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The relationship between high-affinity noncatalytic binding of snake venom phospholipases A_2 to brain synaptic plasma membranes and their central lethal potencies

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The basic phospholipase A_2 from *Naja nigricollis* (African spitting cobra) snake venom is enzymatically less active but more toxic than the acidic phospholipase A_2 from *Naja naja atra* (Taiwan cobra) snake venom, following injection into the right lateral ventricle of the brain of rats. When radiolabeled with ^{125}I, these phospholipases A_2 retained enzymatic activities and lethal potencies. Both enzymes bound with high affinity and specificity to brain synaptic plasma membrane preparations in vitro even in the absence of calcium, suggesting a non-catalytic binding. The acidic enzyme, in a calcium-free medium, had two binding components with K_d values of $1 \cdot 10^{-10}$ and $2.75 \cdot 10^{-8}$ M and B_{max} values of $6 \cdot 10^{-13}$ and $3.4 \cdot 10^{-11}$ mol/mg, respectively. Multiple specific and nonspecific binding components were observed for each phospholipase A_2; saturability for all of the binding sites was conclusively demonstrated only for the *N. naja atra* phospholipase A_2 in a calcium-free medium ($B_{max} = 3.4 \cdot 10^{-11}$ mol/mg). The levels of specific and total binding were 150 pmol/mg and 450 pmol/mg, respectively, for the comparatively toxic enzyme and 15 pmol/mg and 35 pmol/mg, respectively, for the comparatively nontoxic enzyme at a concentration of $2.5 \cdot 10^{-8}$ M. These levels of binding (both total and specific) were directly correlated with the intraventricular lethal potencies of the phospholipases A_2 (0.5 and 5.0 μg/rat for the *N. nigricollis* and *N. naja atra* phospholipases A_2, respectively), suggesting a possible relationship between binding and lethal potency. Carbamylation of lysines reduced the levels of binding and the lethal potencies of both enzymes to a greater extent than their enzymatic activities. Pretreatment with high temperature, proteinases, phospholipases A_2 or C suggested that radiolabeled phospholipase A_2 binds to phospholipids rather than proteins. However, only the *N. naja atra* phospholipase A_2 manifested a strict dependence on a divalent cation (Ca^{2+} or Sr^{2+}) for most of its binding. The *N. nigricollis* enzyme demonstrated a much lower rate of dissociation from synaptic plasma membranes than did *N. naja atra* phospholipase A_2, suggesting that hydrophobic interactions are more important in the binding of the more toxic enzyme as compared to the less toxic enzyme. It is proposed that differences in the extent of high-affinity noncatalytic binding to membrane phospholipids may be at least partly responsible for the marked difference in central toxicities of these two phospholipases A_2.

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Introduction

Phospholipases A_2 isolated from snake venoms have been widely used as probes for studying the organization and function(s) of membrane phospholipids [1–4]. The amino acid sequences and tertiary structures of many pancreatic and snake venom phospholipases A_2 have been determined [5], yielding information regarding the structural requirements of the active site and the catalytic mechanism of action of these enzymes. However, the precise role of phospholipid hydrolysis in the pharmacological and toxicological activities of snake venom phospholipases A_2 has not yet been clarified.

We previously found that *Naja nigricollis* phospholipase A_2, which is similar in amino acid sequence to *Naja naja atra* phospholipase A_2, has only one-half the enzymatic activity of the *atra* enzyme in vitro on purified substrates; $V_{max} = 250$ and 500 microequivalent free fatty acids liberated per min per mg protein, respectively [6], but has 10-times the lethal potency of the *atra* enzyme following injection into the right lateral ventricle of the brain of rats ($LD_{50} = 0.5$ and 5.0 μg/rat, respectively [6]). We also found that the levels of phospholipid hydrolysis in rat brains produced by different venom phospholipases A_2, following intraventricular injection, were not correlated with the varying lethal potencies of the enzymes [7]. Similarly in the heart it was shown that cardiotoxic potency was neither correlated with extent of phospholipid hydrolysis nor with production of hydrolytic products (lysophospholipids or free fatty acids [8–10]). We have also shown that carbamylation of lysine residues in the basic phospholipase A_2 from *N. nigricollis* snake venom and the acidic enzyme from *N. naja atra* snake venom greatly reduced their intraventricular lethal potencies in rats, their in vitro hemolytic and anticoagulant activities and their capacities to penetrate to and hydrolyze substrates in vivo [11]. In contrast, their enzymatic activities were not markedly affected by lysine modification [11]. Conversely, we have demonstrated that modification of the carboxylate functions of aspartate and glutamic acid residues decreases enzymatic activity to a considerably greater extent than pharmacological activity [8]. These results suggest that catalytic activity is not essential to the in vivo action of the enzymes and implicate the involvement of a second site, which is separate from the enzymatic active site, in their biological effects.

It has been suggested that the effects of β-bungarotoxin, a specific presynaptic neurotoxin from snake venom also possesses phospholipase A_2 activity, on neurotransmitter release are derived not from its catalytic function per se but from its selectivity of binding to the nerve terminal [12]. Several reports have indicated that β-bungarotoxin binds with high affinity to synaptosomal membrane proteins [13–17]. However, no information is available concerning the binding to brain fractions of phospholipase A_2 enzymes which are not specific presynaptic toxins. Therefore, we have radioiodinated and analyzed the binding of two structurally homologous venom phospholipases A_2 (which are not specific presynaptic neurotoxins) to rat brain subcellular membranes (both in vivo and in vitro) in order to determine if differences in their potencies can be related to differing extents of binding.

Materials and Methods

Phospholipases A_2 from *N. nigricollis* venom were separated into three fractions (CMS-5, CMS-6 and CMS-9) chromatographically using a CM-Sephadex C-25 Column [18]. The p*I* values of CMS-5, CMS-6 and CMS-9 were determined by isoelectric focusing to be 7.6, 8.3 and 10.6, respectively. The most basic and toxic fraction, CMS-9, was further purified on a DEAE-Sephacel column [18] and used in our studies. The acidic phospholipase A_2 from *N. naja atra* venom was isolated by successive chromatography on SP-Sephadex C-25, DEAE-Sephacel and CM-Sephadex C-25 [19]. The major phospholipase A_2 (p$I = 5.20$ isoenzyme was separated from the minor isoenzyme p$I = 4.7$) on a SP-Sephadex C-25 column. The homogeneity of these enzymes was evaluated using disc electrophoresis on a 7% polyacrylamide gel [20], which confirmed the existence of only a single band for each enzyme [18,19].

Lysines were carbamylated with potassium thiocyanate at a protein : reagent molar ratio of 1': 400 in 0.5 M *N*-ethylmorpholine acetate buffer (pH 8.0) following the procedures of Stark [21]

and Karlsson et al. [22] as described by Yang et al. [19,23]. The carbamylated *N. naja atra* phospholipase A_2 was separated into eight different peaks by chromatography on a DEAE-Sephacel column and the carbamylated *N. nigricollis* enzyme yielded five different peaks when chromatographed on a column of SP-Sephadex C-25, with each peak revealing a single band on disc electrophoresis [19,23]. Fraction 5 (containing four of five lysines modified) of the *N. naja atra* enzyme and fraction d (containing 8 of 10 lysines modified) of the *N. nigricollis* enzyme [11] were used in this study. The number of lysines carbamylated was determined by amino acid analysis [19,23]. The enzymatic, pharmacological and toxicological properties of these modified enzymes have been described previously [11].

