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COMPARISON OF ENZYMATIC AND PHARMACOLOGICAL ACTIVITIES OF LYSINE-49 AND ASPARTATE-49 PHOSPHOLIPASES A_2 FROM *AGKISTRODON PISCIVORUS PISCIVORUS* SNAKE VENOM

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Abstract—The basic Lys-49 phospholipase A_2 (PLA_2) from *Agkistrodon piscivorus piscivorus* venom is homologous to the basic Asp-49 PLA_2 from the same venom as well as other snake venom PLA_2 enzymes. It differs, however, in several respects, most important being replacement of the previously invariant Asp-49 at the calcium binding site by Lys, resulting in a reversed order of addition of calcium and phospholipid, phospholipid binding first. Although the preferences for phospholipid substrates of the two enzymes are identical, the apparent V_{max} of the Lys-49 PLA_2 was only 1.4 to 3% that of the Asp-49 enzyme. Similarly, the Lys-49 PLA_2, compared to the Asp-49 PLA_2 had <3% of the intraventricular lethal potency and 4% of the anticoagulant activity. The intravenous lethal potency of the Lys-49 enzyme was 20% that of the Asp-49 PLA_2 and both had little direct hemolytic activity. In contrast, both enzymes were approximately equipotent on the phrenic nerve–diaphragm preparation and on the isolated ventricle strip of the heart. On the cardiac and neuromuscular preparations, the effects of the Asp-49 PLA_2 were accompanied by hydrolysis of phosphatidylcholine and phosphatidylethanolamine, whereas no phospholipid hydrolysis was observed with the Lys-49 PLA_2. Evaluation of the present results, along with earlier findings using Asp-49 PLA_2 enzymes from *Naja nigricollis*, *Hemachatus haemachatus* and *Naja naja atra* venoms, allows us to conclude that: (1) The *A. p. piscivorus* Asp-49 PLA_2 enzyme resembles the Asp-49 enzymes fron *N. n. atra* and *H. haemachatus*. In contrast, the *A. p. piscivorus* Lys-49 PLA_2 has much lower enzymatic and anticoagulant activities than the Asp-49 enzymes, but equal cardiotoxic and junctional effects. (2) In contrast to some previous suggestions, basic PLA_2 enzymes are not necessarily more toxic than neutral or acidic enzymes. (3) Pharmacological effects upon the heart and phrenic nerve-diaphragm preparation correlate neither with *in vitro* measurements of PLA_2 activity nor with actual levels of phospholipid hydrolysis in the heart or diaphragm. This suggests that PLA_2 enzymes exert effects independent of phospholipid hydrolysis.

Snake venom PLA_2¶ show a remarkable degree of homology in amino acid sequence, yet differ markedly in their enzymatic activities and pharmacological potencies [1–3]. For example, some purified venom PLA_2 enzymes have high lethal potencies, convulsant activities, cardiotoxicities, myonecrotic actions, and hemolytic and anticoagulant activities, and cause a presynaptic blockade of neuromuscular transmission [1, 4–14], without necessarily being more active catalytically than the less toxic enzymes. However, some have suggested that phospholipid hydrolysis and pharmacological potency are directly related [4–6, 15–20], noting for example that *p*-bromophenacylation or methylation of one histidine at the enzymatic active site of the enzyme causes a complete loss of both pharmacological and enzymatic activities [1–6, 21–28]. This is not conclusive evidence, however, since we found that other chemical modifications, especially of lysine residues and carboxyl groups caused, respectively, a selective loss of either pharmacological or enzymatic activity [29–32]. In addition, it was noted in those studies that the extent of *in vivo* phospholipid hydrolysis did not correlate with pharmacological potencies of these native or chemically modified PLA_2. Findings such as these led us to question whether phospholipid hydrolysis is essential for the pharmacological actions of PLA_2 and to suggest that these enzymes may have direct actions independent of phospholipid hydrolysis [2, 7–14, 29–32].

