospholipases on purified phospholipids in mixed micelles with Triton

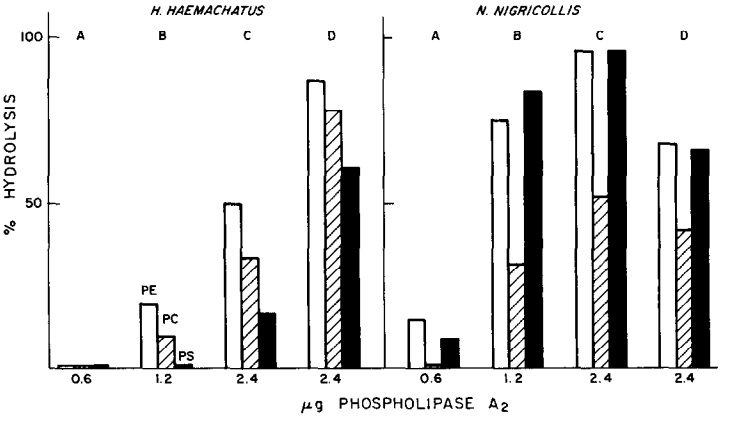

Fig. 7. Phospholipid hydrolysis induced by phospholipases A_2 in red cell membranes modified by Triton X-100 or by storage. Assay conditions as in Fig. 6, except that samples A, B and C were solubilized with Triton X-100 in Triton/phospholipid at an approximate molar ratio of 18:1. Sample D contained no Triton, but the membrane suspensions were stored for 3 days at +4°, prior to enzyme treatment. Symbols: (open bars) phosphatidylethanolamine (PE); (hatched bars) phosphatidylcholine (PC); and (solid bars) phosphatidylserine (PS).

at 2:1 or 4:1 detergent to lipid ratios, at which hydrolysis rates are maximal [9, 24–29]. With purified egg phosphatidylcholine as substrate, the two enzymes were similarly affected by inhibitors and activators, temperature and pH. Both on purified egg phosphatidylcholine and on native egg yolk lipoprotein. *H. haemachatus* enzyme exhibited V_{max} values 2- to 4-fold higher than the *N. nigricollis* phospholipase.

Studies with detergent-free phospholipid liposomes revealed that phospholipases A_2 from various sources respond differently to thermotropic phase transitions of the substrates [30, 31]. *Naja naja* phospholipase A_2, tested on mixed micelles of phospholipid with Triton X-100 in a 2:1 detergent to lipid ratio, exhibited a 7-fold increase in hydrolytic rate at temperatures above the transition point [24]. Similarly, our results show that both *N. nigricollis* and *H. haemachatus* enzymes hydrolyzed two saturated phosphatidylcholines in mixed micelles with Triton, at 4- to 7-fold higher rates at elevated temperatures where the substrates are in the liquid-crystal phase. The increased rates above the transition temperature observed in Triton-containing systems of low Triton/lipid ratios are interpreted by Dennis [24] as reflecting mainly an increased ability of 'melted' phospholipid to incorporate itself into mixed micelles, as opposed to phospholipid in the gel phase.

The comparison of *N. nigricollis* and *H. haemachatus* phospholipases A_2 on purified phospholipids, taken either singly or in various combinations, revealed significant differences in substrate specificity, and documented the fact that the hydrolysis of one substrate is modified by the presence of other phospholipid species. Activation of hydrolysis of one lipid by another is best illustrated with PS, a substrate which is not hydrolyzed by either of the two phospholipases when tested singly, while appreciable hydrolysis by the *N. nigricollis* enzyme is promoted in various mixtures. In all cases, the ability of the *H. haemachatus* enzyme to hydrolyze PS was much lower than that of the *N. nigricollis* enzyme.

The inability of a phospholipase A_2 from another venom, that of *N. naja*, to hydrolyze PS was related to the fact that, in media containing 10 mM Ca^{2+}, this acidic phospholipid precipitates from solution [28]. The fact that we obtained high levels of PS hydrolysis by *N. nigricollis* phospholipase in mixed substrates at 10 mM Ca^{2+} concentrations suggests a redistribution of Ca^{2+} with maintenance of PS in the micellar state. The same might apply also to the promotion of PI hydrolysis by *N. nigricollis* when in the presence of PC. The high levels of PS hydrolysis, obtained by *N. nigricollis* enzyme in the conditions described above, indicate that the low activity of the *H. haemachatus* enzyme (under similar conditions) reflects a true difference in substrate specificity.

Previous studies have demonstrated that the rate and extent of phospholipase hydrolysis of membrane-embedded substrate depends not only on specific preference of the enzyme but also on the degree of substrate exposure and the nature and amount of the other phospholipid species available [29, 32, 33]. In turn, the exposure of a substrate varies with the state of the membrane, in terms of permeability, energy level within the cell, etc. [32, 34–36]. Therefore, we compared the abilities of *N. nigricollis* and *H. haemachatus* enzyme to hydrolyze substrates in intact red cell membranes and in membranes modified by prolonged storage, osmotic hemolysis, or disintegration with Triton X-100.

