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Biochemical and biological properties of phospholipases A_2 from *Bothrops atrox* snake venom

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Abstract

Phospholipases A_2 (PLA_2s), of molecular mass 13–15 kDa, are commonly isolated from snake venom. Two myotoxins with PLA_2 activity, $BaPLA_2I$ and $BaPLA_2III$, with estimated molecular masses of 15 kDa were isolated from the venom of *Bothrops atrox* using Sephacryl S-100-HR and reverse-phase chromatography. $BaPLA_2I$ was basic, with a pI of 9.1, while $BaPLA_2III$ was neutral with a pI of 6.9. On a molecular basis, $BaPLA_2III$ exhibited higher catalytic activity on synthetic substrates than $BaPLA_2I$. Comparison of the N-terminal residues of $BaPLA_2I$ with other PLA_2 proteins from snake venoms showed that it has the highest homology (94%) with *B. asper* myotoxin II and homology with a PLA_2 Lys^{49} from *B. atrox* (89%). In contrast, $BaPLA_2III$ demonstrated 75, 72, and 71% homology with PLA_2 from *Vipera ammodytes meridionalis*, *B. jararacussu*, and *B. jararaca*, respectively. $BaPLA_2I$ and $BaPLA_2III$ were capable, *in vitro*, of inducing mast cell degranulation and, *in vivo*, of causing creatine kinase release, edema, and myonecrosis typical of PLA_2s from snake venoms, characterized by rapid disruption of the plasma membrane as indicated by clumping of myofilaments and necrosis of affected skeletal muscle cells. $BaPLA_2I$- and $BaPLA_2III$-specific monoclonal and polyclonal antibodies, although incapable of neutralizing PLA_2 edematogenic activity, blocked myonecrosis efficiently in an *in vivo* neutralization assay. The results presented herein suggest that the biological active site responsible for edema induction by these two PLA_2 enzymes is distinct from the myonecrosis active site and is not dependent upon the catalytic activity of the PLA_2 enzyme.

Keywords: Phospholipase A_2; Myotoxin; Edema; Snake venom

1. Introduction

PLA_2s (EC 3.1.1.4) catalyze the hydrolysis of *sn-2* ester bonds in phospholipid substrates. The hydrolysis products of the PLA_2 reaction are free fatty acids and lysophospholipids. The fatty acids released by PLA_2, such as arachidonic acid and oleic acid, can be important stores of energy. Arachidonic acid can also function as a second messenger and as the precursor of eicosanoids, which are potent mediators of inflammation and signal transduction. Lysophospholipid, the other product of PLA_2 action, is important in cell signaling, phospholipid remodeling, and membrane perturbation [1,2].

PLA_2s are ubiquitous in nature and have different molecular masses, localization, and calcium requirements for enzymatic activity. Secreted PLA_2s, of 14 kDa, are present in mammals at high concentrations in pancreatic juice and in inflammatory exudates of synovial fluid [3,4]. They are also components of snake, bee, lizard, and scorpion venoms and can be divided into several types based on amino acid sequence and disulfide bond arrangement [3,4]. Type II secreted PLA_2 enzymes include those from *Viperidae* snake venoms and the mammalian secretory type II enzyme that is found in inflammatory exudates.

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Abbreviations: PLA_2, phospholipase A_2; $BaPLA_2I$, *Bothrops atrox* PLA_2I; $BaPLA_2III$, *Bothrops atrox* PLA_2III; pI, isoelectric point; mAb, monoclonal antibody; IEF, isoelectric focusing; and CK, creatine kinase.

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From sequence analyses of the primary structure of type II PLA_2s, it was demonstrated that this group can also be divided into at least two subclasses: (i) the D-49 enzymes, which have an aspartate residue at position 49 and high catalytic activity on artificial phospholipid substrates, and (ii) the K-49 enzymes, which have a lysine residue at position 49 and very low or no hydrolytic activity on artificial substrates. The presence of an aspartic acid at position 49 is crucial for calcium binding and Ca^{2+} is essential for catalytic activity [5]. Despite the lack of an aspartate residue at position 49, the K-49 PLA_2 proteins are very active in the induction of myonecrosis by one or more, yet to be discovered, mechanisms [6].

We report, in this paper, the isolation and biochemical characterization of two PLA_2s from *Bothrops atrox* venom, $BaPLA_2I$ and $BaPLA_2III$, with molecular masses of 15 kDa and pIs of 9.1 and 6.9, respectively. Based on amino-terminal sequence information, catalytic capacity to hydrolyze specific synthetic substrates, and pIs, $BaPLA_2I$ is a K-49 PLA_2, whereas $BaPLA_2III$ is a D-49 PLA_2. In spite of this difference, both enzymes exhibit strong myonecrotic, edematogenic, and mast cell degranulation activities and cause the release of CK.

2. Materials and methods

2.1. Snake venoms

Venoms from adult specimens of *B. atrox* were provided by the Laboratório de Herpetologia, Instituto Butantan. The venoms were filtered through a 0.45 μm membrane, lyophilized, divided into 10 mg aliquots, and stored at −20°.

2.2. Animals

BALB/c mice were from the Isogenics Mouse Animal House at the Instituto de Ciências Biomédicas, Universidade de São Paulo. CF1 mice and RA rats were from the Animal House at the Universidade Federal Fluminense. Animals were bred and reared under strict ethical conditions according to international recommendations.