Most of our studies have been carried out using only three native enzymes and their chemically modified derivatives, i.e. the basic PLA_2 from *Naja nigricollis* venom, the neutral PLA_2 from *Hemachatus haemachatus* or the acidic PLA_2 from *Naja naja atra* snake venoms. The question arises therefore as to whether this separation between enzymatic and pharmacological potencies which we observed applies also to other PLA_2 enzymes. Recently, a new class

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¶ PLA_2 = phospholipase(s) A_2.

of snake venom PLA_2 was described in which Asp-49*, heretofore thought to be an invariant amino acid, which serves as part of the calcium binding site is replaced by Lys-49 [33–35]. The complete amino acid sequences of two homologous PLA_2 enzymes from *Agkistrodon piscivorus piscivorus* venom were determined [35], one having Asp and the other Lys at the 49 position. The availability of these two enzymes, which differ markedly in their enzymatic properties, allowed a better understanding of the groups involved in the enzyme–substrate and enzyme–calcium catalytic complexes [33–35]. A comparative enzymatic and pharmacological study of these enzymes was undertaken to determine whether differences in mechanisms and rates of enzymatic activity are associated with corresponding differences in pharmacological properties.

MATERIALS AND METHODS

Isolation and purification of PLA_2. Lyophilized venom from *A. p. piscivorus* (cottonmouth moccasin) was obtained from the Miami Serpentarium Laboratories (Salt Lake City, UT). The Asp-49 and Lys-49 PLA_2 were purified as previously described [33, 36]. Purification of these basic monomeric enzymes involves column chromatographic separations on Sephadex G-50 followed by SP-Sephadex C-50. Their complete amino acid sequences and many of their enzymatic properties have already been described [33–35].

Substrates and methods for enzymatic activity determinations. Mixed micelles of L-α-phosphatidylcholine plus Triton X-100 in a 1:2 molar ratio were formed in 0.1 M Tris–HCl, 10 mM Ca^{2+}, pH 8.5 buffer (Tris–Ca^{2+} buffer) at a concentration of 12 mg phospholipid/ml as previously described [7]. Egg yolk substrate was prepared by diluting egg yolk 5-fold with Tris–Ca^{2+} buffer. This dilution contained about 12 mg phospholipid/ml. Heart and diaphragm homogenates were prepared by removing the tissue from rats anesthetized with ether. The tissues were washed with ice-cold saline, blotted, weighed, and homogenized (Kinematica Polytron homogenizer, Lucerne, Switzerland) in Tris–Ca^{2+} buffer at a concentration of 500 mg wet weight/ml. Heart homogenates contained about 5 mg phospholipid/ml and diaphragm homogenates about 4 mg phospholipid/ml. The percent distribution of individual phospholipids in egg yolk, heart and diaphragm was described previously [32, 37].

All substrates were serially diluted in Tris–Ca^{2+} buffer and incubated with the PLA_2 enzymes for 10 min at 37°. The liberated free fatty acids were extracted and titrated according to the method of Dole [38], and maximal activities were calculated by extrapolation from Lineweaver–Burk plots to infinite substrate concentrations.

Phospholipid analysis. Following exposure of intact phrenic nerve–diaphragm preparations and right ventricle muscle to PLA_2, the tissues were homogenized (Polytron homogenizer) in 2–5 ml of 10 mM EDTA solution to stop enzymatic activity, as Ca^{2+} is essential for PLA_2 activity [3]. Homogenates were extracted with chloroform–methanol, 1:3 [39], followed by 2:1 [40], and the phospholipids were separated by two-dimensional thin-layer chromatography [41]. Individual phospholipids were detected with iodine vapour and ninhydrin spray, and the phosphorus content of each separated spot was measured [42]. Phospholipid hydrolysis was estimated from the phosphorus values in the lysophospholipid and parent phospholipid spots. In some cases, homogenates of heart or diaphragm muscle or egg yolk were exposed to phospholipase A_2 with individual phospholipids analyzed as described above.

Contractions of phrenic nerve–diaphragm preparations. The effects of PLA_2 on the directly and indirectly induced contractions of the mouse (Swiss–Webster, male, 30–40 g) phrenic nerve–diaphragm preparation were carried out as previously described [9] for a rat preparation using the method of Bulbring [43]. We used the mouse rather than the rat diaphragm in the present study because the preparation from the mouse is smaller. We only had limited amounts of enzyme available and thus needed to use the smallest possible volume of incubation solution (15 ml for the mouse versus 30 ml for the rat preparation). In addition, the potencies of other PLA_2 enzymes (*N. nigricollis, N. n. atra*) were found to be similar in the rat [9] and mouse [30] phrenic nerve–diaphragm preparations. Preparations were bathed in Tyrode's solution [44], bubbled with oxygen, and kept at 37° in a 25-ml organ bath. Muscle twitches were recorded on a Physiograph (Narco Biosystems Inc.) following supramaximal stimulation (0.5 msec, 0.5 Hz) of either the phrenic nerve (indirect stimulation) or the diaphragm (direct stimulation).

