Biochemical Pharmacology, Vol. 32, No. 23, pp. 3525–3530, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

DISSOCIATION OF PHARMACOLOGICAL AND ENZYMATIC ACTIVITIES OF SNAKE VENOM PHOSPHOLIPASES A_2 BY MODIFICATION OF CARBOXYLATE GROUPS

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(*Received* 31 *December* 1982; *accepted* 28 *April* 1983)

Abstract—The carboxylate groups in an acidic and in a basic phospholipase A_2 (PLA_2) enzyme, purified, respectively, from *Naja naja atra* and *Naja nigricollis* snake venoms, were modified with carbodiimide and semicarbazide. The derivatives modified at pH 3.5 and pH 5.5 had less than 1% (*N. nigricollis*) or 2% (*N. n. atra*) residual enzymatic activity, whereas 12–16% enzymatic activity remained following modification at pH 5.5 in the presence of Ca^{2+}. In marked contrast, these derivatives retained variable, but significantly greater, levels of lethal potency, hemolytic and anticoagulant activities, and abilities to block indirectly and directly induced contractions of the diaphragm. By this modification of aspartic and glutamic acid residues we have, for the first time, obtained derivatives of PLA_2 which selectively retain greater pharmacological activity relative to enzymatic activity. Previously, we had found that modification of lysine and arginine residues produced derivatives which retain enzymatic activity but show a loss of pharmacological properties. These findings suggest that some pharmacological effects of snake venom PLA_2 enzymes are due to a non-enzymatic action, suggesting two distinct but perhaps overlapping active sites.

Many basic phospholipase A_2 enzymes (PLA_2, EC 3.1.1.4), isolated from snake venoms, have potent pharmacological effects such as cardiotoxicity, myonecrosis, hemolysis, anticoagulant action and blockade of neurotransmission by a presynaptic action [1–9]. In contrast, other venom PLA_2 enzymes, usually acidic or neutral, are much weaker pharmacological agents, even though their enzymatic activities, as measured in various *in vitro* systems, are often much greater than those of the basic enzymes [1–9]. Despite this apparent lack of correlation, it has been suggested that the pharmacological properties of PLA_2 are due to phospholipid hydrolysis [1–4, 10–15]. This conclusion was strengthened by the observation that *p*-bromophenacylation or methylation of one histidine at the enzymatic active site causes a loss of both pharmacological and enzymatic activities [1–4, 16–22]. Other chemical modifications, however, such as carbamylation, ethoxyformylation or guanidination of lysines or diphenylglyoxylation of arginines in PLA_2 cause, under certain conditions, a greater loss of pharmacological than of enzymatic activity [23–27]. However, this may not be a critical test as to whether enzymatic activity is essential for pharmacological properties since modification of the basic lysine or arginine groups may have altered additional properties such as tissue binding, distribution, or stability of the enzyme. All these factors have been suggested as possibly being responsible for the differences in pharmacological potencies observed with proteins having PLA_2 activity [27–32]. A selective loss of enzymatic activity would be much stronger evidence for an enzymatic independent action(s) of PLA_2 but has, up until now, never been reported. We have, however, now found that, by modification of the carboxylate groups, the basic and relatively toxic *Naja nigricollis* PLA_2 and the acidic and less toxic *Naja naja atra* PLA_2 [5–9] exhibit a marked loss of enzymatic activity which is associated with a smaller loss, no loss, or even an increase in various pharmacological properties.

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MATERIALS AND METHODS

Isolation and purification of PLA_2. Lyophilized *N. nigricollis* (spitting cobra) venom was obtained from the Miami Serpentarium Laboratory, Miami, FL, and lyophilized *N. n. atra* (Taiwan cobra) venom was collected in Kaohsuing, Taiwan, by one of the authors (C.-C.Y.). The most basic PLA_2 (pI 10.6) from *N. nigricollis* venom was separated on a CM-Sephadex C-25 column and further purified on a DEAE-Sephacyl column as previously described [33]. The major acidic PLA_2 (pI 5.2) from *N. n. atra* venom was isolated and purified by successive chromatography on SP-Sephadex C-25, DEAE

Sephacyl and CM Sephadex C-25 columns as previously described [34, 35]. The homogeneity of these two enzymes was verified using disc electrophoresis on a 7% polyacrylamide gel [36].