2.3. Protein purification

Venom samples (50 mg) dissolved in 2.5 mM Tris–HCl, 150 mM NaCl buffer, pH 8.0, were applied to a Sephacryl S-100-HR (Pharmacia) column previously equilibrated with the same buffer. This buffer was also used for the elution step at a flow rate of 1.25 mL/min; the protein contents were monitored spectrophotometrically at 280 nm, and PLA_2 activity was assayed on a fluorescent synthetic substrate as described below. All fractions exhibiting PLA_2 activity were pooled and freeze-dried. The PLA_2 freeze-dried pooled fractions were dissolved in water and applied to an HPLC reverse-phase C4 column (Pharmacia) previously equilibrated with solution A (0.08% trifluoracetic acid). The elution was performed with solution B (0.08% trifluoracetic acid, 80% acetonitrile) in three steps with a flow rate of 0.5 mL/min. The first step consisted of a 20-min elution with a 0–35% gradient; the second step, from min 20–50, was performed with a 35–55% gradient; and the third step, from min 50–55, was done using a 55–100% gradient. The protein content of each fraction was monitored at 280 nm, and PLA_2 activities were assayed.

2.4. PLA_2 assays

PLA_2 activity in fractions following column chromatography was assayed with 1-acyl-2-{6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]-caproyl} phosphatidylcholine (C6-NBDPC) (Avanti Polar Lipids) as a fluorometric substrate. The assay mixture contained 50 mM Tris–HCl (pH 8.0), 3 mM $CaCl_2$, 100 mM NaCl, and an appropriate amount of each fraction in a final volume of 2.0 mL. The reaction was started by the addition of 5 μL of a solution of 1 mg/mL of C6-NBDPC, and fluorescence was monitored continuously as a function of time, with excitation at 460 nm and emission at 534 nm. Protein fractions with PLA_2 activity were lyophilized for storage.

2.5. Electrophoresis

The samples were subjected to 12% polyacrylamide gel electrophoresis according to the method of Laemmli [7], and protein bands were stained with Coomassie brilliant blue. All gels were subjected to computerized densitometric analysis [8].

2.6. N-terminal sequence determination

N-terminal sequences of the purified proteins were analyzed on a Shimadzu PPSQ-10 Automated Protein Sequencer by Edman degradation. PTH-amino acids were detected at 269 nm after separation on a reverse phase C18 column (4.6×250 mm) under isocratic conditions, according to the instructions of the manufacturer. Sequence homology and alignments were performed using the BLAST NCBI algorithm [9].

2.7. Monoclonal antibody (mAb) and polyclonal antibody production

BALB/c mice were immunized with 10 μg of a pool of $BaPLA_2I$ and $BaPLA_2III$ associated with 3 mg of $Al(OH)_3$ as adjuvant. Sixteen and 23 days later, boosters were administered with the corresponding antigens without adjuvant. Spleen cells were harvested on day 25 and fused with A_2 myeloma cells [10]. The hybridoma culture supernatants were screened for the presence of $BaPLA_2I$ and

$BaPLA_2III$ monoclonal antibodies using ELISAs. These supernatants were also screened for the ability to block myonecrosis or catalytic activity of the PLA_2 enzyme. For the polyclonal antibody, a rabbit was primed with 100 μg of PLA_2 emulsified with complete Freund adjuvant (CFA). Thirty days later, three boosters containing 100 μg of both enzymes without adjuvant were administered s.c. at 10-day intervals. Three days after the final booster, serum was collected and IgG was purified by the combined methods of caprylic acid and ammonium sulfate precipitation.

2.8. Immunoprecipitation

One hundred micrograms of a PLA_2-enriched protein fraction containing 100 μg of a monoclonal antibody mixture (clones 12DG6, A85/9, A176/1, and 19/1) and 30 μL of protein G Sepharose beads (Gibco BRL, USA) in 0.5 mL of Low RIPA buffer (150 mM NaCl, 2 mM EDTA, 20 mM Tris–HCl, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100) was incubated for 16 hr at 4° with gentle agitation. The sample was washed once with Low RIPA buffer followed by three washes with PBS. The protein G Sepharose beads were resuspended in 50 μL of SDS–PAGE sample buffer, boiled at 100° for 10 min, and applied to a 15% polyacrylamide gel. Protein bands were detected by Coomassie blue staining.

2.9. Myonecrosis

The myotoxic activity of $BaPLA_2I$ and $BaPLA_2III$ was assayed in CF1 mice. Twenty-five micrograms of either enzyme was injected directly into the gastrocnemius muscles of the mice in the test group, while control mice received an equal volume of saline. Mice were killed by CO_2 inhalation at various time intervals, and blood and muscle samples were collected for CK determination and histopathologic analysis, respectively.

2.10. Edema-forming activity

Edema was induced in CF1 mice by injecting 0.5 μg of the purified $BaPLA_2I$, $BaPLA_2III$, or crude venom (3 μg) into one paw. As a control, an equal volume of saline was injected into the contralateral paw. After different time periods, swelling was measured with a caliper [11]. In some cases, mice were killed by CO_2 inhalation so that both paws could be removed at the tibiotarsal articulation and weighed [12]. The swelling of the paw injected with PLA_2 was compared with the contralateral paw injected with saline, and the results were expressed as a percent of control.

2.11. Mast cell degranulation

RA rats were killed by CO_2 inhalation, and the mesenteries were removed, washed with Tyrode's solution, and divided into pieces. Individual pieces were incubated in 5 mL of Tyrode's solution containing 10–20 μg/mL of $BaPLA_2I$ or $BaPLA_2III$ for 1 hr at 37° with agitation. Control pieces were incubated in Tyrode's solution. After incubation, the pieces of mesentery were transferred to a fixative mixture for the preservation of mast cells, as described by Mota and Dias da Silva [13]. Mast cell degranulation was quantified by counting 300 cells containing metachromatic granules enumerating those showing granules outside the cell membrane, and the percentage of degranulating cells was calculated.

2.12. Histological examinations

$BaPLA_2I$ or $BaPLA_2$ III (25 μg) was injected into the gastrocnemius muscles of CF1 mice. After 1 hr, the mice were killed by CO_2 inhalation, and the muscles were removed and fixed in 5% neutral *p*-formaldehyde. The tissues were dehydrated in ascending concentrations of acetone (30–100%) and embedded in paraffin. Sections were stained with hematoxylin and eosin.