Intracellular recording and contractile activity of the isolated rat ventricle. PLA_2-induced effects on electrical and mechanical function were evaluated on hearts removed from male Sprague–Dawley rats (150–250 g), that had been anesthetized with ether, using procedures similar to those previously described [37]. The rat preparation is widely used because of the relative ease of intracellular recording. The right ventricular wall was removed from the heart and placed with the endocardial surface facing upward, in a 4-ml bath containing Tyrode's solution [44] at 37°, and bubbled with oxygen. Contractile activity was monitored on one channel of a dual trace Tektronix model 564 storage oscilloscope using a Narco Biosystems F-60 force transducer. Electrical activity of the preparation was measured [45] using intracellular glass microelectrodes filled with 3 M potassium chloride (10–20 M Ω) and a bath reference electrode. The membrane potential, measured with a WPI 750 high impedance unity gain electrometer, was displayed on the second channel of the oscilloscope. The preparation was field stimulated with a 5-msec square wave pulse at 2.5 times threshold using a Narco Biosystems S1-10 stimulator and silver electrodes. After a 30-min non-stimulated equilibration period, the bathing solution was replaced

* Numbering of residues in PLA_2 is in keeping with that of pancreatic PLA_2 [3]. In order to preserve homology, a gap is introduced at position 20 of the venom. Thus, His-47 and Asp-48 in the venom enzymes are designated as His-48 and Asp-49 [33].

Table 1. V_{max} values and phospholipid preference, using various substrates, of lysine-49 and aspartate-49 PLA_2 enzymes from *A. p. piscivorus* venom*

			% Phospholipid hydrolysis‡							
	V_{max}		2 μg Asp-49 PLA_2				30 μg Lys-49 PLA_2			
Substrate	Asp-49	Lys-49	PC	PE	PS	PI	PC	PE	PS	PI
PC:Triton	732 ± 61 (100%)	10 ± 1 (1.4%)								
Egg yolk	2350 ± 150 (100%)	61 ± 2 (2.6%)	44 ± 2	45 ± 1		34 ± 3	37 ± 1	37 ± 1		12 ± 7
				PC = PE > PI				PC = PE > PI		
Heart homogenate	867 ± 32 (100%)	25 ± 0 (2.9%)	75 ± 2	63 ± 3	34 ± 7	20 ± 4	52 ± 3	40 ± 3	27 ± 3	12 ± 3
				PC > PE > PS > PI				PC > PE > PS > PI		
Diaphragm homogenate	500 ± 0 (100%)	15 ± 1 (3.0%)	68	52	54	43	55 ± 3	41 ± 2	46 ± 4	18 ± 6
				PC > PS = PE > PI				PC > PS = PE > PI		

The percent distribution of phospholipids was: Egg yolk—LPC 2.8; SM 2.3, LPE 3.1, PI 1.7, PC 68, PE 22; Heart homogenates—LPC 2.1, SM 2.6, LPE 3.2, PI 4.4, PS 3.7, PC 41, PE 34, CL + PA 10.1 (CL of heart homogenates was not hydrolyzed by Lys- or Asp-49 PLA_2); Diaphragm homogenates—LPC 2.9, SM 3.0, LPE 3.0, PI 5.4, PS 3.4, PC 48, PE 30, Cl + PA 4.6.

* Results are means ± SE. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; CL, cardiolipin; and PA, phosphatidic acid.

† V_{max} = μequivalents of free fatty acids hydrolyzed/min/mg protein. N = 2 except for PC:Triton where N = 4 and 5, respectively, for Asp-49 and Lys-49. The Lys-49 V_{max} values as percentages of the corresponding Asp-49 values are shown in parentheses.

‡ N = 4 (Lys-49 PLA_2, all substrates and Asp-49 PLA_2, heart homogenates); N = 3 (Asp-49 PLA_2, egg yolk); N = 2 (Asp-49 PLA_2, diaphragm homogenate).

with fresh Tyrode's solution and the preparation was stimulated at a frequency of 2 Hz for 30 min. The bathing solution was again changed, and control measurements were made. PLA_2 was then added and recordings were made, at intervals of time, of resting potential, action potential height, and peak force of contraction. Results are reported as percent of initial control measurements and are averages of at least three different determinations.

Anticoagulant activity. Anticoagulant activity was estimated from recalcification times of platelet-rich plasma prepared from freshly drawn citrated rabbit blood as previously described [10, 46]. Methods for preparing lipid extracts of the plasma, thin-layer chromatographic separation of the phospholipids, analysis of phosphorus on the separated spots, and calculations of percent phospholipid hydrolysis were identical to those previously described [10, 46].