Modification of carboxylate groups in PLA_2. Modification reactions were carried out using water soluble carbodiimide and semicarbazide, reagents previously found to be suitable for specific modification of carboxyl groups in proteins [37–39]. Procedures utilized for modification of the PLA_2 enzymes, protein desalting, and regeneration of tyrosine residues were performed essentially as described by Fleer *et al.* [40] and may be summarized as follows. PLA_2 (5 mg/ml) was dissolved in 0.25 M cacodylate buffer (pH 5.5) containing 1 M semicarbazide. When the reaction was carried out at pH 3.5, 1 M semicarbazide–HCl was used as buffer. After determination of the initial enzyme activity, the reaction was started by the addition of solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide to a final concentration of 0.1 M. Addition of the carbodiimide (5 mg/ml) was repeated every 30 min to compensate for its decomposition in water. When no further change in enzymatic activity occurred, the reaction was terminated by the addition of solid sodium acetate (75 mg/ml) and the protein was desalted immediately on a column of Sephadex G-25 equilibrated with 0.02 M ammonium bicarbonate. To regenerate tyrosyl residues [41], the protein was treated with 1 M hydroxylamine for 3 hr at pH 7.5 and 25°, followed by desalting and lyophilization. Modified proteins from *N. n. atra* PLA_2 were purified on an SP-Sephadex C-25 column (2.8 × 35 cm) equilibrated with 0.05 M phosphate buffer (pH 7.5) and elution was performed with a linear NaCl gradient (0 to 0.8 M over 2400 ml) in the same buffer. Carboxyl modified fractions from *N. nigricollis* PLA_2 were purified on a column (2 × 27 cm) of DEAE-Sephacyl equilibrated with 0.01 M ammonium bicarbonate buffer (pH 8.9). The buffer was eluted by a non-linear gradient from 300 ml of 0.01 M (pH 8.9) to 600 ml of 0.05 M ammonium bicarbonate (pH 7.8). Purified fractions were eluted as single peaks (pI > 11) and were free of native enzyme.

Modification reactions were carried out at pH 3.5 and 5.5 in the absence of Ca^{2+} and at pH 5.5 in the presence of 1 M Ca^{2+} [40, 42]. Under these three conditions, all carboxylate groups in bovine pancreatic PLA_2 are modified [40] except the invariant residue Asp 99 at pH 3.5, Asp 99 and 39 at pH 5.5, and Asp 99, 39 and 49 at pH 5.5 + Ca^{2+}. Since *N. nigricollis* PLA_2, having nine carboxylates, and the *N. n. atra* enzyme, with fifteen carboxylates, show a considerable degree of homology with the pancreatic enzyme (including Asp groups equivalent to those noted above) and have enzymatic sites with similar properties [43], it is assumed that the modifications of the invariant carboxylates in our venom enzymes are similar.

Determination of enzymatic and pharmacological properties. Enzymatic activity was measured on purified lecithin:triton (molar ratio 1:2) mixed micelles at pH 8.5 in the presence of 10 mM Ca^{2+} with lecithin hydrolysis assayed by titration of fatty acids that were liberated at short time intervals in which only a small percentage of the substrate is hydrolyzed [5]. V_{max} values were derived from Lineweaver-Burk plots at various substrate concentrations.

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 [6, 21, 23].

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 used for *N. nigricollis* phospholipase consisted of 0.2 ml of a 6% red cell suspension and 0.1 ml enzyme, incubated for 1.5 hr at 37° with shaking. For the much less hemolytic *N. n. atra* phospholipase, 0.2 ml of a 3% red cell suspension was incubated with 0.2 ml enzyme. Hemolysis was stopped by addition of 1.5 ml of cold saline followed by a rapid centrifugation. Hemoglobin in the supernatant fraction was estimated at 540 nm. Anticoagulant potency was estimated from recalcification times of platelet-rich plasma prepared from freshly drawn citrated rabbit blood as previously described [8, 24]. 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 [8, 24].

PLA_2 effects on directly and indirectly induced contractions of the mouse (male Swiss–Webster) phrenic nerve-diagram preparation were tested as previously described [7] for a rat preparation. Preparations, bathed in Tyrode's solution [7], were kept at 35° in a 15 ml organ bath, with muscle twitches of the diaphragm being recorded on a desk model Physiograph (Narco Biosystems Inc.) following supramaximal stimulation (0.5-msec duration) of either the phrenic nerve (indirect stimulation) or the diaphragm (direct stimulation). All preparations were equilibrated for 60 min prior to the addition of PLA_2.