3. Results

The *B. atrox* venom was fractionated on Sephacryl S-100-HR, the protein content was monitored spectrophotometrically at 280 nm, and the PLA_2 activity was assayed using a fluorescent synthetic substrate. Fig. 1A shows that PLA_2 activity was associated with the last protein peak and that it remained high even as the protein level dropped. The fractions with PLA_2 activity were pooled and concentrated and were resolved into several protein peaks by HPLC on a reverse-phase C4 column (Fig. 1B). Of these peaks, one eluted with 51% solution B ($BaPLA_2I$) and another at 55% ($BaPLA_2III$). $BaPLA_2I$ exhibited very low PLA_2 activity, whereas $BaPLA_2III$ had a high activity; both peaks were chosen for characterization.

Protein peaks were rechromatographed on the same column under the same conditions, and their homogeneity was confirmed (data not shown). An SDS–PAGE analysis (Fig. 2A) of these protein peaks showed the presence of a prominent single band of 15 kDa corresponding to lanes containing $BaPLA_2I$ or $BaPLA_2III$. The HPLC-purified $BaPLA_2I$ protein was more homogeneous and of lesser mass than the $BaPLA_2III$ protein.

Analysis of the two preparations by IEF not only confirmed the homogeneity of $BaPLA_2I$ and $BaPLA_2III$ but demonstrated that they had distinct pIs, $BaPLA_2I$ being basic with a pI of 9.1 and $BaPLA_2III$ being neutral with a pI of 6.9 (Fig. 2B).

N-terminal amino acid analysis revealed that $BaPLA_2I$ has a lysine at position 49 and $BaPLA_2III$ possesses an aspartic acid at the same position, as predicted by the catalytic activity observed. $BaPLA_2I$ also has an insertion of two amino acid residues, a lysine and an asparagine at

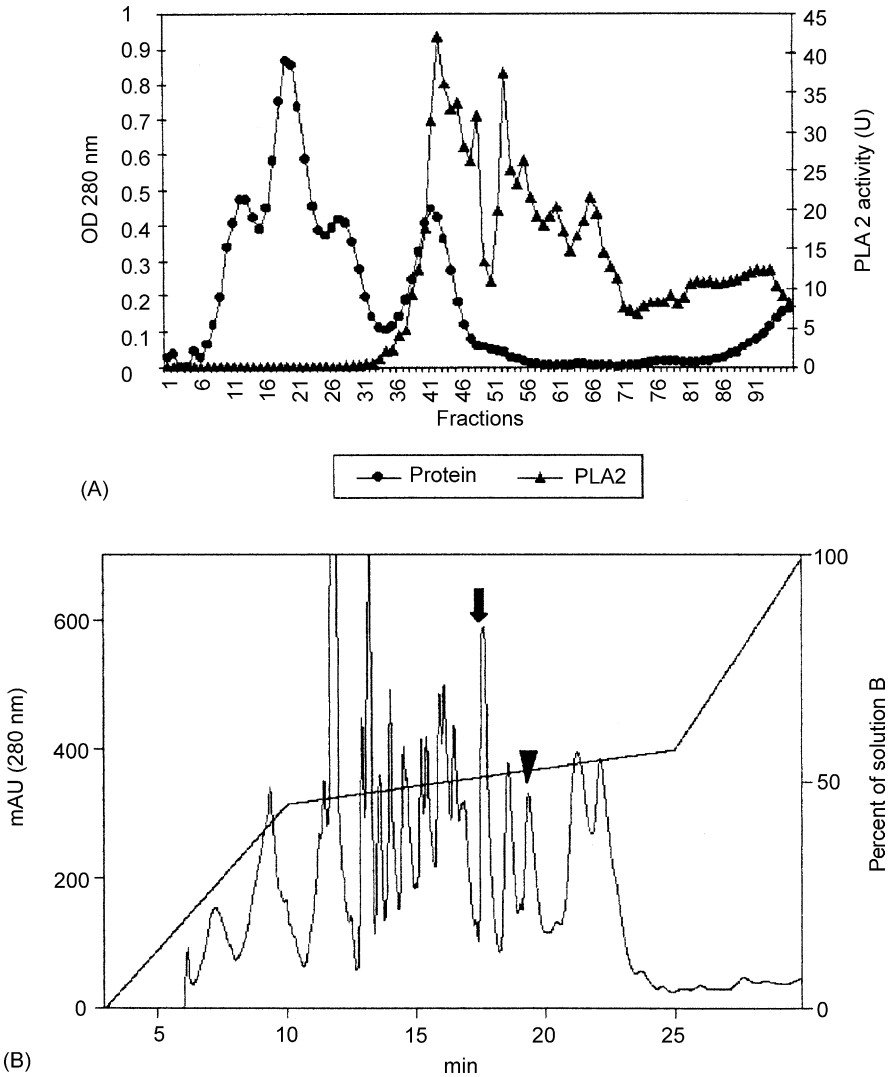

Fig. 1. (A) Size exclusion chromatography of crude *B. atrox* venom. Fifty milligrams of dissolved venom was applied on a Sephacryl S-100-HR column (2.5 × 67.0 cm) as described in Section 2. Relative protein concentrations of collected fractions were monitored at 280 nm (●). PLA_2 activity was determined using C6-NBDPC fluorogenic substrate (▲). (B) Reverse-phase HPLC of pooled fractions with PLA_2 activity obtained by size exclusion chromatography. The arrow indicates $BaPLA_2I$, and the arrowhead indicates $BaPLA_2III$. mAU = milliabsorbance units.