Hemolytic activity. Hemolytic activity was measured on guinea pig red blood cells obtained by heart puncture. The red cells were washed repeatedly in isotonic saline buffered at pH 7.4 with 10 mM Tris–HCl and were resuspended in the same saline–Tris solution. The assay system consisted of 0.2 ml of a 6% red cell suspension and 0.1 ml of enzyme incubated for 45 min at 37° with shaking. Hemolysis was stopped by the addition of 1.5 ml of cold saline followed by a rapid centrifugation. Hemoglobin in the supernatant fraction was estimated at 540 nm.

Determination of lethal potency. Determination of LD_{50} values following enzyme injection into the right lateral ventricles of the brain in male Sprague–Dawley rats (300–400 g) and intravenous injection into Swiss–Webster mice (22–25 g) were performed as previously described [8, 27, 29]. There was insufficient enzyme to allow determination of intravenous LD_{50} in the rat. The rat was used for determination of intraventricular LD_{50} values because of the ease and greater consistency of injections. In addition, only relatively small amounts of enzyme were required, even in the rat, by this route [8, 9, 27, 29, 30].

RESULTS

Enzymatic activity using various in-vitro *substrates.* Both the Asp-49 and Lys-49 PLA_2 showed the highest V_{max} values using egg yolk as the substrate (Table 1). The Lys-49 PLA_2 had much lower activity on all substrates than the Asp-49 enzyme, although it had relatively greater activity on the egg yolk and tissue homogenates (2.6 to 3.0% of the Asp-49 PLA_2) than on the synthetic PC:Triton mixed micelles (1.4%). When used in amounts causing similar percent hydrolysis, we see that the sequence of phospholipid hydrolysis in the three natural substrates was the same for both enzymes (Table 1). The difference in sequences of preferred phospholipids between the three natural substrates is not unexpected, since their phospholipid composition differs and the rate of hydrolysis of any one phospholipid is influenced by the other phospholipids present [3, 7].

Table 2. Phospholipid hydrolysis and anticoagulant activity, in rabbit platelet rich plasma, of Asp-49 and Lys-49 PLA_2 enzymes

PLA_2	μg	PL*	% Hydrolysis† at (min): 0.75	4.8	7.1	30	Clotting time‡ (min)
Asp-49	1.25	PC	12, 14		58, 62	79, 82	
		PE	15, 25		47, 51	64, 70	7.1
		PI	14, 26		29, 41	34, 56	
	2.5	PC			73, 76	95, 95	
		PE			50, 61	80, 82	>30
		PI			35, 47	61, 85	
Lys-49	30	PC	20, 21	51, 52		86, 88	
		PE	13, 14	38, 40		72, 80	4.8
		PI	0, 0	14, 21		50, 52	
	60	PC	30, 31	66, 68		90, 92	
		PE	26, 28	57, 62		78, 79	>30
		PI	16, 41	13, 17		51, 56	

* PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LPC lysophosphatidylcholine; and SM, sphingomyelin. The control phospholipid distribution in rabbit platelet rich plasma is 73% PC, 13% LPC, 8% PE, and 6% SM [10].

† Individual values are shown (N = 2).

‡ Control clotting time = 1.8 min; all clotting times are averages of two determinations which were in excellent agreement.

Lethal potency. Following intravenous injection into mice, the LD_{50} values for the Asp-49 and Lys-49 enzymes were, respectively, 5 and 25 mg/kg. Following injection into the right lateral ventricle of the rat brain, the LD_{50} values for the Asp-49 and Lys-49 enzymes were, respectively, 5 and >160 μg/rat. The greater lethal potency of the Asp-49 PLA_2 is at least qualitatively in agreement with its greater *in vitro* enzymatic activity on phospholipid substrates (Table 1).

Hemolytic and anticoagulant activities. Both enzymes had very little hemolytic activity. Even when used in concentrations of 100 μg per incubation mixture (0.3 ml), the Asp-49 and Lys-49 enzymes caused, respectively, only 30 and 7% hemolysis. This is in contrast to *N. nigricollis* PLA_2, a hemolytic enzyme which causes about 70% hemolysis at a concentration of 1 μg/0.3 ml [37].