Radioiodinations were performed using the iodine monochloride (ICl) procedure of MacFarlane [24] as modified by Vogel et al. [25]. ICl (27 nmol), dissolved in 33 nM HCl, was added in a volume of 10 μl to 100 μl of a 33 mM HCl/170 mM NaCl solution containing 1 mCi of $Na^{125}I$. The solution was gently swirled, left at 0–4°C for 4 min and transferred to 150 ml of a 0.4 M NH_4Cl (previously adjusted to pH 8.9 with concentrated NH_4OH) solution containing 13 nmol (169 μg) of phospholipase A_2. The reaction mixture was stirred for 2 min at 0–4°C. The reaction was terminated by the addition of 20 μl of a 0.1 M $Na_2S_2O_3$ solution followed by the addition of a 0.1 M KI solution [25]. The final volume of the reaction mixture was brought to 1 ml with 50 mM sodium phosphate buffer (pH 7.0) and dialyzed, to remove unreacted ^{125}I, at 4°C against 2 liters of buffer solution several times until the amount of radioactivity in the dialyzate reached a constant low value. Specific activities ranged from 50–125 Ci/mmol. Specific activities were based on measurements of protein and radioactivity of preparations immediately following dialysis. Binding activities were consistent from preparation to preparation regardless of the specific activity.

The in-vitro enzymatic activities of unlabelled and ^{125}I-phospholipases A_2 (native and modified) toward L-α-phosphatidylcholine from egg yolk were measured at a single substrate concentration following the procedure of Condrea et al. [26]. Substrate was solubilized by sonication of a buffer solution containing 0.1 M Tris-HCl (pH 8.5) and 10 mM Ca^{2+} to which was added Triton X-100 detergent in a 3:1 Triton to phospholipid molar ratio. Phospholipase A_2 (2 μg) was then added in a volume of 0.1 ml to a 1 ml aliquot of the above buffer solution containing 12 mg phospholipid per ml and incubated at 37°C for 10 min. Hydrolyzed fatty acids were extracted with a solution containing 100 parts isopropyl alcohol, 25 parts *n*-heptane and 2.5 parts concentrated H_2SO_4 and titrated with alkali under a N_2 stream with Nile blue used as an indicator and palmitic acid used as a standard [26].

Unlabelled and radioiodinated phospholipase A_2 in 50 μl of artificial cerebrospinal fluid [27] were injected into the right lateral ventricle of the brain of male albino Sprague-Dawley rats (250–350 g) following the method of Noble et al. [28].

Brain synaptosomes and myelin fractions were prepared following the method of Booth and Clark [29]. Whole brains (approx. 2 g each) from male albino Sprague-Dawley rats (250–350 g) were homogenized by hand (20% w/v) in cold 0.32 M sucrose/10 mM Tris-HCl/1 mM EDTA (pH 7.4) (isolation medium). The homogenate was diluted to 20 ml with cold isolation medium, vortexed gently and centrifuged in a Sorvall refrigerated centrifuge (4°C) at $1100 \times g$ for 5 min. The supernatant was then centrifuged (4°C) at $17000 \times g$ for 10 min. In some cases, the supernatant obtained from this centrifugation (microsomal fraction) was collected. The pellet (crude mitochondrial pellet) from the $17000 \times g$ centrifugation was resuspended and gently homogenized to a final volume of 1.2 ml with cold isolation medium, diluted with a 5-fold volume of cold 12% (w/w) Ficoll in 0.32 M sucrose/50 μM EDTA (pH 7.4) and layered underneath 7.5% (w/w) Ficoll in 0.32 M sucrose/50 μM EDTA (pH 7.4) on top of which was layered isolation medium. After centrifugation at $99000 \times g$ for 30 min at 4°C, synaptosomes were collected at the 7.5%/12% Ficoll interface. In some cases myelin membranes were also collected at the 7.5% Ficoll/isolation medium interface [29].

Brain synaptic plasma membranes and intrasynaptosomal mitochondria were prepared following the procedure of Cotman and Mathews

[30]. The synaptosomal fraction obtained above was diluted with approximately a 4-fold volume of cold isolation medium, mixed and centrifuged at $78000 \times g$ for 30 min at 4°C. The pellet was resuspended in cold isolation medium and synaptosomes were osmotically lysed by dilution with a 5-fold volume of cold 6 mM Tris-HCl (pH 8.1) followed by incubation at 4°C for 90 min. Lysed synaptosomes were centrifuged at $54150 \times g$ for 15 min at 4°C, resuspended and homogenized in 1.2 ml of cold 0.32 M sucrose and applied over a gradient containing steps of 25%/32.5%/35%/38% (w/v) sucrose. After centrifugation at $63600 \times g$ for 90 min at 4°C, synaptic plasma membranes were collected at the 25%/32.5% (w/w) sucrose interface. In some cases, intrasynaptosomal mitochondria were collected at the 35%/38% (w/w) sucrose interface. The collected synaptic plasma membranes were diluted with an approximately 4-fold volume of 0.1 mM EDTA and centrifuged at $78000 \times g$ for 30 min at 4°C [30]. The pellet was resuspended in artificial cerebrospinal fluid (with calcium replaced with a molar equivalent of strontium) containing aprotinin, at a concentration of 0.5 units/g tissue, to inhibit proteolysis and stored at −20°C. Electron microscopic examination of this fraction revealed predominantly plasma membranes, while measurements of negligible levels of fumarase enzyme activity indicated minimal contamination by mitochondria.

Prior to binding experiments, control rat brain synaptic plasma membranes were pelleted at $20000 \times g$ for 10 min in three volumes of an artificial cerebrospinal fluid solution [27] containing 0.1 mM EDTA, 2 mg/ml bovine serum albumin (Sigma, Fraction V, essentially fatty acid free), and 1.8 mM Sr^{2+} replacing 1.8 mM Ca^{2+}. The pellet was resuspended in 1 ml of the same solution.

10 μg (in 10 μl) of membrane protein was added to a polystyrene microfuge tube (Sarstedt) containing 1 ml of either the above artificial cerebrospinal fluid solution or a similar solution containing Ca^{2+} instead of Sr^{2+} plus varying concentrations of ^{125}I-phospholipase A_2. The solution containing Ca^{2+} did not contain EDTA. The membranes were incubated with phospholipases A_2 for 5 min at room temperature. Incubations were routinely run in triplicate at each concentration of ^{125}I-phospholipase A_2. Membranes were pelleted at $12000 \times g$ for 6 min and the supernatant was carefully removed with a Pasteur pipette. Protein assay indicated that all of the membranes were pelleted under these conditions. Membranes were washed once by resuspension in 1 ml of the appropriate (Sr^{2+} or Ca^{2+} containing) solution, vortexed and repelleted immediately as above. The supernatant was removed and pellet radioactivity measured in a solid scintillation counter. Membrane binding was corrected for non-specific binding to the centrifuge tube, measured in the absence of membranes. Some tubes contained a concentration of unlabelled phospholipase A_2 which was 100-times that of the highest concentration of labelled phospholipase A_2 in order to measure nonspecific (non-displaceable) binding. Nonspecific binding was subtracted from the binding observed in the absence of unlabelled enzyme (total binding) to obtain a measure of specific (displaceable) binding. The nonspecific and specific binding of radioiodinated enzymes to control myelin membranes was also determined as described above.

