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Structural and functional characterization of an acidic platelet aggregation inhibitor and hypotensive phospholipase A_2 from *Bothrops jararacussu* snake venom

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Abstract

An acidic (pI ~ 4.5) phospholipase A_2 (BthA-I-PLA_2) was isolated from *Bothrops jararacussu* snake venom by ion-exchange chromatography on a CM-Sepharose column followed by reverse phase chromatography on an RP-HPLC C-18 column. It is an ~13.7 kDa single chain Asp49 PLA_2 with approximately 122 amino acid residues, 7 disulfide bridges, and the following N-terminal sequence: ^{1}SLWQFGKMINYVM–GESGVLQYLSYGCYCGLGGQGQPTDATDRCCFVHDCC^{51}. Crystals of this acidic protein diffracted beyond 2.0 Å resolution. These crystals are monoclinic and have unit cell dimensions of a = 33.9, b = 63.8, c = 49.1 Å, and β = 104.0°. Although not myotoxic, cytotoxic, or lethal, the protein was catalytically 3–4 times more active than BthTX-II, a basic D49 myotoxic PLA_2 from the same venom and other *Bothrops* venoms. Although it showed no toxic activity, it was able to induce time-independent edema, this activity being inhibited by EDTA. In addition, BthA-I-PLA_2 caused a hypotensive response in the rat and inhibited platelet aggregation. Catalytic, antiplatelet and other activities were abolished by chemical modification with 4-bromophenacyl bromide, which is known to covalently bind to His48 of the catalytic site. Antibodies raised against crude *B. jararacussu* venom recognized this acidic PLA_2, while anti-Asp49-BthTX-II recognized it weakly and anti-Lys49-BthTX-I showed the least cross-reaction. These data confirm that myotoxicity does not necessarily correlate with catalytic activity in native PLA_2 homologues and that either of these two activities may exist alone. BthA-I-PLA_2, in addition to representing a relevant molecular model of catalytic activity, is also a promising hypotensive agent and platelet aggregation inhibitor for further studies.

Keywords: *Bothrops jararacussu*; Acidic phospholipase A_2; N-terminal sequence; X-ray crystallography; Platelet aggregation inhibition; Hypotensive effect

1. Introduction

PLA_2s (EC 3.1.1.4) are small stable calcium-dependent proteins that hydrolyze the *sn*-2 acyl groups of phospholipids to liberate fatty acids and lysophospholipids. Extracellular PLA_2s occur abundantly in mammalian pancreatic juice as well as in snake and insect venoms, constituting a large family of homologous proteins, which are the major components of snake venoms [1].

PLA_2s from snake venoms have been classified as groups I and II on the basis of their primary structure and disulfide bridge pattern [1,2]. In addition to their primary catalytic role, snake venom PLA_2s show other important toxic/pharmacologic effects including myonecrosis, neurotoxicity,

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Abbreviations: PLA_2, phospholipase A_2; BthA-I-PLA_2, acidic phospholipase A_2 from *Bothrops jararacussu* venom; PRP, platelet-rich plasma; WRP, washed rabbit platelets; BPB, 4-bromophenacyl bromide; i.c.v., intracerebroventricular.

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cardiotoxicity, and hemolytic, hemorrhagic, hypotensive, anticoagulant, platelet aggregation inhibition and edema-inducing activities [3–5].

Several PLA_2 enzymes that strongly affect platelet aggregation have been purified and characterized from snake venoms [5–9]. PLA_2 enzymes that affect platelet aggregation have been classified into three major classes: class A contains PLA_2 enzymes that induce platelet aggregation, class B contains PLA_2 enzymes that inhibit platelet aggregation, and class C contains PLA_2 enzymes that act both as inducers and inhibitors [9].

A great number of basic PLA_2s have been purified from the venoms of snakes of the genus *Bothrops* and have been characterized structurally and functionally [10–20]. However, little is known about their acidic PLA_2s [21–25]. Two basic myotoxic PLA_2 homologues, named bothropstoxin-I (Lys49 BthTX-I) and II (Asp49 BthTX-II), have been isolated from *Bothrops jararacussu* venom and characterized [14,26–29]. We now report the isolation, biochemical/pharmacological characterization, partial amino acid sequence, crystallization, and X-ray diffraction data of an acidic platelet aggregation inhibitor and hypotensive PLA_2 from this same venom.

2. Materials and methods

2.1. Materials

B. jararacussu venom was collected from several snakes kept in the Serpentarium of the Faculdade de Medicina, Universidade de São Paulo, and then was vacuum-dried. ADP, BSA, bovine thrombin, EDTA, BPB, a CK-UV kinetic kit, molecular weight protein standards, and acrylamide were obtained from the Sigma Chemical Co. Collagen (Type I) from bovine tendons was purchased from the Chrono-Log Corp. All other reagents were of analytical or sequencing grade.