The Lys-49 PLA_2 had only about 4% of the anticoagulant potency of the Asp-49 enzyme, as esti-

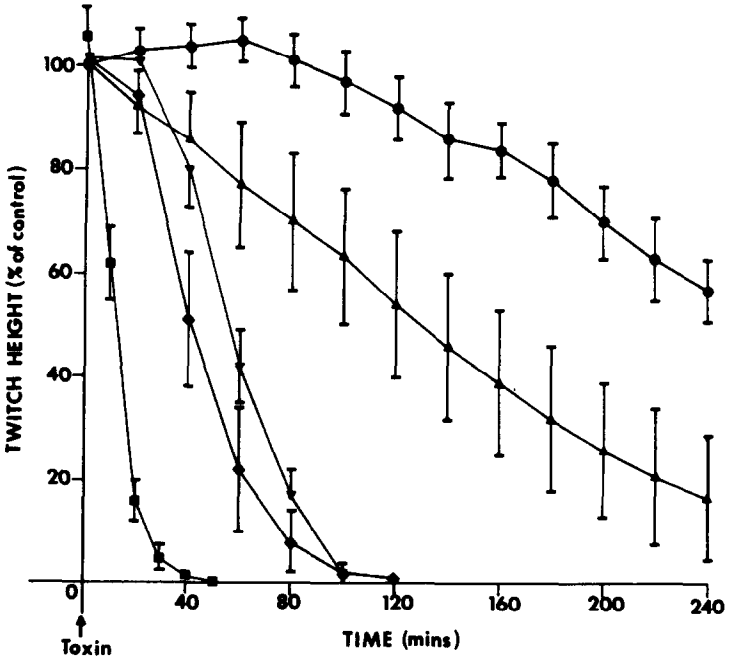

Fig. 1. Effects of 10 (▲) and 35 (■) μg/ml Lys-49 PLA_2 and 10 (◆) and 35 (▼) μg/ml Asp-49 PLA_2 from *A. p. piscivorus* venom on the heights of indirectly stimulated twitches of the mouse phrenic nerve–diaphragm preparation. (●) = Control. Each value is the mean ± SE of four or five separate determinations.

mated by comparing amounts of enzyme causing an approximately equal delay in recalcification times (Table 2). Even the Asp-49 enzyme was, however, only about one-tenth as potent as the strongly anticoagulant *N. nigricollis* PLA_2. It only had about the same potency as the weakly anticoagulant *N. n. atra* PLA_2 [10]. The percent phospholipid hydrolysis with both enzymes was greater than that observed with *N. nigricollis* PLA_2 but less than that with *N. n. atra* PLA_2 [10].

Effects on heart and neuromuscular junction. The effects of the enzymes on the indirectly evoked contractions of the diaphragm in the mouse phrenic nerve–diaphragm preparation are shown in Fig. 1. Identical results were obtained when directly evoked contractions were measured (not shown), suggesting that these enzymes were not acting specifically at the presynaptic terminal or the end plate region. At 10 μg/ml, the Asp-49 PLA_2 had its maximal effect, as noted by the slightly lesser effect of 35 μg/ml. In contrast, 35 μg/ml of the Lys-49 PLA_2 enzyme had a much greater effect than 10 μg/ml and also greater than both 10 and 35 μg/ml of the Asp-49 enzyme. These results suggest that the Lys-49 PLA_2 may have a greater intrinsic activity (efficacy), but a lower affinity, than the Asp-49 PLA_2, although additional concentrations would have to be tested to verify this suggestion.