Lethal dosages (5 μg; 120 Ci/mmol) of ^{125}I-*N. nigricollis* or ^{125}I-*N. naja atra* phospholipase A_2 were injected intraventricularly. Brains were removed at the time of death and whole brain subcellular fractions were prepared as described above. Aliquots were removed and assayed for protein, radioactivity, and total binding.

Protein determinations for aqueous solutions of phospholipases A_2 were performed following the procedure of Bradford [31]. Insoluble proteins in tissue samples were assayed following the procedure of Lowry et al. [32] as modified by Markwell et al. [33]. Bovine serum albumin was used as a standard in both methods.

Lyophilized *Naja nigricollis* snake venom was obtained from Miami Serpentarium Laboratories (Miami, FL); lyophilized *Naja naja atra* snake venom was collected by one of the authors (C.C.Y.). Sodium dodecylsulfate (SDS), crystalline bovine serum albumin (BSA, Fraction V, essentially fatty acid-free), trypsin, chymotrypsin, neuraminidase (from *Clostridium perfringens*), phospholipase C (from *Clostridium perfringens*), purified egg L-α-phosphatidylcholine, β-bungarotoxin,

cytochrome *c* and Ficoll were purchased from Sigma Chemical Co. (St. Louis, MO). Protein dye reagent was obtained from Bio-Rad (Richmond, CA). Disodium EDTA was purchased from Fisher Scientific Co. (Fair Lawn, NJ). $Na^{125}I$ (specific activity = 2200 Ci/mmol) was purchased from New England Nuclear. All other reagents were of analytical grade.

Results

Biological activities of radioiodinated phospholipase A_2

Radioiodinated phospholipases A_2 were routinely stored at −80°C. Iodinated preparations were used within two weeks after iodination. The iodination procedure did not significantly diminish the intraventricular lethal potency of either the *N. nigricollis* or *N. naja atra* enzymes, since intraventricular injection of an amount corresponding to twice the LD_{50} of their respective unlabelled enzymes was lethal to all of the animals [3] injected. Also both enzymes retained 70–100% of their respective enzymatic activities when measured on purified phosphatidylcholine in vitro up to two weeks following iodination (each preparation assayed in duplicate incubation; results not shown). No changes in the binding of a given preparation were observed within this time period.

Binding to synaptic plasma membranes and meylin fractions in vitro

The total binding in vitro of both the ^{125}I-*N. nigricollis* and ^{125}I-*N. naja atra* phospholipases a_2 (5 nM) to synaptic plasma membranes in the presence or absence of calcium was linear with respect to membrane concentration (10–100 μg membrane protein) (results not shown). The binding of either enzyme in normal calcium media was the same at 27 and 37°C and was also the same at incubation times of 1, 5, 10 and 30 min at concentrations between 1 and 10 nM. The latter results indicate a rapid rate of association of enzymes with membranes (results not shown).

To determine whether or not enzymatic activity was required for binding, experiments were performed in the presence and absence of calcium; in the latter case calcium was replaced by a molar equivalent of strontium. Phospholipase A_2 demonstrates an absolute requirement for calcium for its catalytic activity although the active site of the enzyme can bind substrate in the presence of strontium [34]. The extensive washing of membranes with an EDTA-containing solution during the isolation procedure and the addition of 0.1 mM EDTA to the binding incubation medium to chelate residual calcium further insured that enzyme catalysis was prevented in the calcium-free media. Both the *N. nigricollis* and *N. naja atra* phospholipases A_2 were found to be enzymatically inactive in vitro toward phosphatidylcholine-Triton mixed micelles in a Ca^{2+}-free, strontium containing Tyrodes solution [35].

The total and specific binding of each of the radioiodinated phospholipases A_2 in a calcium-free medium was measurable at concentrations as low as 0.1 nM (Fig. 1A). An intraventricular dosage of 1 μg/rat (twice and one-fifth the LD_{50} values of the *N. nigricollis* and *N. naja atra* enzymes, respectively) should give a concentration in vivo in 1 ml of cerebrospinal fluid of approx. $8 \cdot 10^{-8}$ M for these enzymes, each of which has a M_r of approx. 13000. We found that at a concentration of $2.5 \cdot 10^{-8}$ M the relative levels of total and specific binding for the two phospholipases A_2 were in close correspondence with the 10 fold difference in their relative intraventricular lethal potencies; 460 and 150 pmol/mg, respectively, for the comparatively toxic enzyme and 35 and 15 pmol/mg, respectively, for the comparatively nontoxic enzyme (Fig. 1). The *N. naja atra* phospholipase A_2 reached saturation in both total and specific binding at a concentration of 80 nM, while the *N. nigricollis* phospholipase A_2 did not demonstrate saturability with respect to either measure of binding at any concentration up to 1000 nM (Fig. 1A). However, the rate of increase of the specific binding of this enzyme with increasing concentrations of free enzyme declined between $1 \cdot 10^{-7}$ M and $1 \cdot 10^{-6}$ M (Fig. 1A, inset). Limited amounts of enzyme available prohibited the use of higher concentrations at which saturation might have been revealed.

Enzymatic activity was not a prerequisite for binding which occurred even in the total absence of phospholipid hydrolysis. In a normal calcium medium, the levels of total and specific binding of the phospholipases A_2 also correlated with their