2.2. Purification procedure

The acidic PLA_2 was purified by ion-exchange chromatography on a CM-Sepharose column at pH 8.0 as previously described [14], followed by reverse phase-high performance liquid chromatography (RP-HPLC). Basically, the desiccated venom (250 mg) was dispersed in 50 mM ammonium bicarbonate buffer (pH 8.0), cleared by centrifugation (480 *g* for 10 min at 25°), and applied on a 2.0 × 20.0 cm CM-Sepharose column. A gradient was applied up to 0.5 M buffer, and fractions of 3.0 mL/tube were collected at a flow rate of 25 mL/hr. The eluted acidic PLA_2 fraction was lyophilized, dissolved in 5% (v/v) acetonitrile in 0.1% (v/v) trifluoracetic acid (TFA), and rechromatographed on a Shimadzu C18 RP-HPLC column (2.0 × 25.0 cm). Elution followed a 30–100% (v/v) continuous gradient of 60% (v/v) acetonitrile in 0.1% TFA at a flow rate of 1.0 mL/min for 70 min. All steps of the purification procedure were carried out at room temperature (25°). The PLA_2 was lyophilized and used for pharmacological characterization, amino acid sequence determination, and crystallization trials.

2.3. Biochemical characterization

PAGE for acidic proteins and SDS–PAGE followed previously described methods [30]. Isoelectric focusing was run according to Vesterberg [31]. Buffalyte, pH range 3.0–9.0 (Pierce), was used to generate the pH gradient. The determination of the amino acid composition followed the procedure described by Spackman *et al.* [32]. For protein determination, the micro-biuret method of Itzhaki and Gill [33] was followed.

2.4. N-terminal sequence

BthA-I-PLA_2 (1.8 mg) was dissolved in 250 mM Tris–HCl buffer, pH 8.2, containing 0.1% (w/v) EDTA. For each mol of Cys, 30 mol of dithiothreitol was added. After being flushed with N_2, the solution was incubated for 4 hr at 50° in the dark. Then excess vinylpyridine was added, and incubation proceeded overnight. The sample was purified on a C_{18} RP-HPLC column, and its N-terminal sequence was determined up to the 50th residue, using a model 491 Perkin-Elmer Procise Sequenator (Applied Biosystem Division) with on-line identification of the corresponding phenylthiohydantoin derivatives [34]. The N-terminal amino acid sequence of BthA-I-PLA_2 was aligned with sequences available in the GenBank and Swiss-Prot databases using the CLUSTAL program [35].

2.5. Crystallization of BthA-I-PLA_2

The sparse-matrix method [36] was used to perform initial screening of the crystallization conditions. The crystals of the acidic PLA_2 were obtained by the conventional hanging drop vapor diffusion method [37] at 293 K. Initially, small single crystals were obtained after 1 week, with the protein solution equilibrated against a reservoir containing 0.2 M ammonium sulphate and 30% (w/v) polyethylene glycol 8000. These conditions were optimized, and the volume of the drops was increased to 4 μL. Large single crystals were obtained from a solution containing 0.2 M ammonium sulphate and 22% (w/v) polyethylene glycol 6000, after approximately 1 month. X-ray diffraction data were collected using a Rigaku RU200 X-ray generator operating at 90 mA and 50 kV and an MAR Research 180 imaging plate detector, at room temperature. The image plate was operated in the 180 mm scanning mode, and crystal-detector distance was 80 mm. Two hundred images were collected using an oscillation range of 0.8°. Initially, the crystal diffracted until 1.9 Å resolution. However, due to radiation damage, diffraction

gradually decreased during data collection. The data were processed to 2.1 Å resolution using the DENZO program and scaled by the SCALEPACK program [38].

2.6. Enzymatic activities

Phospholipase activity of the purified acidic PLA_2 upon egg yolk emulsion, which contains phosphatidylcholine as a substrate, was assayed as described by de Haas *et al.* [39]. The recalcification time test of citrated bovine plasma was used for determination of anticoagulant activity [40].