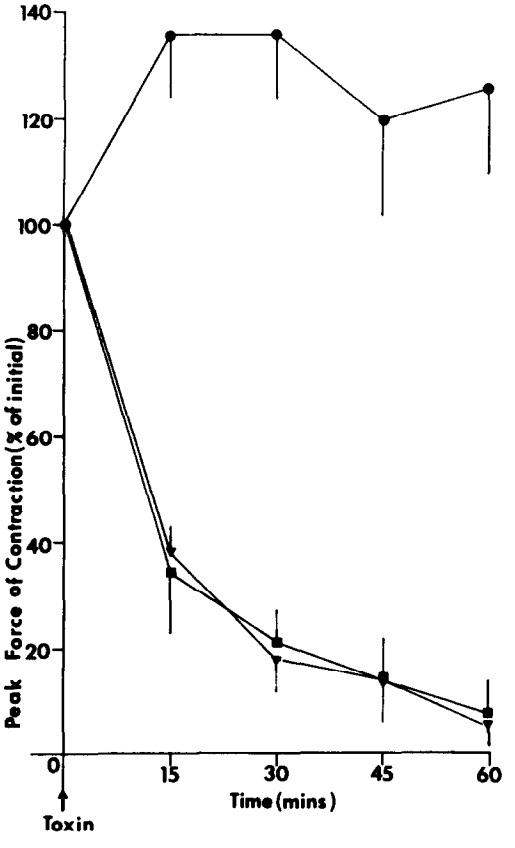

Fig. 2. Effects of Lys-49 PLA_2 (▼) and Asp-49 PLA_2 (■) from *A. p. piscivorus* venom, at a concentration of 70 μg/ml, on peak force of contraction of the isolated rat right ventricular wall preparation. (●) = Control. Each value is the mean ± SE of at least three separate determinations, each made in triplicate.

The effects of the two PLA_2 enzymes (70 μg/ml) on the peak force of contraction, the action potential and the resting potential in the right ventricular wall preparation of the rat heart are shown in Figs. 2–4. Both enzymes caused marked decreases in these electrophysiological parameters of cardiac function. They were equipotent in exerting a negative inotropic effect, causing a 50% decrease in force of contraction in less than 15 min (Fig. 2). The times to 50% decrease of the action potential by 70 μg/ml of the enzymes were very similar (20–25 min), although complete block was reached in 60 min with the Asp-49 PLA_2 whereas the Lys-49 PLA_2 had not caused complete block in 75 min (Fig. 3). The Asp-49 PLA_2 was somewhat more rapid in its effects on the resting potential than the Lys-49 PLA_2, causing a 50% decrease in about 20 min as compared to 40 min being required for the Lys-49 PLA_2 (Fig. 4). Likewise, on the action potential the Asp-49 PLA_2 caused complete block in 60 min, whereas the Lys-49 PLA_2 had not caused complete block in 75 min. At 7 μg/ml neither enzyme had any effect on the heart preparation.

The diaphragm and heart tissues used in the physiological experiments described above were homogenized at the end of the experiments, and phospholipids were analyzed (see Materials and Methods). The Lys-49 PLA_2 did not cause any significant hydrolysis of the two major phospholipids in these tissues, whereas the Asp-49 enzyme caused 28–49% hydrolysis (Table 3).

DISCUSSION

Most PLA_2 enzymes have aspartic acid at the 49 position, and during the formation of the catalytic complex they have an ordered addition of first calcium and then phospholipid [3] some pancreatic enzymes may have a random addition of calcium and phospholipid [47]. In contrast, the few PLA_2 enzymes which have lysine at the 49 position show an ordered addition of first phospholipid and then calcium [33–35]. Although the Lys-49 enzymes have differences at several residues which are invariant in the Asp-49 enzymes, these two groups of enzymes are homologous. They have the same pattern of half cystine residues in the molecule, have hydrophobic residues at the active site, and conserve Asp-99 and His-48 which are associated with the catalytic reaction [35]. Our previous studies using both native and chemically modified Asp-49 PLA_2 enzymes have shown a lack of correlation between enzymatic and pharmacological activities [2, 7–14, 21, 27–32, 37, 46, 48, 49]. For example cardiotoxicity of these enzymes did not correlate with *in vitro* enzymatic activity, *in vivo* phospholipid hydrolysis or with production of lysophosphatides or prostaglandins. Nor could enzymatic activity differences explain the varied lethal potencies, and direct hemolytic or anticoagulant activities, of the native and chemically modified PLA_2 enzymes. Our previous studies, however, were limited to three snake venom PLA_2