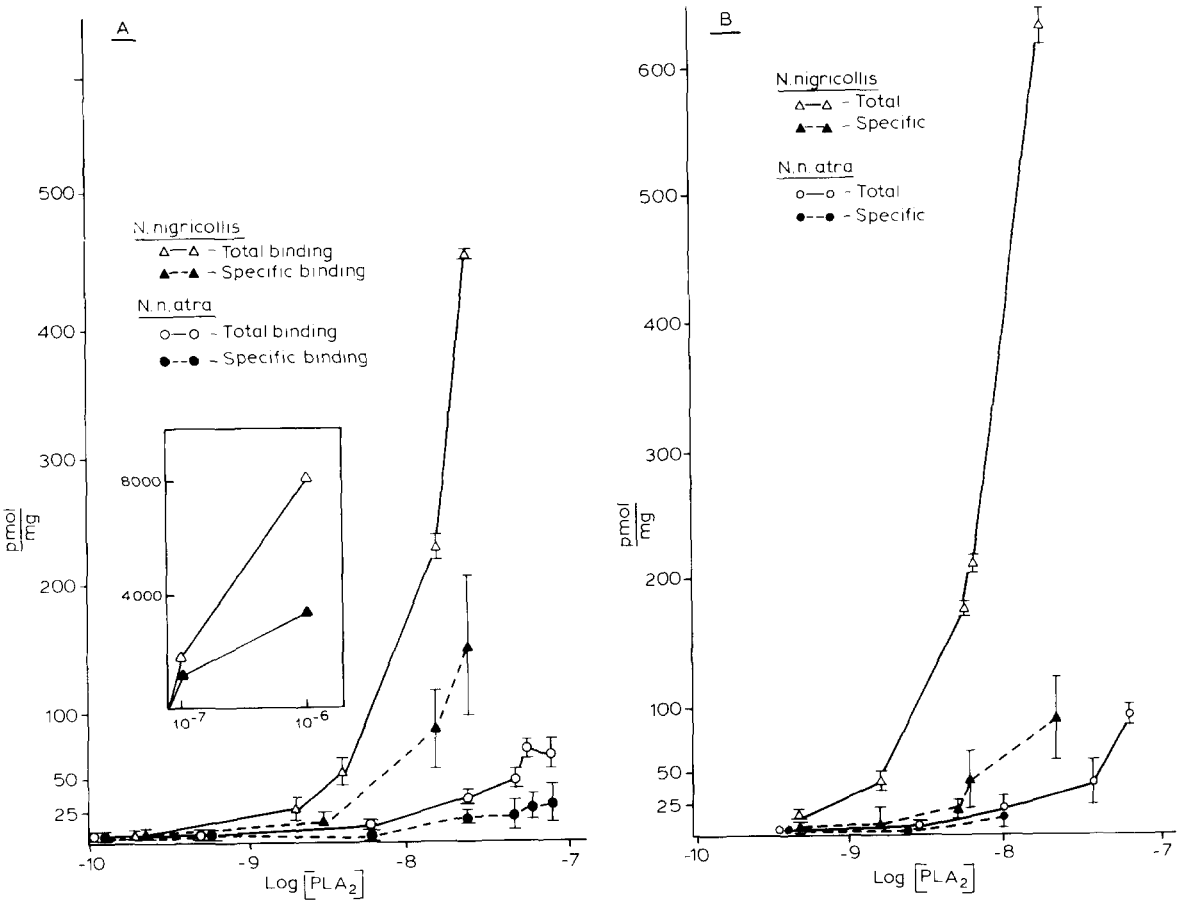

Fig. 1. Total and specific binding of ^{125}I-*N. nigricollis* and ^{125}I-*N. naja atra* to whole brain synaptic plasma membranes. 10 μg of membrane protein were incubated in calcium-free medium (A) or a normal calcium medium (B) as described in Methods. Values are presented as mean ± S.E. ($N = 3$). △, ▲: *N. nigricollis*; ○, ●: *N. naja atra*; open figures, total binding; closed figures, specific binding. Inset: Total and specific binding of ^{125}I-*N. nigricollis* at a concentration of 10^{-7} and 10^{-6} M. Conditions described above.

intraventricular lethalities (Fig. 1B). The levels of specific binding with both enzymes in the presence of calcium were similar to those measured in the calcium-free medium (Figs. 1A and B). However, the levels of total binding in the calcium-free medium, were slightly lower than those observed in normal calcium, indicating a slight enhancement by calcium of nonspecific binding perhaps due to a greater exposure of receptor sites resulting from hydrolysis of phospholipids.

The levels of both total and specific binding of the phospholipases A_2 to myelin fractions in the presence or absence of calcium were similar to those determined with synaptic plasma membranes, with the *N. nigricollis* enzyme exhibiting greater binding under all conditions (results not shown). These results indicate that neither of the phospholipases A_2 has a subcellular preference for greater binding to nerve terminals relative to myelinated axons.

Scatchard analysis of the specific binding of the ^{125}I-*N. naja atra* phospholipase A_2 in a calcium-free medium revealed both high- and low-affinity components (Fig. 2) which were resolved by the method of Rosenthal [36]. Multiple binding components ($K_d = 10^{-9}$–10^{-7} M), revealed by Scatchard analyses, were obtained for both phospholipases A_2 in either a normal calcium or calcium-free medium (results not shown).

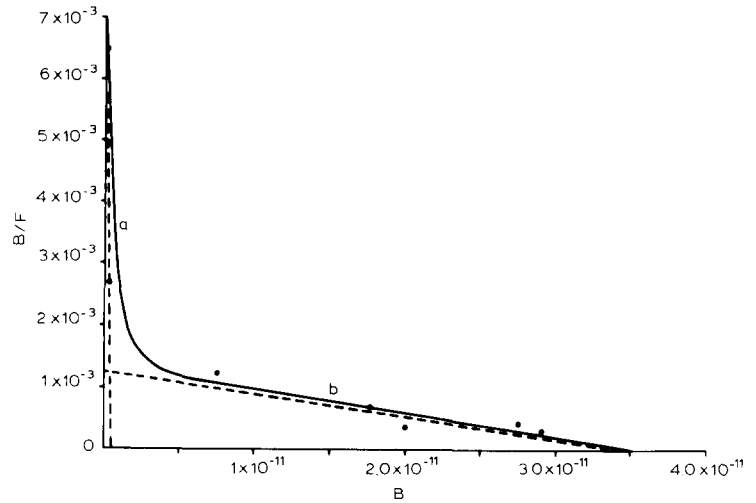

Fig. 2. Scatchard plot with Rosenthal [36] analysis for specific binding of ^{125}I-*N. naja atra* phospholipase A_2 in a calcium-free medium. Data for specific binding (pmol/mg) taken from Fig. 1A. *B*, phospholipase A_2 bound (mol/mg); *F*, molar concentration of free phospholipase A_2. High-affinity component (a) yielded a K_d and B_{max} value of $1 \cdot 10^{-10}$ M and $6 \cdot 10^{-13}$ mol/mg, respectively; corresponding values for the low-affinity component (b) were $2.75 \cdot 10^{-8}$ M and $3.4 \cdot 10^{-11}$ mol/mg, respectively.

Binding to brain subcellular fractions following intraventricular injection

Lethal dosages of radioiodinated phospholipases A_2 were applied intraventricularly, brains removed at the time of death and brain subcellular fractions isolated. From 3–15-times more of the ^{125}I-*N. nigricollis* enzyme was bound to each fraction (except for the microsomal fraction) compared to the ^{125}I-*N. naja atra* enzyme (specific activity of both preparations = 115 Ci/mmol) (Table I). (Radioactivity in the microsomal fraction probably represents primarily free ^{125}I-labeled enzyme.) In other experiments (not shown), synaptic plasma membranes incubated with radiolabeled *N. nigricollis* or *N. naja atra* phospholipase A_2 were then incubated with a non-labeled crude brain homogenate and subcellular fractions isolated. Since, under these conditions, some dissociation and migration of the labelled enzymes occurred during the isolation of subcellular fractions (results not shown), the values shown in Table I may not accurately represent the exact levels of binding to each subcellular region occurring in vivo. However, these results together with the in vitro data indicate that a comparatively toxic phospholipase A_2 binds to subcellular fractions from brain to a greater degree than does a comparatively nontoxic phospholipase A_2, with neither enzyme exhibiting any marked subcellular region specificity.