2.7. Platelet aggregation inhibition effect

In these experiments, the procedure described by Fuly *et al.* [41] was used. PRP was prepared from citrated rabbit blood (0.31%, w/v) by centrifugation (~360 *g*/12 min) at room temperature. WRP were prepared from blood collected in 5 mM EDTA (final concentration). PRP samples obtained as above were centrifuged at ~1370 *g* for 20 min, and the platelet pellets were suspended in a calcium-free Tyrode's solution containing 0.35% (w/v) BSA and 0.1 mM EGTA (final concentration), pH 6.5, and washed twice by centrifugation (1370 *g* for 20 min at 0°). The final pellet was then suspended in Tyrode-BSA, pH 7.5, without EGTA. The suspension was adjusted to give $3–4 \times 10^5$ platelets/μL. Platelet aggregation was measured turbidimetrically using a Whole Blood Lumi-Aggregometer (Chrono-Log Corp.). Assays were performed at 37° in siliconized glass cuvettes using 200 μL of PRP or WRP, with stirring, and aggregation was triggered after preincubation for 2 min with aliquots of acidic PLA_2 in the presence of 1 mM $CaCl_2$ (final concentration). Control experiments were done using the platelet agonists alone (ADP, collagen, or thrombin). The inhibitory concentration (IC_{50}) was determined as the amount of PLA_2 that produces 50% inhibition of platelet aggregation.

2.8. Hypotensive effect

Male Wistar rats (260 ± 20 g body weight) were anesthetized with urethane (1.2 g/kg, i.p.). The carotid artery and jugular vein were isolated surgically and cannulated using polyethylene catheters for blood pressure recording and i.v. drug injection, respectively. Blood pressure was recorded using a blood pressure signal amplifier (7450A, HP) connected to a computerized data acquisition system (di190, Dataq). BthA-I-PLA_2 was dissolved in saline, and different doses (1–200 μg/kg) were injected i.v. to generate a dose-effect curve. Data were submitted to non-linear regression analysis, and an ED_{50} value was calculated using Prism graphic and statistical software (GraphPad). The effects of BthA-I-PLA_2 were compared to those of the BPB-modified protein.

2.9. Edema-inducing activity

Groups of five Swiss male mice (18–22 g) were injected in the subplantar region with the BthA-I-PLA_2 (50 μg/50 μL). After 0.5, 1, and 3 hr, paw edema was measured with the aid of a low pressure spring caliper (Mitutoyo) [17–19]. The zero time values were then subtracted, and the differences reported as median percent ± SD.

2.10. Myotoxic activity

The assay for creatine kinase (CK) was carried out using a CK-UV kinetic kit from Sigma. BthA-I-PLA_2 (5 μg/μL) was injected (i.m., 50 μL) in male Swiss mice of 18–22 g (N = 6). The control was given 0.15 M PBS. After 3 and 6 hr, blood from the tail was collected in heparin-coated tubes and centrifuged (3020 *g* for 10 min at 0°) for separation of the plasma. The amount of CK was then determined in 4 μL plasma, by incubation with 1.0 mL of the CK Reagent for 3 min at 37° according to the Sigma kinetic CK-UV protocol. Activity was expressed in units per liter, 1 unit resulting from the phosphorylation of 1 μmol of creatine/min at 25°.

2.11. Cytotoxic activity

Toxic activity was determined on tEnd cells and C2C12 myoblasts/myotubes, as previously described [42]. In brief, doses of 50 μg of acidic PLA_2 were diluted in the assay medium (Dulbecco's Modified Eagle's Medium supplemented with 1% fetal bovine serum), and then added to cells grown in 96-well plates, in a total volume of 150 μL/well. Controls for 0 and 100% toxicity consisted of the assay medium and 0.1% Triton X-100 in the assay medium, respectively. After 3 hr of incubation, a supernatant aliquot of 100 μL was collected for the determination of lactic dehydrogenase activity released from damaged cells, using a colorimetric end-point assay (Sigma No. 500).

2.12. Lethality (LD_{50})

Lethality induced by the BthA-I-PLA_2 was evaluated by i.p., i.v., or i.c.v. injections of samples at different concentrations, in groups of Swiss mice of 18–22 g (N = 6), within 48 hr.

2.13. Chemical modification and inhibition

Modification of His48 with BPB was carried out as previously described [43]. About 3 mg of the BthA-I-PLA_2 was dissolved in 1 mL of 0.1 M ammonium bicarbonate, pH 8.0, and 150 μL of BPB at 0.8 mg/mL ethanol was added. The mixture was incubated for 24 hr at 25°. Excess reagent was usually removed from the acidic PLA_2 preparations by ultrafiltration through an Amicon YM3 membrane, followed by lyophilization. Inhibition by 1 mM

EDTA (final concentration) was evaluated after incubation for 30 min at 37°. After that, the samples were assayed for phospholipase activity and the inhibitory effects on platelet aggregation induced by collagen (10 μg/mL). Control experiments were performed in parallel with non-treated PLA_2 assayed under the same conditions.