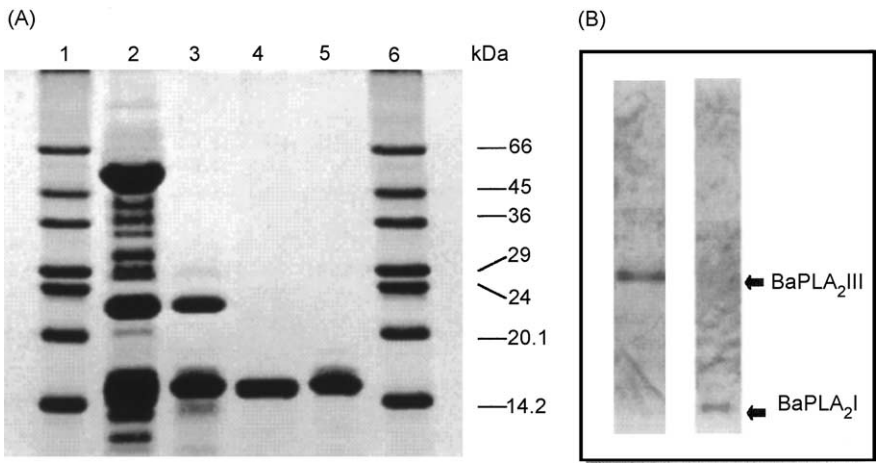

Fig. 2. (A) SDS–PAGE of purified $BaPLA_2I$ and $BaPLA_2III$. Lanes 1 and 6, MW markers; lane 2, venom (75 μg); lane 3, pooled fractions from a size exclusion column (24 μg); lane 4, $BaPLA_2I$ (8 μg); and lane 5, $BaPLA_2III$ (8 μg). (B) IEF analysis of purified $BaPLA_2I$ and $BaPLA_2III$. Ampholites with a pH range of 3.5–9.5 were used. $BaPLA_2I$ and $BaPLA_2III$ are indicated by arrows.

Table 1
N-terminal amino acid sequence of purified $BaPLA_2I$ and $BaPLA_2III$ and alignment with other PLA_2s

P1	–	S	L	***V***	E	L	G	K	M	I	L	Q	E	T	G	K	N	P	*V*	***T***	S	Y	G	*A*	Y	G
A	–	S	L	***F***	E	L	G	K	M	I	L	Q	E	T	G	K	N	P	*A*	***K***	S	Y	G	*A*	Y	G
B	–	S	L	***V***	E	L	G	K	M	I	L	Q	E	T	G	K	N	P	*L*	***T***	S	Y	G	*V*	Y	G
P1	–	C	N	C	G	V	***L***	***G***	R	***G***	K	P	K	D	***A***	T	*N*	***E***	C	C	Y	***K***	***N***	V	H	**K**
A	–	C	N	C	G	V	***L***	***G***	R	***G***	K	P	K	D	***A***	T	*D*	***R***	C	C	Y	–	–	V	H	**K**
B	–	C	N	C	G	V	***G***	***S***	R	***H***	K	P	K	D	***D***	T	*D*	***R***	C	C	Y	–	–	V	H	**K**
P1	–	C	C	Y	K	K	L																			
A	–	C	C	Y	K	K	L																			
B	–	C	C	Y																						
P3	–	*N*	L	*W*	Q	F	*G*	***K***	M	I	N	***E***	***E***	***M***	G	***K***	*F*	***A***	***F***	***L***	N	Y	*V*	*S*	Y	G
C	–	*N*	L	*F*	Q	F	*A*	***K***	M	I	N	***G***	***K***	***L***	G	***A***	*F*	***S***	***V***	***W***	N	Y	*I*	*S*	Y	G
D	–	*D*	L	*W*	Q	F	*G*	***Q***	M	I	***L***	***K***	***E***	***T***	G	***K***	***L***	***P***	***F***	***P***	***Y***	Y	***T***	*T*	Y	G
E	–	*D*	L	*W*	Q	F	*G*	***Q***	M	***M***	N	***D***	***V***	***M***	***R***	***E***	*Y*	***V***	***V***	***F***	N	Y	*L*	***Y***	Y	G
P3	–	C	Y	C	G	W	G	G	G	G	***Q***	P	*R*	D	A	T	D	R	C	C	F	V	H	**D**	C	C
C	–	C	Y	C	G	W	G	G	G	G	***T***	P	*K*	D	A	T	D	R	C	C	F	V	H	**D**	C	C
D	–	C	Y	C	G	W	G	G	G	G	***Q***	P	*K*	D	A	T	D	R	C	C	F	V	H	**D**	C	C
E	–	C	Y	C	G	W	G	G	***I***	G	***K***	P	*R*	D	A	T	D	R	C	C	F	V	H	**D**	C	C
P3	–	Y	G	*R*	*V*	T	G																			
C	–	Y	G	*R*	*V*	***R***	G																			
D	–	Y	G	*K*	*L*	T	***N***																			
E	–	Y	G	*K*	*V*	T	G																			

P1 corresponds to $BaPLA_2I$ and P3 to $BaPLA_2III$; (A) *B. asper* myotoxin II [14]; (B) *B. atrox* Lys^{49} [15]; (C) *Vipera ammodytes meridionalis* PLA_2 [16]; (D) *B. jararacussu* PLA_2 [17]; and (E) *B. jararaca* PLA_2 [18]. Homologue amino acid substitutions are set in italics; different amino acid substitutions are highlighted in bold and italic; amino acids K and D at position 49 are in bold.

positions 45 and 46, respectively. A high degree of homology was seen between these isolated PLA_2s and other PLA_2s already described (Table 1).

Fig. 3A shows that both $BaPLA_2I$ and $BaPLA_2III$ were edematogenic at concentrations of 0.5 μg when injected into the paws of mice. Both proteins had comparable edema-inducing activities. Maximal activity was attained 1 hr after crude venom, $BaPLA_2I$, or $BaPLA_2III$ injection, declining thereafter. The level of edema induced by 0.5 μg of each PLA_2 and 3 μg of crude venom was similar. The edematogenic activity of both enzymes, however, was not blocked when the mice were pretreated with purified anti-PLA_2 rabbit IgG (Fig. 3B). The same result was observed when monoclonal antibodies were used in these experiments instead of polyclonal antibodies (data not shown). In addition, $BaPLA_2I$ and $BaPLA_2III$ were capable of inducing mast cell degranulation in a concentration-dependent fashion (Fig. 3C).