TABLE I

BINDING TO BRAIN SUBCELLULAR FRACTIONS FOLLOWING INTRAVENTRICULAR INJECTION OF PHOSPHOLIPASES

Binding of ^{125}I-*N. nigricollis* and ^{125}I-*N. naja atra* phospholipases A_2 in whole brain subcellular fractions isolated at the time of death of rats receiving lethal intraventricular dosages (5 μg/rat). Four experiments (animals) are shown with each subcellular fraction; two with each phospholipase A_2. Aprotinin proteolytic inhibitor was present in homogenizing media at a concentration of 0.05 units/g tissue. C.M.P., crude mitochondrial pellet; S.P.M., synaptic plasma membranes.

Subcellular fraction	Binding (pmol/mg protein)	
	N. nigricollis	*N. naja atra*
Nuclear	0.73, 0.66	0.15, 0.18
Microsomal	0.28, 0.28	0.35, 0.35
C.M.P.	0.34, 0.36	0.13, 0.11
Synaptosomes	0.37, 0.28	0.07, 0.07
Myelin	0.35, 0.23	0.02, 0.04
S.P.M.	0.36, 0.34	0.02, 0.02
Mitochondria	0.3, 0.23	0.05, 0.04

Binding of radioiodinated carbamylated phospholipases A_2

We have shown previously that the carbamylation of 8.2 out of the 10 lysine residues in the *N. nigricollis* phospholipase A_2 produced a decrease of 46% in the enzymatic activity and 73% in the intraventricular lethal potency of the native enzyme [11]. Similarly, carbamylation of the *N. naja atra* phospholipase A_2 (3.2 out of 5 lysines mod-

ified) reduced these parameters by 13% and over 98%, respectively [11]. Since this demonstrated a marked dissociation between the two properties, these particular fractions were radioiodinated and their total binding to synaptic plasma membranes was measured in a normal calcium and in a calcium-free medium (Figs. 3A and B). It is evident that in parallel with the loss of lethality, there was also a large decrease in total binding for both enzymes which exceeded the reduction in in-vitro enzymatic activity produced by this modification.

Reversibility of binding

Any possible relationship between the reversibility of the binding of the phospholipases A_2 and their biological potencies was also investigated. Membranes were incubated in either a normal calcium or a calcium-free medium, as described in Methods, with ^{125}I-*N. nigricollis* (2–4 nM) or ^{125}I-*N. naja atra* (6–7 nM) phospholipase A_2. Membranes were pelleted, supernatants removed, pellet radioactivity counted and membranes resuspended in the same medium (1 ml) without phospholipase A_2 and incubated at room temperature for various periods of time prior to centrifugation and recounting of pellet radioactivity. Controls did not contain membranes and were treated in the same manner.

The overall rate of dissociation was much lower for the *N. nigricollis* phospholipase A_2, compared to the less toxic enzyme, in both the normal calcium or calcium-free medium (Fig. 4). Graphs of per-

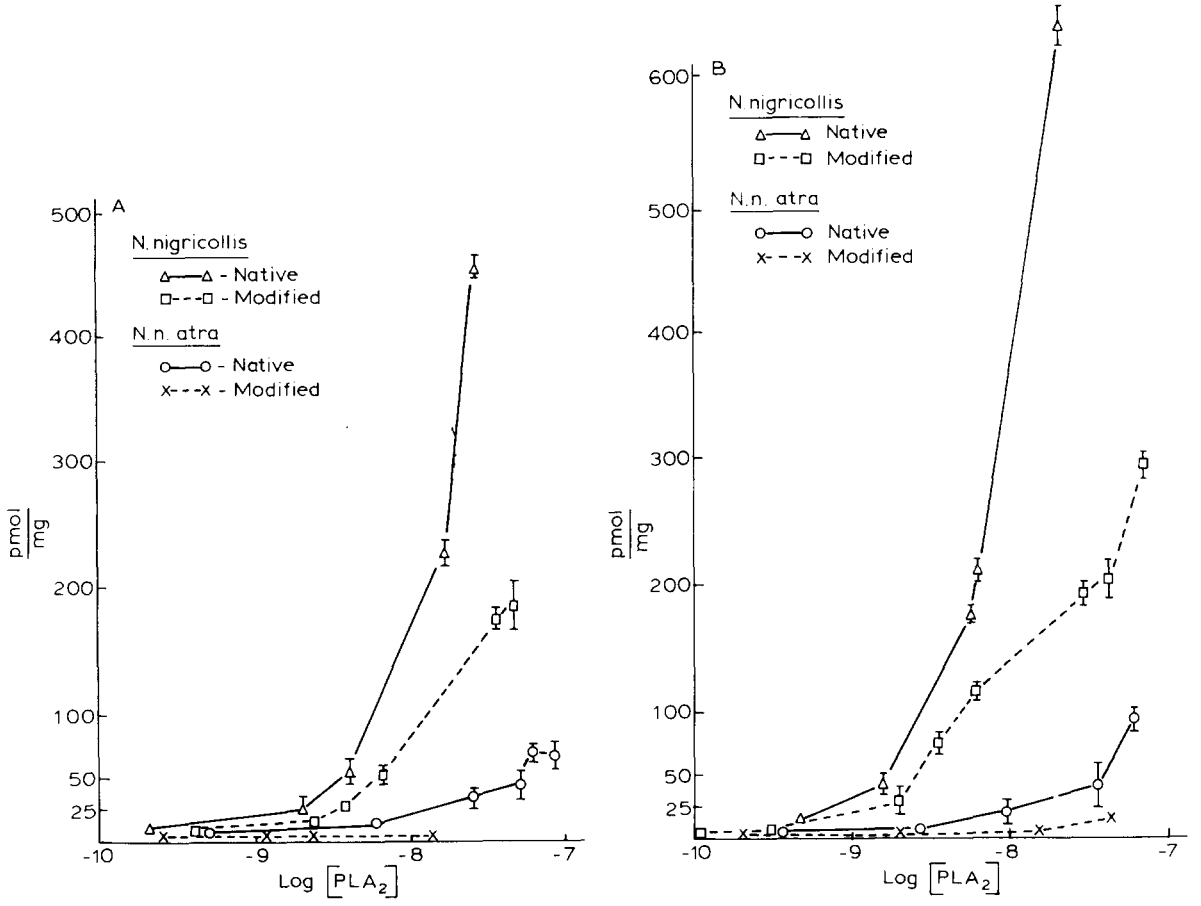

Fig. 3. Total binding of native and carbamylated ^{125}I-*N. nigricollis* (8.2 of 10 lysines modified) and *N. naja atra* (3.2 of 5 lysines modified) phospholipases A_2 to synaptic plasma membranes. 10 μg of membrane protein were incubated in a calcium-free medium (A) or a normal calcium medium (B) as described in Methods. Data for native enzymes taken from Fig. 1. Values are presenied as mean ± S.E. ($N = 3$). Δ, native *N. nigricollis*; □, carbamylated *N. nigricollis*; O, native *N. naja atra*; ×, carbamylated *N. naja atra*.