2.14. Enzyme-immunoassays

Microplates (Dynatech Laboratories) were coated with purified acidic PLA_2 at 0.2 μg/well by overnight incubation in 0.1 M Tris, 0.15 M NaCl, pH 9.0 buffer. After five washes with solution A (50 mM Tris, 0.15 M NaCl, 20 μM $ZnCl_2$, 1 mM $MgCl_2$, pH 7.4 buffer), the plates were air-dried and stored at 4°. Purified rabbit antibodies to *B. jararacussu* (BthTX-I and BthTX-II), B. moojeni (MjTX-II), all produced in this laboratory, B. asper (Basp-II) and anti-C-terminus peptide (115–129) from Basp-II, supplied by Dr. B. Lomonte, Instituto Clodomiro Picado, Universidad de Costa Rica, were added to triplicate wells, diluted in solution A (1:12,500) containing 2% (w/v) BSA, and incubated at room temperature for 2 hr. After five washes with solution A, bound antibodies were detected with anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma), diluted 1:2000 in solution A–BSA and incubated for 90 min. After washing, color was developed with *p*-nitrophenylphosphate, and absorbances were recorded on a microplate reader at 410 nm [44]. Normal rabbit serum was utilized as a negative control and crotamine was included as an unrelated antigen.

3. Results

3.1. Structural characterization of the acidic PLA_2 from *B. jararacussu* snake venom

Fractionation of *B. jararacussu* venom (250 mg) by ion-exchange chromatography on a CM-Sepharose column produced six major protein peaks (Fig. 1A). Fraction JIb, showing high PLA_2 activity, was fractionated further by reverse phase chromatography on a C_{18} HPLC column