Consistent with previous data on a number of purified phospholipases [20, 32], the *N. nigricollis* and *H. haemachatus* enzymes do not hemolyze human red cells isolated from fresh blood, and the hydrolysis they induce reflects the phospholipid exposure in the membrane outer layer. For PC, this amounts to about 70 per cent of the total [20]. When assayed on permeable human red cell membranes prepared by osmotic hemolysis, the phospholipid substrates were hydrolyzed in a sequence of preference which was PE > PC > PS for both enzymes. The osmotic ghosts are considered to have their phospholipids freely exposed to the enzyme so that sequences of substrate hydrolysis are taken to reflect enzyme specificity. Our data, however, indicate that PS in the osmotic ghosts is still partially shielded from the enzyme. We based our conclusion on a comparison of the sequences obtained on osmotic ghosts, on Triton-disintegrated ghosts, and with lipid mixtures mimicking the red cell membrane. *N. nigricollis* phospholipase, which has a high ability to hydrolyze PS in lipid mixtures and shows the sequences PS = PC, regained this characteristic property only when permeable ghosts were disintegrated with a high concentration of Triton. Obviously, this would not be apparent when using a phospholipase with intrinsically low activity on PS such as the *H. haemachatus* enzyme tested by us or the *N. naja* enzyme employed by Adamich and Dennis [29].

The possibility that Triton specifically promotes high PS hydrolysis is ruled out by the finding that unmasking of this lipid was also achieved with prolonged storage of the ghosts. Since PS is located entirely within the membrane inner half, its shielded state in the presumably 'freely permeable' ghosts is not surprising. Our previous finding that PS is not hydrolyzed by *Vipera palestinae* phospholipase in freshly prepared ghosts but becomes susceptible to hydrolysis after spectrin-depletion and storage of the membranes [37] agrees with the present data. The effect of Triton on membrane phospholipid hydrolysis is dual; on one hand it exposes the substrates by disintegrating the membrane structure and on the other hand, at the relatively large ratios used for membrane disruption (Triton to phospholipid molar ratio of 18:1), it exerts an inhibiting effect on hydrolysis. This was observed in experiments with *H. haemachatus* but not with the *N. nigricollis* enzyme. Further investigation would be necessary to establish why the two enzymes respond differently to inhibition by excess Triton.

The most striking difference between the two phospholipases resides in the ability of the *N. nigricollis* enzyme to hemolyze and hydrolyze phospholipids of fresh guinea pig cells and of human red cells separated from stored outdated blood, in contrast to the phospholipase of *H. haemachatus* venom. Previous reports on a direct lytic effect of the *N. nigricollis* phospholipase have appeared in the literature. Thus, Dumarey *et al.* [38] observed lysis of

human and horse erythrocytes following pre-heating at 45° for 30 min, and Lee *et al.* [39] reported on lysis of guinea pig red cells at 37°. Another basic phospholipase from *Agkistrodon halys blomhofii* venom directly hemolyzed and induced phospholipid hydrolysis in human red cells when the reaction was carried out at elevated pH or at increased Ca^{2+} concentration (up to 40 mM) [34]. In these conditions the enzymically induced hemolysis was associated with low intracellular ATP levels [34]. The *A. h. blomhofii* phospholipase was also able to hemolyze guinea pig red cells in the same experimental conditions as for human erythrocytes [34]. Our results on erythrocytes from stored outdated blood confirm the association of direct hemolysis with low energy levels in the cells. However, we found that *N. nigricollis* phospholipase directly hemolyzes fresh guinea pig cells at neutral pH and 1 mM Ca^{2+}, conditions in which fresh human red cells resist hemolysis.

The direct lytic and phospholipid splitting ability of the *N. nigricollis* enzyme resembles the synergistic effects induced by a combination of direct lytic factor and phospholipase A_2 [23]. It seemed an attractive speculation that the *N. nigricollis* phospholipase, a protein with both positive charge and enzymatic activity, combines the properties of the direct lytic factor and phospholipase activity in the same molecule. However, the fact that heparin, a direct lytic factor-inhibitor [23], failed to prevent direct hemolysis by *N. nigricollis* enzyme does not give support to this analogy. The question of whether the direct lytic property is unique to basic phospholipases has been asked before. Reports by Martin *et al.* [34] and by Haest and Deuticke [40] document the fact that an acidic phospholipase isolated from *N. naja* is also capable of inducing direct hemolysis and phospholipid hydrolysis of human erythrocytes at elevated pH and 40 mM Ca^{2+} [34] or at neutral pH and 10 mM Ca^{2+} following preincubation of the cells for 24 hr in glucose-free media [40]. Both the *Crotalus adamanteus* enzyme and the acidic phospholipase from *A. h. blomhofii* were inactive in the above conditions [34].

We found, in conclusion, that the toxic phospholipase A_2 from *N. nigricollis* venom differs from the non-toxic *H. haemachatus* phospholipase both in substrate specificity and in ability to attack susceptible membrane structures. Whether these differences are relevant to their pharmacological effects is the object of further studies, some completed [1] and others in progress, in which both native enzymes and enzymes chemically modified at defined sites are compared for changes in toxicity and/or enzymatic parameters.

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