The intramuscular injection of 25 μg of $BaPLA_2I$ or $BaPLA_2III$ increased the basal level of serum CK activity from less than 0.5 U/mL to 4 and 2 U/mL, respectively, at 1 hr. In contrast, the injection of crude venom increased CK activity to 2.5 U/mL at 3 hr (Fig. 4A). The myotoxic activity of these enzymes, in contrast to the edematogenic activity, was neutralized completely by the 12DG6 and A85/9 mAbs (Fig. 4B) as well as by a rabbit polyclonal IgG raised against the purified enzymes (data not shown). Immunoprecipitation assays (Fig. 4C) showed that mAbs 12DG6, A85/9, A176/1, and 19/1 were able to recognize and precipitate PLA_2 from Sephacryl-enriched fractions.

Fig. 5 shows light micrographs of gastrocnemius muscles injected with 25 μg of $BaPLA_2I$ or $BaPLA_2III$ or saline. Exposure to $BaPLA_2I$ extensively altered cellular morphology as denoted by hypercontracted and disorganized myofibrils, hallmarks of extensive myonecrosis. Edema and inflammatory cells can be observed surrounding the altered striated muscle cells (Fig. 5A). $BaPLA_2III$ induced an intense infiltration of inflammatory cells (mainly polymorphonuclear leukocytes), edema, and the degeneration of striated muscle cells as characterized by an increase in cell volume, the presence of intracellular vacuoles, and the loss of myofibrils (Fig. 5B).

4. Discussion

Since the discovery that PLA_2 and crotapotin are non-covalently linked subunits of the crotoxin molecule [19,20], a potent neurotoxin first isolated by Slotta and Fraenkel-Conrat [21], growing evidence has ascribed PLA_2 to be one of the most toxic snake venom components. Over fifty different PLA_2s have been isolated and characterized from different snake venoms and some have been cloned and expressed [22]. Accumulating evidence strongly implicates venom PLA_2s as being among the mediators of myonecrosis [23,24], hemolysis [25], platelet aggregation [26], mast cell degranulation [27], and edema formation [28]. The precise involvement of particular domains or specific amino acid sequences in the mediation of each of these activities will require further molecular

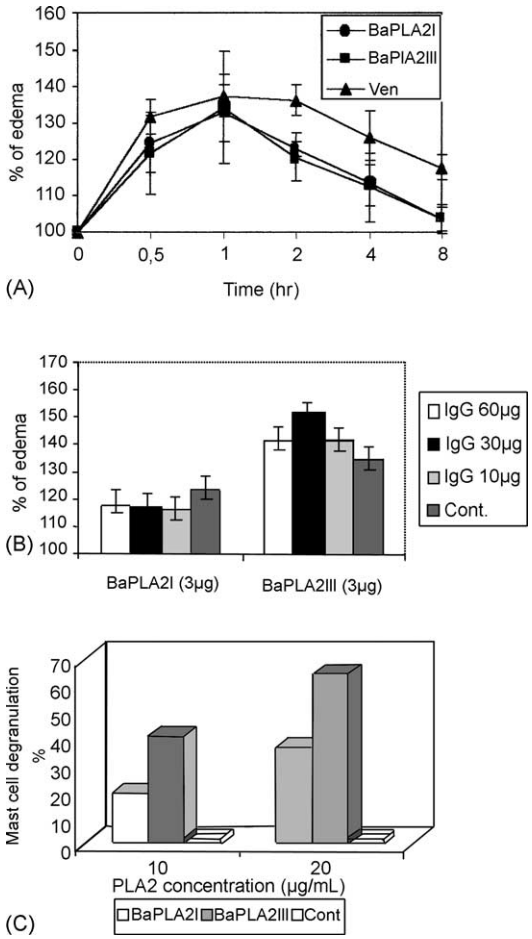

Fig. 3. (A) Edema induced by $BaPLA_2I$, $BaPLA_2III$, and crude venom in CF1 mice. $BaPLA_2I$ (0.5 μg), $BaPLA_2III$ (0.5 μg), and crude venom (3 μg) were injected s.c. into the right footpads of CF1 mice (20–25 g). In the left footpad 50 μL of saline was injected. Edema was measured at various time intervals and expressed as the percentage of the increase in the thickness of the envenomed footpad in comparison with control. Values are means ± SD of 3 or 4 mice at each time point. (B) Inhibition of edema formation by polyclonal anti-PLA_2 IgGs. Three micrograms of $BaPLA_2I$ or $BaPLA_2III$ preincubated with 10, 30, or 60 μg of rabbit anti-PLA_2 IgGs was injected into the paws of CF1 mice. After 1 hr, edema was measured by weighing the legs. Values are means ± SD of 4 mice in each group. (C) Rat mast cell degranulation. Different concentrations of $BaPLA_2I$ and $BaPLA_2III$ enzymes were incubated with rat mesentery. After incubation, the mesenteries were fixed in Tyrode's solution, stained, and mounted on a microscope slide. The percent of mast cell degranulation was determined by scoring 300 cells in at least five microscopic fields (400×).