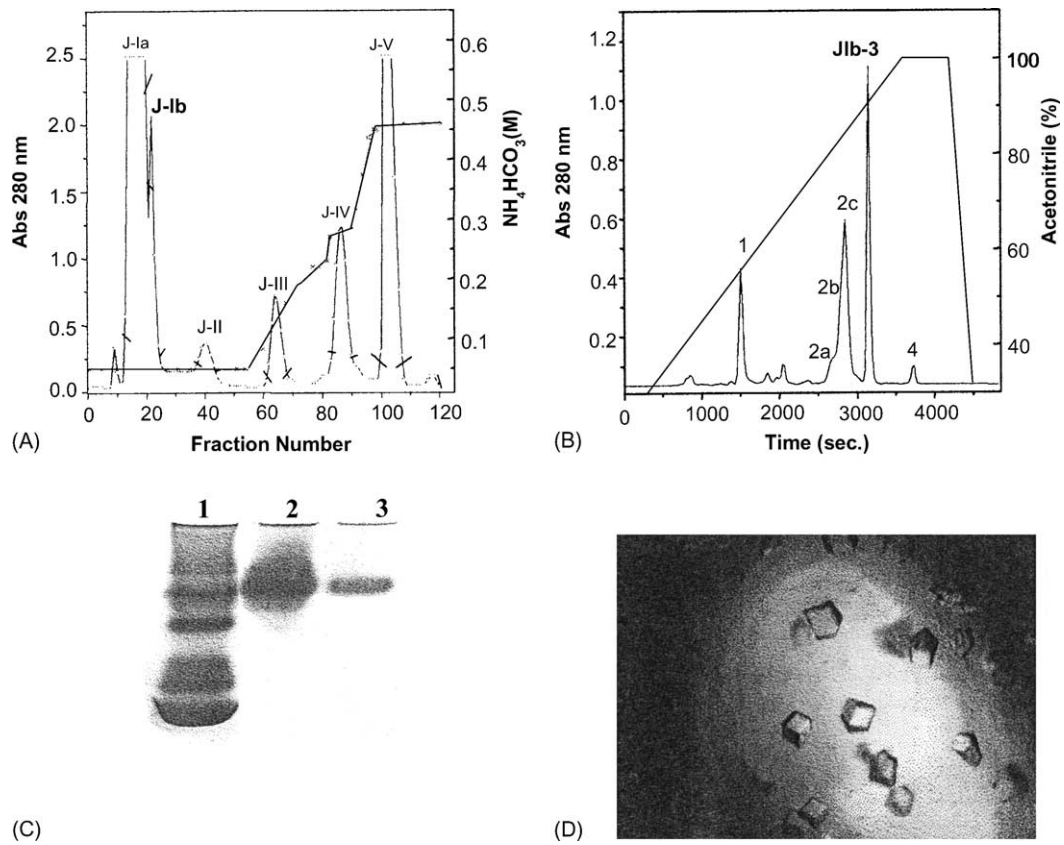

Fig. 1. Isolation of *B. jararacussu* acidic phospholipase A_2 (BthA-I-PLA_2). (A) Ion-exchange chromatography of crude venom on a CM-Sepharose column equilibrated with 50 mM ammonium bicarbonate buffer, pH 8.0. A gradient was applied up to 0.5 M buffer, and fractions of 3.0 mL/tube were collected at a flow rate of 25 mL/hr. (B) PLA_2-enriched fraction (JIb) obtained by ion-exchange chromatography was fractionated further by reverse phase chromatography on a C_{18} column run on an HPLC apparatus. Elution was carried out using a linear gradient from 18-60% (30–100% of a 60% solution) (v/v) acetonitrile in trifluoracetic acid (0.1%, v/v) at a flow rate of 1.0 mL/min for 70 min. Protein elution was monitored at 280 nm, and the fraction was assayed for phospholipase activity. The subfraction JIb-3 eluted at 88% acetonitrile displayed PLA_2 activity, being denoted BthA-I-PLA_2. (C) Native-PAGE for acidic proteins: lane 1, *B. jararacussu* venom; lane 2, fraction JIb; and lane 3, BthA-I-PLA_2 (JIb-3). (D) BthA-I-PLA_2 crystals with dimensions of $0.4 \times 0.2 \times 0.1$ mm.

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                                                  1                                                  50
                                                  |                                                  |
gi|2117957|pir||A48188                           GLWQFENMIIKVV-KKSGILSYSAYGCYCGWGGRGKPKDATDRCCFVHDCC
gi|129417|sp|P06859|PA21_TRIFL                  GLWQFENMIIKVV-KKSGILSYSAYGCYCGWGGRGKPKDATDRCCFVHDCC
gi|422653|pir||A46169                           GLWQFENMIIKVV-KKSGILSYSAYGCYCGWGGRGKPTDATDRCCFVHDCC
gi|2117960|pir||I51190                          -LFQFEKLIKKMT-GKSGMLWYSAYGCYCGWGGQGRPKDATDRCCFVHDCC
gi|400721|sp|P31854|PA2_VIPBB                   NLFQFGNMINHMV-GKHAVWSYLSYGCYCGWGGQGKPQDATDRCCFVHDCC
gi|3914258|sp|P81243|PA21_BOTJ                  DLWQFGQMMNDVM-REYVVFNYLYYGCYCGWGGIGKPRDATDRCCFVHDCC
BthA-I-PLA2                                     SLWQFGKMINYVM-GESGVLQYLSYGCYCGLGGQGQPTDATDRCCFVHDCC
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Fig. 2. Multiple alignments of N-terminal amino acid sequences of BthA-I-PLA_2 and other acidic PLA_2s from snake venoms using the CLUSTAL program. The sequences were taken from: *Trimeresurus flavoviridis* isoforms of PLA_2s (gi2117957, gi129417, and gi422653); *Agkistrodon piscivorus* PLA_2 (gi2117960); *Vipera berus berus* PA2-VIPBB (gi400721); and *B. jararaca* BJ-PLA_2 (gi3914258). According to adopted numbering for homology studies (gap between Met13 and Gly14), the last sequenced residues were His48-Asp49-Cys50-Cys51.

and resolved into several protein peaks (Fig. 1B). Subfraction JIb-3 was found to display PLA_2 activity and was named BthA-I-PLA_2. Homogeneity of this fraction was demonstrated further by native-PAGE (Fig. 1C), SDS–PAGE (data not shown), and N-terminal sequencing (Fig. 2). The purified protein consisted of a single polypeptide chain with an isoelectric point at ~4.5 and an apparent approximate molecular weight of 13,700 as estimated by SDS–PAGE.

BthA-I-PLA_2 crystals with approximate dimensions of $0.4 \times 0.2 \times 0.1$ mm were mounted in a quartz capillary (Fig. 1D). The crystallographic data for the BthA-I-PLA_2 are shown in Table 1. A total of 112,423 reflections were measured of which 11,962 were unique. The merging of all equivalent reflections resulted in a data set that is about 95.7% complete at 2.1 Å with $R_{merge} = 12.8\%$. The crystals belong to the monoclinic system, space group P2 or $P2_1$, and have average unit cell dimensions of a = 33.91, b = 63.75, c = 49.14 Å, and $\beta = 104.04°$.

Table 1
X-ray diffraction data of BthA-I-PLA_2 from *B. jararacussu* snake venom

Space group	$P2_1$ or P2
Cell dimensions	a = 33.91, b = 63.75, c = 49.14 Å, $\beta = 104.04°$
Unique reflections	11,962 (1110)[a]
Resolution ranges (Å)	50–2.1 (2.18–2.10)[a]
Completeness (%)	95.7 (93.4)[a]
Average I/σ(I)	5.4 (2.2)[a]
R_{merge} (%)[b]	12.8 (48.1)[a]

[a] Numbers in parentheses are for the highest resolution shell, 2.18–210 Å.