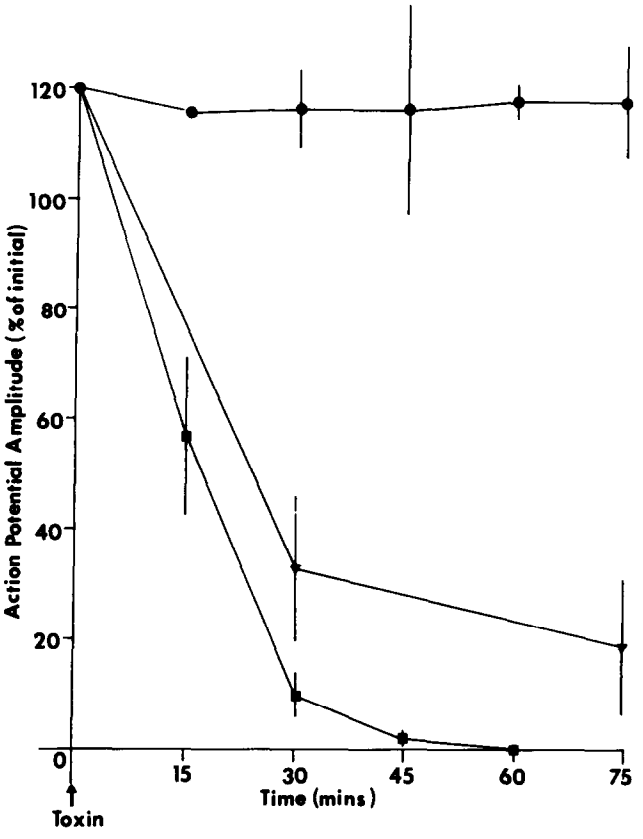

Fig. 3. Effects of Lys-49 PLA_2 (▼) and Asp-49 PLA_2 (■) from *A. p. piscivorus* venom, at a concentration of 70 μg/ml, on the action potential amplitude of cardiac cells in the rat right ventricular wall preparation. (●) = Control. Each value is the mean ± SE of at least three separate determinations, each made in triplicate.

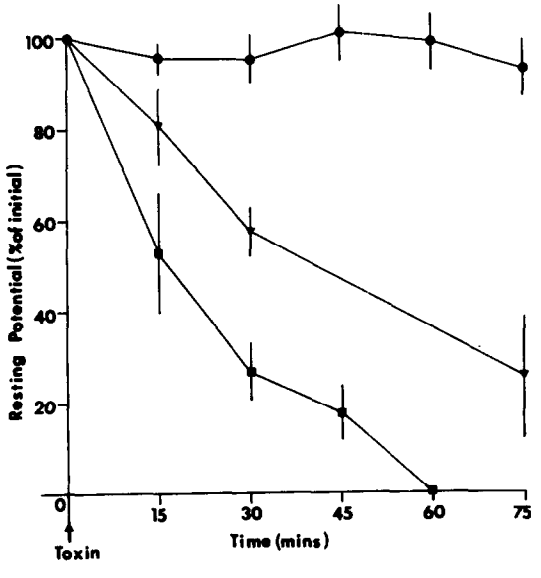

Fig. 4. Effects of Lys-49 PLA_2 (▼) and Asp-49 PLA_2 (■) from *A. p. piscivorus* venom, at a concentration of 70 μg/ml, on resting membrane potentials of cardiac cells in the rat right ventricular wall preparation. (●) = Control. Each value is the mean ± SE of at least three determinations, each made in triplicate.

enzymes, and their chemically modified derivatives, all of the Asp-49 type. It was thus of interest to extend our studies to other PLA_2 enzymes, especially to those having a modified mechanism of catalysis, e.g. the Lys-49 enzymes, to determine whether this class of enzymes may also have a pharmacological active site distinct from that of the enzymatic site.

While the rate constant (k_{cat}/K_m) of the Asp-49 PLA_2 enzyme was only slightly greater than that of the Lys-49 enzyme (measured on lecithin monolayers) [33], the apparent V_{max} of this Asp-49 PLA_2 is 33- to 73-fold greater than that of the Lys-49 enzyme (measured on a synthetic and several natural substrates; Table 1). In many respects, however, both enzymes are similar; for example, both are highly basic enzymes that require calcium for catalysis [33] and both show similar phospholipid substrate preferences (Table 1).

It has been noted that basic PLA_2 enzymes show greater toxicity and pharmacological activity than acidic or neutral enzymes (see review [1] for references). Our studies [31, 32], however, had shown that isoelectric points cannot be used to predict the lethal potencies of these enzymes. In this present study, we also found that, while both enzymes were strongly basic, the *A. p. piscivorus* Asp-49 enzyme had intravenous and intraventricular lethal potencies

Table 3. *In vivo* phospholipid hydrolysis in diaphragm and heart by the Asp-49 and Lys-49 PLA_2

Preparation	PLA_2	Concn (μg/ml)	% Hydrolysis* PC	% Hydrolysis* PE
Diaphragm	Asp-49	35	44 ± 9	49 ± 6
	Lys-49	35	4 ± 4	2 ± 4
Heart	Asp-49	70	28 ± 4	34 ± 2
	Lys-49	70	1 ± 2	4 ± 2

* Results in mouse phrenic nerve–diaphragm and rat right ventricle are expressed as mean ± SE (N = 3 or 4). All values are corrected for the level of hydrolysis in control tissues. Asp-49 PLA_2 values are significantly ($P < 0.05$) different from corresponding Lys-49 PLA_2 values.