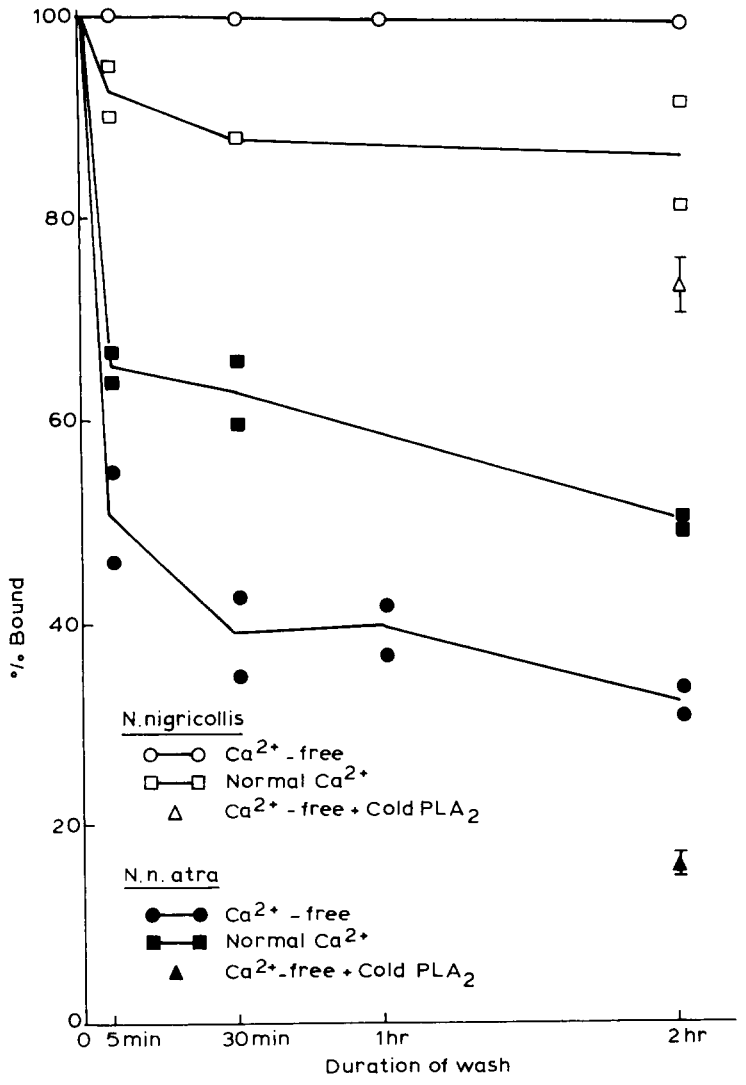

Fig. 4. Reversibility of total binding of native ^{125}I-phospholipases A_2 to whole brain synaptic plasma membranes in vitro. The incubation media contained artificial cerebrospinal fluid and was either calcium-free (○, ●) or contained 1.8 mM calcium (□, ■). In some experiments, membranes were incubated with labelled enzyme in a calcium-free medium, and resuspended in the same medium containing 2 μM unlabelled enzyme without labelled phospholipase A_2 (△, ▲). Open figures: *N. nigricollis* (membranes incubated with 2–4 nM labelled enzyme). Closed figures: *N. naja atra* (membranes incubated with 6–7 nM labelled enzyme). Individual data points are shown, except for △ and ▲, which represent means ± S.E. ($N = 3$).

cent of ^{125}I-phospholipase A_2 remaining bound vs. time of wash (Fig. 4) suggest the presence of both high- and low-affinity binding components, for both phospholipases, since the rate of dissociation of bound enzyme was rapid for the first 5 min (except for the *N. nigricollis* enzyme in a calcium-free medium) while after 5 min it was much lower. This is consistent with the multiple binding components revealed by Scatchard analyses for both enzymes. A greater fraction of the total binding of the *N. nigricollis* enzyme was comprised of the slowly dissociating component compared to *N. naja atra* (Fig. 4) which is perhaps related to the higher levels of binding shown by the more toxic enzyme (Fig. 1).

The presence of an excess of unlabelled enzyme in the calcium-free wash medium appeared to increase the dissociation of both phospholipases A_2 presumably by the displacement of specifically bound molecules. The percent of bound ^{125}I-*N. nigricollis* or *N. naja atra* enzymes displaced by an excess of cold ligand (Fig. 4) was either similar to or slightly less than the fraction of the total binding which is specific within this concentration range. Therefore, it would appear that an excess of either enzyme can displace (the same) bound en-

zyme molecules from specific binding sites, indicating that this fraction of the total binding is reversible for both enzymes.

Divalent cation requirements for binding

^{125}I-phospholipase A_2 total binding to synaptic plasma membranes was measured, as described in Methods, in an artificial cerebrospinal fluid solution containing a physiologically normal concentration of calcium (1.8 mM), high calcium (6 mM) or zero calcium medium (+0.1 mM EDTA) with or without strontium (1.8 mM). Membranes were incubated with 3.5 nM enzyme in the high calcium medium (comparatively low concentrations were used to limit the increase in phospholipid hydrolysis stimulated by the high calcium condition) or 35 nM enzyme in the other media. The incubates were pelleted and washed in 1 ml of the calcium-free solution prior to counting of radioactivity as described in Methods. The binding of the *N. nigricollis* phospholipase A_2 was not affected to as great a degree as was that of the *N. naja atra* phospholipase A_2 by changes in the ionic composition of the incubation medium (Table II). Although the total binding of the enzymes were not substantially decreased by replacing calcium with an equimolar amount of strontium, there was a significant loss of binding of the *N. naja atra* phospholipase A_2 when both cations were omitted from the medium (Table II). In addition, when the external calcium concentration was raised from 1.8 to 6 mM, a dramatic increase in the binding of the phospholipases was observed especially for the *N. naja atra* enzyme. Even when the binding of the *N. naja atra* enzyme in high calcium was tested at a concentration of $0.8 \cdot 10^{-10}$ M, 0.82 ± 0.1 pmol/mg ($X \pm$ S.E.; $N = 3$) was bound compared to binding of only 0.28 ± 0.11 pmol/mg in normal calcium even at the higher concentration of $4 \cdot 10^{-10}$ M. The results obtained with the calcium-free/strontium-free/EDTA condition suggest that only the less toxic phospholipase A_2 has a binding requirement for calcium or other divalent cations which are known to be able to substitute for calcium physiologically. Calcium, therefore, increases binding of the phospholipase A_2 in addition to being required for enzymatic activity. The potentiating effect of high calcium on the binding of both enzymes may be partly related to an enhancement of phospholipid hydrolysis resulting from the high calcium concentration. However, the greater stimulation of the binding of the *N. naja atra* enzyme suggests that another property of the molecule in addition to its hydrolytic activity may have been affected by the elevation of external calcium concentration.

Competitive binding studies

We were interested in finding other possible

TABLE II

DIVALENT CATION REQUIREMENTS FOR BINDING

Values are presented as means ± S.E. ($N = 3$) for the total binding, expressed as a percentage of the binding measured in normal calcium (1.8 mM) artificial cerebrospinal fluid at the same enzyme concentrations. Membranes were incubated with 35 nM of either enzyme under all conditions except the high calcium condition (3.5 nM). The control total binding of the *N. nigricollis* enzyme was 100 pmol/mg and 650 pmol/mg at 3.5 and 35 nM, respectively; that of the *N. naja atra* enzyme was 6 pmol/mg and 35 pmol/mg at 3.5 and 35 nM, respectively. * $P < 0.05$ and ** $P < 0.005$ as compared to binding in normal calcium medium.