[b] $R_{merge} = \Sigma_{hkl} (\Sigma_i (|I_{hkl,i} - \langle I_{hkl} \rangle|))/\Sigma_{hkl,i} \langle I_{hkl} \rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices *h*, *k* and *l*, and $\langle I_{hkl} \rangle$ is the mean intensity of that reflection calculated for $I > -3\sigma(I)$.

3.2. Functional characterization of the acidic PLA_2 from B. jararacussu snake venom

BthA-I-PLA_2 showed high PLA_2 activity, but low anticoagulant activity on platelet poor plasma. It was also able to induce edema (Table 2). BthA-I-PLA_2 was not toxic when injected i.p. (25 mg/kg), i.v. (7.5 mg/kg), or i.c.v. (0.5 mg/kg) in mice. Doses up to 250 μg did not cause any necrosis and did not release CK into the plasma (results not shown).

BthA-I-PLA_2 inhibited platelet aggregation of rabbit PRP in a dose-dependent manner, and its potency depended on the concentration of agonists used to trigger platelet aggregation (Fig. 3). Similar IC_{50} values (437 and 743 nM for 10 and 25 μg/mL of collagen and 624 and 1248 nM for 4 and 10 μM ADP, respectively) were found.

When WRP were incubated with BthA-I-PLA_2 (25 μg/mL), no inhibition of aggregation induced by collagen or thrombin was detected (results not shown). However, addition of small amounts of rabbit plasma (5%, v/v) or exogenous phosphatidylcholine (80 μg/mL, final concentration) to the washed platelet suspension restored the inhibitory effect of BthA-I-PLA_2 for collagen-induced platelet aggregation (data not shown).

BthA-I-PLA_2 was chemically modified by reacting the protein with BPB and then assayed for phospholipase,

Table 2
Enzymatic and pharmacological activities induced by native or BPB-BthA-I-PLA_2 from *B. jararacussu* snake venom

Enzyme	PLA_2 activity (U/mg)	Cytotoxicity[a] (%)	CK[b] (U/L)	Edema[c] (%)	Platelet aggregation inhibition[d] (%)
BthA-I-PLA_2	119.3 ± 2.44	0.0	0.0	30.6 ± 1.18	100.0 ± 2.00
BPB-BthA-I-PLA_2	0.0	ND[e]	ND	0.5	0.0

Values represent the means ± SD of three experiments.

[a] Cytotoxicity was induced by 40 μg of BthA-I-PLA_2.

[b] Myotoxicity was induced by 250 μg of BthA-I-PLA_2.

[c] Edema was induced by 50 μg of BthA-I-PLA_2, 30 min after inoculation.

[d] The native and modified BthA-I-PLA_2 were assayed for inhibitory effect on collagen-induced platelet aggregation by 20 μg of BthA-I-PLA_2.

[e] ND = not determined.