RESULTS

The enzymatic activities of the native and modified PLA_2 enzymes and their lethal, hemolytic and anticoagulant potencies are shown in Table 1. Enzymatic activity following modification at pH 3.5 or pH 5.5 was depressed to very low levels, which is in good agreement with the loss of activity observed with bovine pancreatic PLA_2 [40]. This drastic decrease is accounted for by the modification of Asp 49, the Ca^{2+} binding site, Ca^{2+} being essential for PLA_2 activity [40, 42, 43]. The addition of Ca^{2+} at pH 5.5 protected the Ca^{2+} binding site on Asp 49 [40, 42] and the residual enzymatic activity increased to 12–16% of the native, which again is in good agreement with the previous finding of 15% using bovine pancreatic PLA_2 [40].

The percentage of enzymatic activity remaining was in all cases markedly less than the percentage of lethal potency and hemolytic activity remaining. Note that even at pH 3.5 and 5.5 there remained 29% of the lethal potency with the *N. n. atra* enzyme

Table 1. Effect of modification of carboxylate groups on various activities of phospholipases A_2*

PLA_2	pI	Enzymatic V_{max} ‡	%	Lethality LD_{50} μg/rat	%	Hemolytic HC_{50} μg/ml	%	Anticoagulant μg/ml	%
N. n. atra									
Native	5.2	500	100	5	100	>300	100	5	100
pH 3.5	9.4	10	2	17	29			100	5
pH 5.5	8.0	10	2	17	29			50	10
pH 5.5 + Ca^{2+}	8.0	60	12	1	500	100	>300	5	100
N. nigricollis									
Native	10.6	200	100	0.5	100	6	100	0.25	100
pH 3.5	>11	<2	<1	9.4	5	28	21	5	5
pH 5.5	>11	<2	<1	9.4	5	28	21	20	1
pH 5.5 + Ca^{2+}	>11	32	16	1.5	33	9	66	0.5	50

* V_{max} values were determined on lecithin–triton (1:2) mixed micelles; LD_{50} values were calculated following injection centrally into the right lateral ventricle; the concentration to cause 50% hemolysis (HC_{50}) was derived from hemolysis enzyme concentration curves using guinea pig red cells. Anticoagulant concentration is that which prolongs recalcification time to greater than 30 min (see Table 2 for further details). Values in the table are the averages of duplicate determinations which were in good agreement. The percent values represent the percent of the native (unmodified enzyme) activity. Of the nine free carboxyls in *N. nigricollis* PLA_2 (7 Asp, 1 Glu, 1-COOH terminal) and the fifteen in *N. n. atra* PLA_2 (10 Asp, 4 Glu, 1-COOH terminal) we would expect all to be modified except Asp 99 at pH 3.5, Asp 99 + 39 at pH 5.5, and Asp 99 + 39 + 49 at pH 5.5 in the presence of Ca^{2+} [39].

‡ Microequivalents of free fatty acids liberated · min^{-1} · mg^{-1}.

Table 2. Effect of modification of carboxylate groups of PLA_2 enzymes on their anticoagulant potency and associated phospholipid hydrolysis in rabbit platelet-rich plasma*

PLA_2	Phospholipids	% Hydrolysis at 45 sec	% Hydrolysis at Clotting time	% Hydrolysis at 30 min	Delay in clotting time
N. n. atra					
Native					
2.5 μg	PC	58	90		
	PE	65	100		3 min 35 sec
	HP	59	91		
5.0 μg	PC	86		95	
	PE	84		100	>30 min
	HP	86		96	
Carboxyl modified					
pH 3.5					
25 μg	PC	9	46		
	PE	32	79		3 min
	HP	11	49		
100 μg	PC	34		97	
	PE	75		100	>30 min
	HP	38		97	
pH 5.5					
12.5 μg	PC	12	29		
	PE	25	67		3 min 15 sec
	HP	13	32		
25 μg	PC	20		85	
	PE	42		95	<30 min
	HP	22		85	
50 μg					>30 min
pH 5.5 + Ca^{2+}					
2.5 μg	PC	11	56		7 min 40 sec
	PE	10	86		
	HP	12	58		
5.0 μg	PC	29		91	
	PE	65		96	>30 min
	HP	34		90	