	Percent binding	
	N. nigricollis	*N. naja atra*
Normal calcium	100	100
High calcium (6 mM)	171 ± 20 *	594 ± 162 *
Sr^{2+}/EDTA (Ca^{2+}-free)	85 ± 4 *	96 ± 6
$-Sr^{2+}$, $-Ca^{2+}$, +EDTA	83 ± 2 **	36 ± 7 *

TABLE III

COMPETITION IN BINDING BETWEEN PHOSPHOLIPASES A_2, β-BUNGAROTOXIN OR CYTOCHROME *c*

Incubates of ^{125}I-phospholipase A_2 and synaptic plasma membranes contained 2 μM of one of the unlabelled 'ligands' listed above in a calcium-free medium. Total binding was measured as described in Methods. Values are presented as means ± S.E. ($N = 3$) for total binding expressed as a percentage of the total binding (35 pmol/mg and 10 pmol/mg for *N. nigricollis* and *N. naja atra*, respectively) measured in calcium-free controls (containing no unlabelled ligand) at the same enzyme concentration. * $P < 0.1$ as compared to control.

Unlabelled 'ligand'	^{125}I-*N. nigricollis* (2.6 nm)	^{125}I-*N. naja atra* (3.2 nM)
β-Bungarotoxin	86 ± 4	88 ± 10
Cytochrome *c*	94 ± 1	95 ± 11
N. nigricollis	64 ± 11 *	95 ± 9
N. naja atra	103 ± 7	61 ± 12 *

ligands for the phospholipase A_2 binding site (Table III). Simultaneous incubation with an excess of β-bungarotoxin (a basic presynaptic neurotoxin with phospholipase A_2 activity) decreased the total binding of the two phospholipases A_2 by only 12–14%, suggesting little or no overlap in binding sites with this snake venom neurotoxin. There is no overlap of binding sites between the two phospholipases A_2, since a 1000-fold excess of one unlabelled enzyme could not displace any of the binding of the other labelled enzyme. In order to determine whether the basicity of the ligand is related to binding, an excess of cytochrome *c* (p*I* = 10.6) was added to incubates, with no effect on the binding of either phospholipase A_2 (Table III).

Effects of heat and enzyme pretreatments on binding

An attempt was also made to indirectly characterize the phospholipase A_2 membrane binding site through the use of heat and enzyme pretreatments of synaptic plasma membranes prior to binding determinations (Table IV). Synaptic plasma membranes were incubated in the normal calcium medium described previously (without bovine serum albumin) for 30 min at 37°C with trypsin, chymotrypsin, neuraminidase, phospholipase C or unlabelled *N. naja atra* phospholipase A_2 at the concentrations shown in Table III or were heated at 94°C for 30 min. Membranes were pelleted and resuspended in the calcium-free medium described previously containing the phospholipase enzymes plus 2 mg bovine serum albumin per ml and total binding was measured as described in Methods. Heating the membranes had no significant effect on binding, indicating that a change in the conformation of membrane proteins does not alter binding. Treatment with trypsin or chymotrypsin was also without significant effect (Table IV). These two results suggest that the high-affinity binding of a phospholipase A_2 to membranes is not dependent upon a receptor type interaction with membrane proteins, although proteins possibly not affected by the proteases used or by heat may play a role in the binding site. The loss of sialic acid residues of gangliosides by neuraminidase also did not influence binding (Table IV).

The only substantial changes observed were in the binding of the *N. nigricollis* enzyme after pretreatment with either phospholipase C or a high concentration of *N. naja atra* phospholipase A_2. The results with phospholipase C suggest that the phosphate group or polar head group of phospholipids may be associated with the binding site for the *N. nigricollis* enzyme. The increase in its binding observed after pretreatment with phospholipase A_2 may be due to a reduction in membrane permeability barriers resulting from phospholipid hydrolysis by this relatively high concentration of phospholipase A_2.

TABLE IV

EFFECTS OF HEAT AND ENZYME PRETREATMENTS OF SYNAPTIC PLASMA MEMBRANES ON IN VITRO (TOTAL) BINDING OF NATIVE PHOSPHOLIPASES A_2

Pretreatment enzyme concentrations (neuraminidase and proteases) chosen to effect 100% hydrolysis of 10 mg substrate (10 mg synaptic plasma membranes protein used in each incubation). Phospholipase C and phospholipase A_2 concentrations chosen to effect 100% hydrolysis of substrate based on Giambalvo and Rosenberg [43] and Condrea et al. [6]. See text for further experimental details. Values are presented as means ± S.E. ($N = 3$) for total binding expressed as a percentage of the binding (23 pmol/mg and 15 pmol/mg for *N. nigricollis* and *N. naja atra*, respectively) measured in controls (calcium-free) at the same enzyme concentration. * $P < 0.1$ and ** $P < 0.05$ as compared to controls.

Treatment	% of control binding	
	N. nigricollis (1 nM)	*N. n. atra* (7 nM)
Heat (94°, 30 min)	85 ± 9	121 ± 29
Trypsin (15.6 units)	99 ± 9	78 ± 29
Chymotrypsin (2.5 units)	80 ± 9	–
Neuraminidase (0.0017 units)	108 ± 9	72 ± 30
Phospholipase C (10 units)	55 ± 12 *	–
N. naja atra phospholipase A_2 (0.38 μM)	185 ± 7 **	94 ± 32

Discussion

Although the mechanism of action of β-bungarotoxin, a specific presynaptically acting snake venom neurotoxin which possesses phospholipase A_2 activity, has been extensively studied

[12,37] that of venom phospholipase A_2 (which are not specific presynaptic neurotoxins) is not as well understood. In our previous studies, we have attempted to relate the pharmacological and toxicological effects of snake venom phospholipases A_2 to the levels of phospholipid hydrolysis they produced both in vitro and in vivo. We initially found that the biological potencies of two snake venom phospholipases A_2 following central administration in rats were not correlated with either pattern or extent of phospholipid hydrolysis in various brain regions measured at the time of death [7]. Other studies involved chemically modifying key amino acids in the phospholipase A_2 molecule in order to see whether it is possible to selectively affect one property or the other and thereby demonstrate that pharmacological and enzymatic activity are unrelated. We have recently found that the carbamylation of lysines greatly lessens a number of the pharmacological and toxicological effects of the *N. nigricollis* and *N. naja atra* phospholipases A_2, without significantly affecting the phospholipases in vitro enzymatic activities [11]. It has also been shown that the in vitro enzymatic activities of these two phospholipases A_2 can be markedly reduced through modifications of the carboxylate groups of aspartate and glutamic acid residues [8] without greatly altering the other biological properties of the enzymes.

These results suggested that the biological effects of venom phospholipases A_2 are mediated to a large extent by a nonenzymatic action which is derived from a site in the molecule separate from the catalytic site. Therefore, this study was undertaken in order to determine whether or not pharmacological and toxicological potencies are related to the extent of the binding of phospholipase A_2 to membranes.