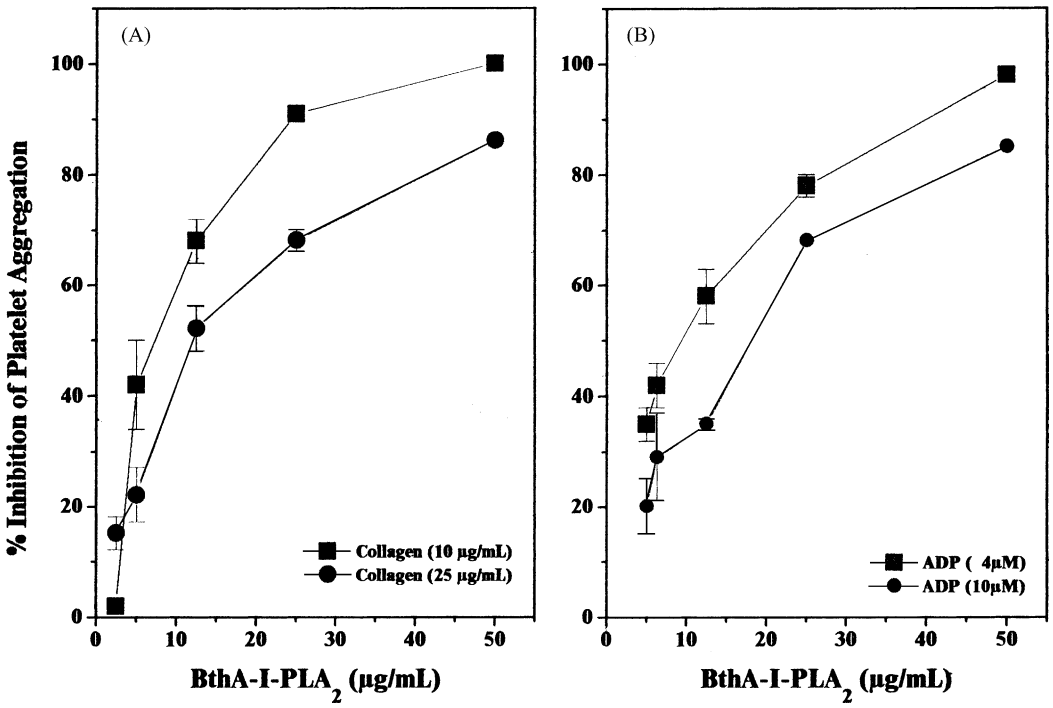

Fig. 3. Inhibitory effect of BthA-I-PLA_2 on platelet aggregation. (A) PRP samples were stirred for 2 min at 37° with BthA-I-PLA_2 (2–50 µg/mL); then platelet aggregation was triggered by adding 10 or 25 µg/mL of collagen. (B) The platelets were preincubated with BthA-I-PLA_2 under the same conditions, and then 4 or 10 µM ADP was added to induce platelet aggregation. Results are reported as means ± SD (N = 3 animals/assay).

edema-inducing and hypotensive activity, and inhibitory effects on collagen-induced platelet aggregation. Amino acid analysis comparisons of native and modified BthA-I-PLA_2 showed that a single His was modified by BPB (results not shown). Table 2 shows that all activities were abolished or reduced. Control BthA-I-PLA_2 incubated with ammonium bicarbonate buffer alone showed no loss of activity.

The i.v. injection of BthA-I-PLA_2 caused an immediate fall in blood pressure. The hypotensive response to the protein was dose-related, with an approximate ED_{50} of 30 µg/kg. Repeated injections of lower doses of the protein caused acute desensitization, indicating occurrence of tachyphylaxis. No significant cardiovascular effects were observed after the i.v. injection of the BPB-modified Bth-I-PLA_2 at the same dose corresponding to the ED_{50} for the intact protein (Fig. 4).

In addition, BthA-I-PLA_2 showed a higher cross-reaction with anti-*B. jararacussu* and anti-BthTX-II antibodies. The anti-C terminus peptide (115–129) from *B. asper* myotoxin II weakly recognized BthA-I-PLA_2 (Fig. 5).

4. Discussion

Snake venom PLA_2 enzymes, in addition to their possible role in the digestion of prey, exhibit a wide variety of pharmacological effects, including effects on platelet aggregation [9,25,41,45]. Several platelet aggregation inhibitors have been purified and characterized from various snake venoms, but data on *Bothrops* snake venoms with respect to platelet aggregation inhibitors are still scant.

BthA-I-PLA_2 is a new antiplatelet and hypotensive acidic PLA_2, isolated from *B. jararacussu* venom by a two-step sequential purification procedure, which was characterized structurally and functionally. The N-terminal sequence analysis of native BthA-I-PLA_2 (Fig. 2) showed that BthA-I-PLA_2 contains all of the PLA_2 conserved residues involved in Ca^{2+} binding (Tyr28, Gly30, Gly32, and Asp49) and in the catalytic network (His48). Some substitutions were found in the hydrophobic channel where Trp19, which is also reported as part of the interfacial binding surface [46], is replaced by a Val19 residue. A comparison of the N-terminal sequence of BthA-I-PLA_2 with other PLA_2s exhibiting platelet-inhibiting activity showed a moderate degree of similarity (68–72%) to class II PLA_2 isolated from snake venoms.

The molecular mass of BthA-I-PLA_2 is approximately 13.7 kDa. The volume of the unit cell is 103,522 $Å^3$, compatible with one or two molecules in the asymmetric unit with a V_M (volume per dalton) value of 3.76 or 1.88 $Å^3$/Da, respectively. Assuming a value of 0.74 cm^3/g for the protein partial specific volume, the calculated solvent content in the crystal is 67.3 or 34.7% [47] for a dimer or a monomer, respectively, in the asymmetric unit. Attempts to solve the structure by the molecular replacement method are underway.