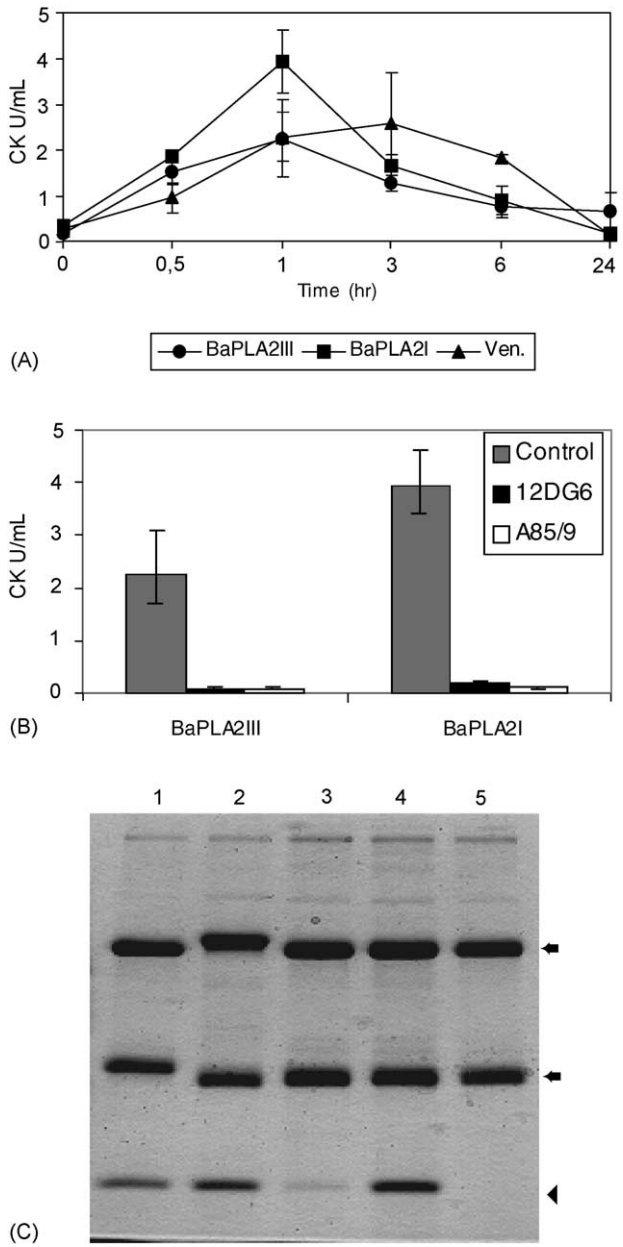

Fig. 4. (A) Myotoxic activity induced by $BaPLA_2I$ and $BaPLA_2III$ as measured by creatine phosphokinase release. $BaPLA_2I$ and $BaPLA_2III$ enzymes were injected into the gastrocnemius muscles of CF1 mice. Controls were injected with 50 μL of saline. At different times, blood was collected, and serum CK levels were measured using a Merck Granutest 2.5. Values are means ± SD of 4 mice at each time point. (B) Inhibition of CK release by anti-PLA_2 mAbs. $BaPLA_2I$ or $BaPLA_2III$ was preincubated for 1 hr at 25° with purified mAbs, at an antigen to antibody ratio of 1:2 before injection into the gastrocnemius muscles of CF1 mice. One hour after injection, serum was collected, and CK levels were determined. Values are means ± SD of 4 mice in each group. (C) Immunoprecipitation of PLA_2 from Sephacryl-enriched fractions with mAbs. The precipitated products were analyzed by SDS–PAGE. Lane 1, mAb 12DG6; lane 2, mAb A85/9; lane 3, mAb A176/1; lane 4, mAb 19/1; and lane 5, control mAb. The arrows indicate the heavy and light chain; the arrowhead indicates the immunoprecipitated PLA_2.

characterization of the PLA_2s. On the other hand, mammalian-secreted PLA_2s are involved in inflammation, host defense, and several inflammatory diseases, and are specifically distributed in several tissues, suggesting that they may play a role in a number of fundamental physiological processes [29]. Studies on the diversity and function of venom PLA_2s will be helpful in determining the functional diversity of mammalian PLA_2s as well as the pathological mechanism of snake envenoming.

Size exclusion chromatography resolved *B. atrox* crude venom into four major protein peaks. PLA_2 activity, as assayed using C6-NBDPC, was associated with the fourth peak and persisted over the rest of the chromatogram, despite a drop in the protein level. This chromatographic profile indicated the existence of more than one protein species containing PLA_2 activity. This hypothesis was, in