Our present results provide further evidence that a noncatalytic property, and not the capacity to hydrolyze phospholipids, determines the biological activities of venom phospholipases A_2. We found a 10-fold difference in total binding to synaptic plasma membranes in vitro (Fig. 1) between the *N. nigricollis* and *N. n. atra* phospholipases A_2, which can be directly correlated with the 10-fold difference in their intraventricular LD_{50} values (0.5 and 5.0 μg/rat, respectively). These findings suggest that even if the actions of the two unmodified phospholipases A_2 on neuronal membranes following central administration were identical, the higher potency of the *N. nigricollis* enzyme could be due simply to its greater binding compared to that of the *N. naja atra* enzyme.

The results with heat and enzyme pretreatments (Table IV) suggest that the binding site for the phospholipases may not be protein. The increase in the binding sites of the *N. nigricollis* phospholipase A_2 following pretreatment with the *N. naja atra* phospholipase A_2 (in the presence of calcium) (Table IV) may implicate either hydrophylic or hydrophobic regions of phospholipids as binding sites for the *N. nigricollis* enzyme. The hydrolysis of one acyl chain of a phospholipid may have loosened the packing of molecules allowing the *N. nigricollis* phospholipase A_2 to more effectively reach and bind to fatty acids and/or polar head groups of externally and/or internally situated phospholipids. A comparatively poorer ability of the *N. naja atra* enzyme to overcome membranal barriers, as will be discussed below, may account for the fact that a similar pretreatment (with unlabelled *N. naja atra* enzyme in the presence of calcium) did not demonstrably increase the binding of ^{125}I-*N. naja atra* enzyme to membranes containing extensively hydrolyzed phospholipids. The finding of decreased binding of the *N. nigricollis* phospholipase A_2 following phospholipase C preincubation (Table IV) also suggests a role for polar head substituents of phospholipids in the binding of this enzyme (*N. naja atra* phospholipase A_2 was not tested). Our failure to demonstrate a protein binding site for the phospholipases A_2 is in direct contrast to several studies with β-bungarotoxin which suggest some involvement of membrane proteins in toxin binding [14–16]. Various preparations of β-bungarotoxin [13,15–17] demonstrated (with Scatchard analyses) K_d values for binding in the nM range and B_{max} values between 0.05 and 13 pmol/mg protein. Although the binding affinities are comparable to those determined for the *N. naja atra* (Fig. 2) and *N. nigricollis* (results not shown) enzymes, their maximum levels of binding were considerably less than the levels of binding we demonstrated (Fig. 1), while lethal potency following central administration of β-bungaroto-

xin is much greater [38]. This suggests a greater selectivity in the binding of β-bungarotoxin relative to that of the venom phospholipase A_2, which would be consistent with a postulated specific neuronal target site, such as neurotransmitter release processes, for the presynaptic neurotoxin [12].

If, as the results of the heat and enzyme pretreatment experiments suggest, the venom phospholipases A_2 bind principally to phospholipids, then their binding may be closely related to the well characterized binding of pancreatic phospholipases A_2 to the lipid/water interface [39]. It has been shown in studies with (1) Triton-phospholipid mixed micelles that binding of pancreatic enzymes to the interface and subsequent hydrolysis of interfacial phospholipids is dependent on the lipophilicity of the N-terminus [5] and (2) in monolayer studies that the surface concentration of enzyme increased with the length of the substrate fatty acyl chains, indicating the importance of hydrophobic interaction in binding [40,41].

Since the binding of the N-terminus of pancreatic phospholipase A_2 to the lipid-water interface at physiological pH has been shown to depend on calcium [39], the distinct differences in divalent cation binding requirements observed between the two enzymes in this study could be due to their (phospholipid) interfacial binding characteristics. The lack of dependence of the binding of the *N. nigricollis* enzyme on calcium or strontium (Table II) suggests either that this molecule may not require the N-terminal-mediated interfacial recognition site in order to bind with high affinity to membranes, or that the interfacial binding of this enzyme may occur by a calcium-independent process. The *N. naja atra* phospholipase A_2, however, manifested dependence on a divalent cation (not including Mg^{2+}) for the majority of its binding to synaptic plasma membranes. The increase in binding observed in a high calcium medium (Table II) only with the *N. naja atra* enzyme may represent a potentiation of this phospholipase's N-terminal interfacial binding. The increase could not have resulted from the higher levels of phospholipid hydrolysis promoted by the high calcium condition, since binding was not enhanced to the same extent for the *N. nigricollis* enzyme (Table II) (which was shown to hydrolyze synaptic plasma membrane phospholipids in vitro as well as the less toxic enzyme [11]. Most importantly, the difference in divalent cation requirements for binding between the phospholipases A_2 may be one of the factors responsible for the greater in vivo potency of the *N. nigricollis* enzyme, especially under conditions of low calcium availability in discrete areas.

Assuming that the binding sites of the phospholipases consist primarily of phospholipids, a greater membrane penetrability of the *N. nigricollis*, compared to the *N. naja atra*, phospholipase A_2 could account for its 10-fold greater levels of both total and specific binding to synaptic plasma membranes in vitro (Fig. 1) as well as the comparatively low rate of dissociation of the *N. nigricollis* enzyme (Fig. 4) (possibly arising from extensive binding to internal membrane components) compared to the *N. naja atra* enzyme. The *N. nigricollis* phospholipase A_2 demonstrates a unique ability to (1) hydrolyze internally oriented phospholipids in vivo in synaptic plasma membranes and in other tissues [11], (2) disrupt membranes as evidenced by its direct hemolytic activity in red blood cells [6] and (3) produce ventricular fibrillation after intravenous administration in mice [35]. This suggests that the *N. nigricollis* enzyme can form extensive interactions with membrane components. *N. naja atra* phospholipase A_2 does not produce ventricular fibrillation [35] and demonstrates the other properties described above to a much lesser degree than does the *N. nigricollis* enzyme [11].

We have found evidence supporting a role for basic (lysine) residues in the membrane disruptive affects of the *N. nigricollis* enzyme, including ventricular fibrillation, hemolytic activity and hydrolysis of internally situated phospholipids in vivo, as well as the intraventricular lethal potencies of both of these phospholipases [11]. These effects were all decreased by carbamylation of lysines while in vitro enzymatic activities were retained to a much greater extent. The latter finding is not unexpected since lysines are not present in the active site region of phospholipases [5]. Forst et al. [42] also attributed the loss of hydrolytic activity of a phospholipase A_2 toward phospholipids of *Escherichia coli* after the carbamylation of lysines to an inability of the modified

enzyme to reach substrate in the membrane, since catalytic rate toward purified phospholipids was unchanged. Furthermore, we found that this particular modification demonstrably reduced both the high-affinity (total) binding of both phospholipases A_2 to synaptic plasma membranes in vitro (Fig. 3) and their intraventricular lethal potencies in vivo to a greater extent than in vitro enzymatic activity.

In this study, we have demonstrated a nonenzymatic property of snake venom phospholipases A_2 (binding) that is more closely correlated with their pharmacological and toxicological activities than is enzymatic activity. Therefore, these and earlier results from our laboratory suggest that the presence of both enzymatic and nonenzymatic actions in the phospholipase A_2 molecule complicates their use as specific biological tools. Chemical modifications of enzymes which selectively eliminate either catalytic activity or noncatalytic effects may yield better probes of membrane structure and function.

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