Table 2—*continued*

PLA_2	Phospholipids	% Hydrolysis at 45 sec	% Hydrolysis at Clotting time	% Hydrolysis at 30 min	Delay in clotting time
N. nigricollis					
Native					
0.125 μg	PC	12	22	59	2 min 35 sec–3 min 15 sec
	PE	19	30	65	
	HP	14	23	60	
0.25 μg	PC	14		84	>30 min
	PE	23		88	
	HP	16		85	
Carboxyl modified					
pH 3.5					
2.5 μg	PC	8	10		1 min 2 sec
	PE	8	27		
	HP	8	12		
5.0 μg	PC	13		81	>30 min
	PE	29		100	
	HP	16		83	
pH 5.5					
10 μg	PC	16	50		1 min 30 sec–3 min 30 sec
	PE	43	77		
	HP	18	52		
20 μg	PC	28		93	>30 min
	PE	59		100	
	HP	31		93	
pH 5.5 + Ca^{2+}					
0.25 μg	PC	6	18	59	2 min–3 min 15 sec
	PE	16	33	65	
	HP	7	21	60	
0.50 μg	PC	10		84	>30 min
	PE	18		88	
	HP	11		85	

* Aliquots (1 ml) of platelet-rich plasma were supplemented with enzyme, recalcified, clotting times or lack of coagulation noted, and the phospholipids extracted at the times indicated. Several different batches of plasma were used, their control recalcification times being between 1.5 and 2.5 min. The hydrolyzable phospholipids (HP) are phosphatidylcholine (PC) plus phosphatidylethanolamine (PE) which constitute 65–77% of the total phospholipids in the plasma. Values shown are the means of duplicate experiments which were in good agreement.

and 21% of the hemolytic potency with the *N. nigricollis* enzyme, even though these preparations showed only 2%, or less than 1%, of the native enzyme activity. The dissociation was even more dramatic with the *N. n. atra* enzyme modified at pH 5.5 in the presence of Ca^{2+} where the lethal and hemolytic potencies were, respectively, 5-fold and more than 3-fold greater than the native unmodified enzyme even though the enzymatic activity was only 12% that of the native enzyme. Because of the limited amounts of derivatives available, it was not possible to determine the intravenous LD_{50} values for all derivatives; however, the intravenous LD_{50} of *N. n. atra* pH 5.5 + Ca^{2+} was 8.6 mg/kg which is the same as that of the native enzyme.

A comparison of the anticoagulant potencies of the derivatives with their residual enzymatic activities (Table 1) indicates an approximately 2- to 8-fold greater retention of the former than of the latter. The anticoagulant potencies of the native and modified enzymes, as well as the associated hydrolysis of phospholipids in rabbit plasma, are shown in detail in Table 2. The anticoagulant potency following modification at pH 5.5 in the presence of Ca^{2+} was unchanged for the *N. n. atra* (5.0 μg/ml preventing coagulation) and about 50% that of the native enzyme for *N. nigricollis* (0.50 μg/ml preventing coagulation). We had found previously [8, 24] that the anticoagulant potencies of *N. nigricollis* and *N. n. atra* PLA_2 did not correlate with the extent of hydrolysis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the two major hydrolyzable phospholipids in plasma. Likewise, in our present studies we found that, although the *N. n. atra* PLA_2, modified at pH 5.5 in the presence of Ca^{2+}, was as potent an anticoagulant as the native enzyme, it only hydrolyzed, at the anticoagulant dose of 5.0 μg/ml, 34% of the available substrate (PC + PE) in 45 sec, whereas the corresponding value for the same amount of native enzyme was 86%. The native *N. nigricollis* PLA_2 was a more potent anticoagulant than the *N. n. atra* enzyme, even though it induces much less phospholipid hydrolysis [8, 24]. Its pH 5.5 + Ca^{2+} derivative, at the anticoagulant concentration of 0.5 μg/ml (Table 2), induced, in 45 sec a low level of PC + PE hydroly-

Table 3. Effect of modification of carboxylate groups of PLA_2 enzymes on their abilities to block directly and indirectly evoked contractions of the mouse phrenic nerve-diaphragm preparation*

PLA_2	Conc (μg/ml)	Time (min) to block contraction			
		Direct	%	Indirect	%
None		503 ± 99	100	409 ± 27	100
N. n. atra					
Native	12	175 ± 39	35	147 ± 32	36
	24	108 ± 8	21	65 ± 22	16
pH 3.5	24	412 ± 75	82	299 ± 83	73
pH 5.5	24	281 ± 100	56	274 ± 110	67
pH 5.5 + Ca^{2+}	12	275 ± 99	55	202	49
N. nigricollis					
Native	6	159	32	139	34
	12	124 ± 52	25	90 ± 35	22
pH 3.5	24	589 ± 67	117	483 ± 102	118
pH 5.5	24	406 ± 112	81	334 ± 114	82
pH 5.5 + Ca^{2+}	12	142	28	97	24
	24	90	18	67	16

* Diaphragm muscle was stimulated either directly or indirectly via stimulation of the phrenic nerve. Times to block of contraction (99% decreases in force of contraction) were compared with the survival in control untreated preparations. Times (min) to block of contractions are shown as mean ± S.D. (two to four experiments) or as single values.

sis (11%), which is comparable to that induced by an anticoagulant concentration of the native enzyme (16% at 45 sec).