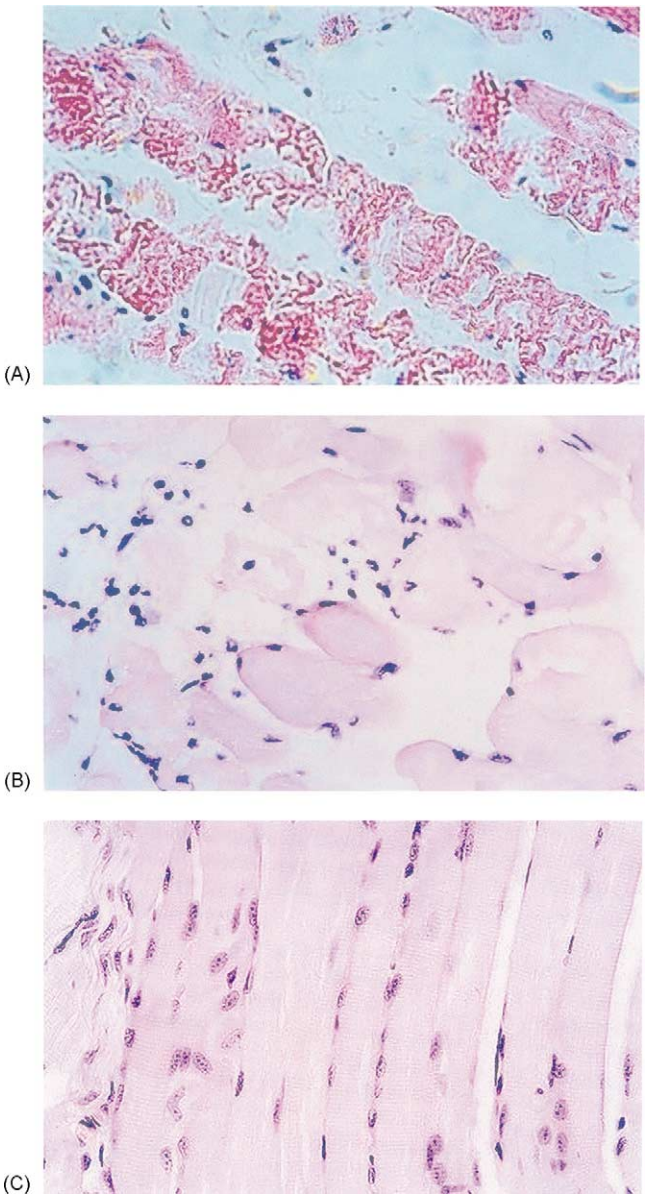

Fig. 5. Histopathology of gastrocnemius muscles from mice locally injected, 1 hr before sacrifice, with: (A) $BaPLA_2I$ (25 μg); (B) $BaPLA_2III$ (25 μg); and (C) control (saline).

fact, confirmed when the fractions containing PLA_2 activity were chromatographed on a reverse-phase C4 column. Several distinct protein peaks were resolved, some of them containing PLA_2 activity. Two peaks with PLA_2 activity, representing $BaPLA_2I$ and $BaPLA_2III$, were concentrated and rechromatographed on the same column, and the corresponding proteins were analyzed. Upon SDS–PAGE analysis, the $BaPLA_2I$ peak was confirmed to be comprised of a homogeneous 15 kDa protein, while the $BaPLA_2III$ peak, in addition to containing a major 15 kDa protein, also contained a minor contaminant of slightly higher molecular mass. Although similar in molecular mass, $BaPLA_2I$ and $BaPLA_2III$ have pIs of 9.1 and 6.9, respectively. The differences between these two PLA_2s were underscored by the observation that $BaPLA_2III$ is over 130 times more catalytic than $BaPLA_2I$. The comparison of the $BaPLA_2I$ N-terminal amino acid sequence with other published sequences showed a high degree of homology with myotoxin II from *B. asper* [14] and PLA_2 Lys^{49} from *B. atrox* [15]. For $BaPLA_2III$, a high degree of homology was also found with PLA_2 from *B. jararacussu* [17], PLA_2 from *B. jararaca* [18], and vipoxin, a PLA_2 from *Vipera ammodytes meridionalis* [16].

The discrepancy in the ability of specific mAbs or polyclonal IgGs to inhibit $BaPLA_2I$ and $BaPLA_2III$ myotoxic activity, but not edematogenic activity, might be explained by the fact that the edematogenic and myonecrotic activities are mediated by distinct proteins. This theory, however, appears to be unfounded, since in all immunochemical assays $BaPLA_2I$ and $BaPLA_2III$ were apparently homogeneous. An alternative possibility may be that each activity is mediated by a distinct enzyme domain in the same molecule. This hypothesis is reinforced by the observation that the antibodies were able to neutralize the myotoxic activity but not the edematogenic activity (Fig. 4B and Fig. 3B, respectively). Therefore, 12DG6 and A85/9 mAbs are specific for epitopes on $BaPLA_2I$ and $BaPLA_2III$ that mediate myonecrosis and do not block domains that increase vascular permeability. This latter hypothesis is reinforced by the observation that all mAbs were also able to immunoprecipitate the enzyme from the enriched Sephacryl fractions (Fig. 4C). A study on the neutralization of *B. asper* myotoxin III, a D-49 PLA_2, showed that myotoxicity was inhibited by heparin, whereas PLA_2 activity was not affected [30]. This observation confirmed the dissociation of enzymatic activity from the myotoxic site [24]. In contrast, our experiments using monoclonal and polyclonal antibodies resulted in the neutralization of myonecrotic activity, without affecting either the catalytic (data not shown) or edematogenic activities of the enzymes. Since snake venom K-49 PLA_2s lack or have very low hydrolytic activity and since the edema induced by these PLA_2s is comparable to that induced by D-49 PLA_2s, it may be postulated that snake venom PLA_2 hydrolytic activity is not related to its edematogenic activity. In addition, Landucci *et al.* [27] suggested that the hydrolysis of phospholipids by PLA_2 is not essential for edema formation. Taken together these results lead us to suggest that edema is mediated by a domain in the PLA_2 molecule that is independent of the domains that mediate myonecrosis and hydrolysis.

The mechanisms underlying the increase of vascular permeability induced by $BaPLA_2I$ and $BaPLA_2III$ have not been addressed. Pre-formed mediators stored in mast cells such as histamine and serotonin or *de novo* synthesized from arachidonic acid metabolism are the most obvious candidates. Mast cell degranulation induced by *B. jararacussu* PLA_2 has been described by Landucci *et al.* [27], who also suggested that the edematogenic activity of these enzymes was mediated by histamine and serotonin. Both enzymes, used at the same concentrations, induced tissue

alterations characterized by inflammatory cell infiltration and edema; however, their effects on skeletal muscle were different. Whereas $BaPLA_2III$ induced a mild form of myofibrillar degeneration that was characterized by cell volume loss and vacuolization, $BaPLA_2I$ induced severe alterations characterized by cellular necrosis, as frequently observed in Lys^{49} PLA_2 myonecrosis. These results suggest that these enzymes exert their effects through different mechanisms.