of 5-fold and greater than 32-fold, respectively, those of the Lys-49 enzyme. The lethal potency of the basic Asp-49 PLA_2 was similar to that of the neutral *H. haemachatus* PLA_2 and the acidic *N. n. atra* PLA_2 but about 10-fold less than that of the basic *N. nigricollis* PLA_2 (compare results in present study with those in Refs. 8 and 29). In contrast, the Lys-49 enzyme was less toxic than any other PLA_2 enzyme we have tested. Differences in intravenous potencies of other PLA_2 enzymes have been related to their differences in cardiotoxicity [9 11], whereas differences in intraventricular potencies have been related to differences in binding to synaptic plasma membranes [13]. The Lys-49 PLA_2 may be unable to bind effectively to brain synaptic plasma membranes, although this has yet to be determined experimentally. The 5-fold difference in intravenous lethal potencies cannot be related directly to differences in cardiotoxicity or blocking action on the phrenic nerve-diaphragm preparation since the Lys-49 enzyme was not markedly weaker than the Asp-49 enzyme in these respects (Figs. 1–4).

The *A. p. piscivorus* Asp-49 enzyme used in this study resembles, in several respects, the *N. n. atra* and *H. haemachatus* Asp-49 enzymes used in our previous work. As noted previously, their lethal potencies are similar. In addition, all three enzymes have little direct hemolytic activity and only weak anticoagulant activity [2, 7, 10, 21, 37; Table 2]. The concentrations of these three enzymes required to affect the isolated ventricle and the phrenic nerve–diaphragm preparation are also very similar [2, 9, 21, 29–31, 37; Figs. 1–4]. The *A. p. piscivorus* Asp-49 enzyme has enzymatic activity about equal to that of the other two enzymes on lecithin–Triton mixed micelles and upon egg yolk, while it has less activity on heart and diaphragm homogenates [32, 37, 49; Table 1]. In contrast to these three enzymes, *N. nigricollis* Asp-49 PLA_2 has much greater lethal, direct hemolytic, anticoagulant and cardiotoxic effects even though it has lower enzymatic activity on all substrates [2, 7–14, 21, 27–32, 37, 46, 48, 49]. The *A. p. piscivorus* Lys-49 PLA_2 differed from the four Asp-49 PLA_2 enzymes in having much lower enzymatic activity (Table 1), lethal potency, and anticoagulant activity (Table 2).

On the phrenic nerve–diaphragm preparation, the Lys- and Asp-49 PLA_2 enzymes from *A. p. piscivorus* venom were similar to the other three enzymes used in previous studies. They are, however, all different from presynaptic acting PLA_2 toxins (β-bungarotoxin, notexin, etc.) in that they have no specific presynaptic action, affecting equally the directly and indirectly evoked contractions of the diaphragm. The two enzymes used in the present study and the three enzymes used in our earlier studies also show qualitatively similar effects upon isolated strips of cardiac tissue, although the *N. nigricollis* enzyme is much more potent (references previously noted). Even though the Lys-49 PLA_2 from *A. p. piscivorus* venom had only 1.4 to 3.0% of the enzymatic activity of the Asp-49 enzyme from the same venom (Table 1), it had the same or only slightly less activity than the Asp-49 enzyme when tested upon the isolated heart or phrenic nerve–diaphragm preparation (Figs. 1–4). This would appear to be another example of a dissociation between enzymatic and pharmacological potencies, reinforcing the conclusion, already noted, of our earlier studies. Of even more direct relevance in this regard are the measurements of phospholipid hydrolysis made upon the tissues at the conclusion of the physiological measurements. The Asp-49 enzyme caused significant hydrolysis of the two major tissue phospholipids (28–49%; Table 3), whereas the Lys-49 enzyme caused no significant hydrolysis (Table 3). While this marked difference in the extent of tissue hydrolysis correlates well with the differences in enzymatic activity observed upon *in vitro* incubation of the enzymes with various substrates (Table 1), it does not correlate with their equipotent effects upon the electrical and/or mechanical properties of these tissues. The Lys-49 enzyme is much more potent than might have been expected, based upon its low enzymatic activity. This suggests that the Lys-49 enzyme, like the *N. nigricollis* Asp-49 PLA_2 enzyme, has pharmacological actions that are independent of tissue phospholipid hydrolysis and that do not correlate with their relatively low enzymatic activities.

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