With the mouse phrenic nerve-diaphragm preparation (Table 3), the results with the native enzymes were similar to earlier findings using a rat diaphragm preparation [7]. The derivative modified at pH 5.5 in the presence of Ca^{2+} was either as potent (*N. nigricollis*) or only slighly less potent (*N. n. atra*) than the native enzyme in blocking directly and indirectly evoked contractions of the diaphragm. Only the pH 3.5 derivative of the *N. nigricollis* enzyme was inactive upon the diaphragm in the concentration used. Once again, a dissociation between pharmacological potency (Table 3) and residual enzymatic activity (Table 1) was observed.

DISCUSSION

These results provide strong evidence for a dissociation between the enzymatic and the pharmacological properties of snake venom PLA_2 enzymes. We have, for the first time, found derivatives of PLA_2 which show retention or even potentiation of pharmacological effects, although enzymatic activity was reduced markedly. Previously reported loss of enzymatic activity induced by modification of histidine at the enzymatic site of PLA_2 was associated with loss of pharmacological potency [16–22], suggesting that these two variables are related. The histidine modifications could, however, also be explained if the histidine at the enzymatic active site (histidine–48) also overlapped or interacted with a separate site responsible for the pharmacological properties of the enzyme. An alternative possibility is suggested by a recent report [44] that not only a histidine participates in the *p*-bromophenacylation of *N. n. atra* PLA_2, but also another group, probably the α-amino group of the N-terminal asparagine residue. *p*-Bromophenacylation could, therefore, cause loss of activity not only at the enzymatic active site but also at a separate pharmacological site.

More direct evidence, in support of our suggestion that the enzymatic and the pharmacological properties of PLA_2 enzymes are not related, is provided by our findings that *in vivo* levels of phospholipid hydrolysis did not correlate with the relative potencies of *N. nigricollis* and *N. n. atra* PLA_2 in inducing cardiotoxicity, blocking contractions in the phrenic nerve-diaphragm preparations, prolonging clotting time of rabbit plasma, or inducing convulsions and death from intraventricular injection [5–9, 21, 23–26]. These same results also showed that the pharmacological effects of these PLA_2 enzymes are not due to the lysophospholipids or free fatty acids liberated in the tissues as a result of PLA_2 enzymatic action. If hydrolytic products were responsible for pharmacological actions, we would have observed some relationship between extent of *in vivo* phospholipid hydrolysis and relative pharmacological potency toward these various preparations. In addition, following chemical modifications of arginine and lysine side chains, pharmacological effects are decreased much more than enzymatic activities [23–26]. It is interesting that similar effects upon pharmacological activities were observed by Visser *et al.* [45] following chemical modification of amino acid side chains in snake venom cardiotoxins (basic proteins with no phospholipase activity). They found that modification of lysine and arginine groups decreases pharmacological actions whereas modification of carboxyl groups has no effect.

Phospholipases A_2 are used extensively as probes with which to study phospholipid function and membrane organization. If, however, as our results suggest, PLA_2 has effects independent of or in addition to phopholipid hydrolysis, then the usefulness of PLA_2 as a membrane probe must be critically reev-

aluated. On the basis of all of our results, we conclude that the toxicity of at least certain snake venom PLA_2 enzymes may be due to a direct effect which does not correlate with levels of phospholipid hydrolysis and that this direct action is prominent in the relatively toxic enzyme while it is less manifest in the less toxic enzymes. The possibility of selectively depressing the catalytic function, with little effect on pharmacological properties, by manipulation of aspartic and glutamic acids, and conversely decreasing the pharmacological effects with conservation of enzymatic activity by alteration of lysines and arginines, suggests two distinct, but perhaps overlapping (at His 48) sites in PLA_2.

Acknowledgements—This work was supported by a U.S. Public Health Service Grant (ROINS 14521) awarded to P. R. We thank Mrs. Lilian Braun for expert technical assistance.

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