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References

[1] Davidson F, Dennis EA. Structure, function and mode of action of snake venom and other phospholipase A_2s. In: Tu AT, editor. Handbook of natural toxins. New York: Marcel Dekker, 1991. p. 107–45.
[2] Six DA, Dennis EA. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim Biophys Acta 2000;1488:1–19.
[3] Dennis EA. History, classification, structure and function of phospholipases A_2. In: Uhl W, Nevaiainem TJ, Buchler MW, editors. Phospholipases A_2: basic and clinical aspects in inflammatory diseases. Basel, Switzerland: S. Karger AG, 1997. p. 1–10.
[4] Kini RM. Phospholipase A_2: a complex multifunctional protein puzzle. In: Kini RM, editor. Venom phospholpase A_2 enzymes: structure, function and mechanism. Chichester: John Wiley, 1997. p. 1–28.
[5] Scott DL, Achari A, Vidal JC, Sigler PB. Crystallographic and biochemical studies of the (inactive) Lys-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. J Biol Chem 1992; 267:22645–57.
[6] Lomonte BJM. A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). Toxicon 1989;27: 725–33.
[7] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[8] Bozzo S, Retamal C. Gel-perfect: geles unidimensionales, un nuevo método densitométrico para computadores personales. Arch Biol Med Exp (Santiago) 1991;24:181–4.
[9] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25: 3389–402.
[10] Köhler J, Milstein C. Continuous culture of fused cells secreting antibody of predefined specificity. Nature 1975;256:495–7.
[11] Yamakawa M, Nozaki M, Hokama Z. Fractionation of Sakishima-habu (*Trimeresurus elegans*) venom and lethal, hemorrhagic and edema-forming activities of the fractions. In: Osaka A, Hayashi K, Sawai Y, editors. Animal, plant and microbial toxins, vol. 1. New York: Plenum Press, 1976. p. 97–109.
[12] Lôbo de Araújo A, Souza AO, Cruz-Höfling MA, Flores CA, Bon C. *Bothrops lanceolatus* (Fer de lance) venom induces oedema formation and increases vascular permeability in the mouse hind paw. Toxicon 2000;38:209–21.
[13] Mota I, Dias da Silva W. Antigen induced damage to isolated sensitized mast cells. Nature 1960;186:145–6.
[14] Francis B, Gutierrez JM, Lomonte B, Kaiser I. Myotoxin II from *Bothrops asper* (Terciopelo) venom is a lysine-49 phospholipase A_2. Arch Biochem Biophys 1991;284:352–9.
[15] Maraganore JM, Merutka G, Cho W, Welches W, Kézdy FJ, Hendrikson RL. A new class of phospholipase A_2 with lysine in place of aspartate 49. Functional consequences for calcium and substrate binding. J Biol Chem 1984;259:13839–43.
[16] Perbandt M, Wilson JC, Eschenburg S, Mancheva I, Aleksiev B, Genov N, Willingmann P, Weber W, Singh TP, Betzel Ch. Crystal structure of vipoxin at 2.0 Å: an example of regulation of a toxic function generated by molecular evolution. FEBS Lett 1997;412:573–7.
[17] Moura-da-Silva AM, Paine MJ, Diniz MR, Theakston RD, Crampton JM. The molecular cloning of a phospholipase A_2 from *Bothrops jararacussu* snake venom: evolution of venom group II phospholipase A_2's may imply gene duplications. J Mol Evol 1995;41:174–9.
[18] Serrano SM, Reichl AP, Mentele R, Auerswald EA, Santoro ML, Sampaio CA, Camargo AC, Assakura MT. A novel phospholipase A_2, BJ-PLA_2, from the venom of the snake *Bothrops jararaca*: purification, primary structure analysis, and its characterization as a platelet-aggregation-inhibiting factor. Arch Biochem Biophys 1999;267: 26–32.
[19] Hendon RA, Fraenkel-Conrat H. Biological roles of the two components of crotoxin. Proc Natl Acad Sci USA 1971;68:1560–3.
[20] Rubsamen K, Breithaupt H, Habermann E. Biochemistry and pharmacology of the crotoxin complex. I. Subfractionation and recombination of the crotoxin complex. Naunyn Schmiedebergs Arch Pharmacol 1971;270:274–88.
[21] Slotta KH, Fraenkel-Conrat HL. Schlangengifte III Mitteilung: Reinigung und Krystallisation des Klapperschlangen-Giftes. Ber Dt Chem 1938;71:1076–81.
[22] Ownby CL, Selistre de Araújo HS, White SP, Fletcher JE. Lysine 49 phospholipase A_2 proteins. Toxicon 1999;37:411–5.
[23] Mebs D, Ownby CL. Myotoxic components of snake venoms: their biochemical and biological activities. Pharmacol Ther 1990;48: 223–36.
[24] Gutiérrez JM, Lomonte B. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. Toxicon 1995;33:1405–24.
[25] Condrea E, Fletcher JE, Rapuano BE, Yang CC, Rosenberg P. Dissociation of enzymatic activity from lethality and pharmacological properties by carbamylation of lysine in *Naja nigricolis* and *Naja naja atra* snake venom phospholipase A_2. Toxicon 1981;19:705–20.
[26] Yuan Y, Jackson SP, Mitchell CA, Salem HH. Purification and characterization of a snake venom phospholipase A_2: a potent inhibitor of platelet aggregation. Thromb Res 1993;70:471–81.
[27] Landucci ECT, Castro RC, Pereira MF, Cintra ACO, Giglio JR, Marangoni S, Oliveira B, Cirino G, Antunes E, Nucci G. Mast cell degranulation induced by two phospholipase A_2 homologues: dissociation between enzymatic and biological activities. Eur J Pharmacol 1998;343:257–63.
[28] Lomonte B, Tarkowski A, Hanson LA. Host response to *Bothrops asper* snake venom, analysis of edema formation, inflammatory cells and cytokine release in mouse model. Inflammation 1993;17:93–105.
[29] Valentin E, Lambeau G. What can venom phospholipase A_2 tell us about the functional diversity of mammalian secreted phospholipase A_2? Biochemie 2000;82:815–31.
[30] Lomonte B, Tarkowski A, Bagge U, Hanson LÅ. Neutralization of the cytolytic and myotoxic activities of phospholipases A_2 from *Bothrops asper* snake venom by glycosaminoglycans of the heparin/heparan sulfate family. Biochem Pharmacol 1994;47:1509–18.