BthA-I-PLA_2 showed a very high PLA_2 catalytic activity (119.3 U/mg) when compared with BthTX-II (32 U/mg), an Asp49 basic and myotoxic PLA_2 isolated from the

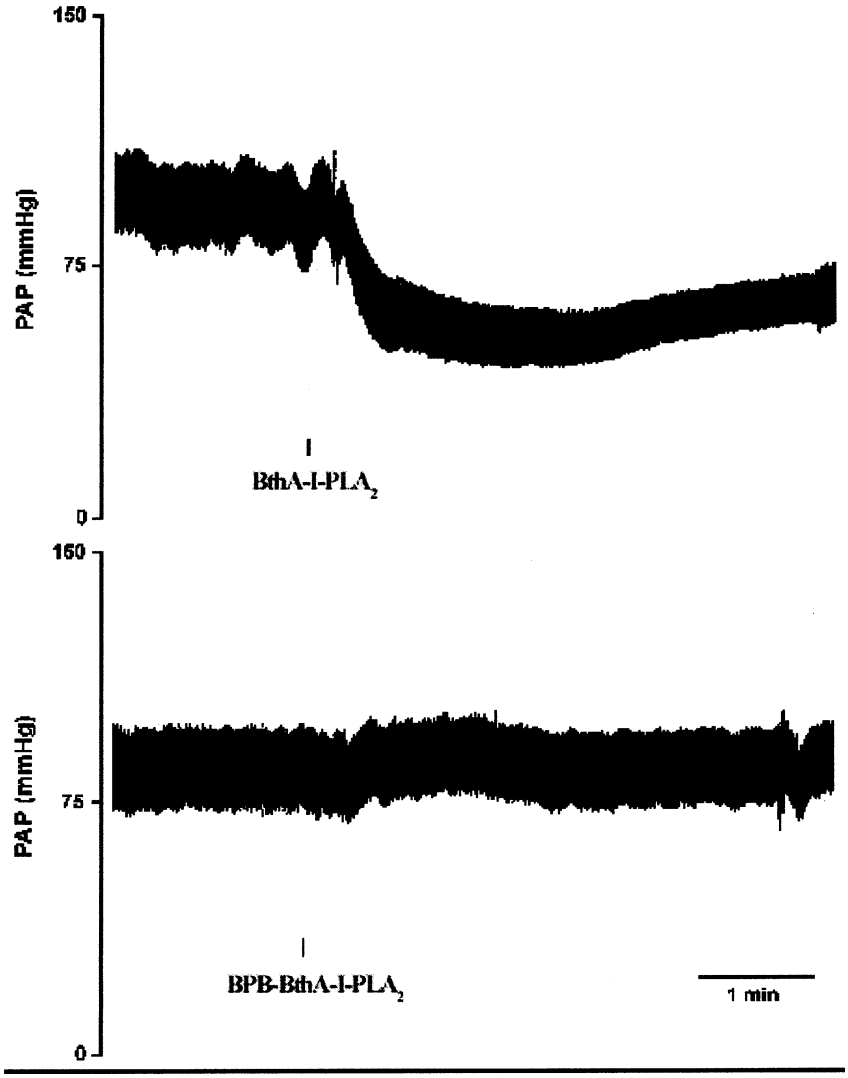

Fig. 4. Hypotension activity of BthA-I-PLA_2. Pulsatile arterial blood pressure (PAP) recordings of urethane-anesthetized rats showing the hypotensive response to the i.v. injection of 30 μg/kg of BthA-I-PLA_2 and the absence of the cardiovascular effects after the injection of the BPB-modified protein (30 μg/kg of BPB–BthA-I-PLA_2), indicating that the hypotensive response is related to enzyme activity.

same venom [14,26–28]. BthA-I-PLA_2 did not induce any myotoxicity or lethality in the mice. Nevertheless, other acidic PLA_2s isolated from *B. alternatus* [48] and from *Lachesis muta* [49] were reported to be able to induce myonecrosis.

Platelet aggregation plays an important role in platelet retraction and wound healing. Any alteration in platelet aggregation could lead to debilitation or death. As observed in "Results," purified BthA-I-PLA_2 displayed a significant inhibitory effect on aggregation of rabbit PRP. BthA-I-PLA_2 showed IC_{50} values of 437 and 743 nM for collagen- (10 and 25 μg/mL) and 624 and 1248 nM for ADP- (4 and 10 μM) induced platelet aggregation. When BthA-I-PLA_2 was preincubated with washed platelets, no inhibitory effect was observed, suggesting the dependence of a plasma substrate for the purified enzyme. When an exogenous source of phospholipids was added to the washed platelet suspension, the inhibitory effect was clearly restored, indicating that phospholipids present in the plasma may be substrates for BthA-I-PLA_2 and/or that the products enzymatically formed are able to elicit such responses on platelets.

The i.v. injection of BthA-I-PLA_2 caused an immediate fall in blood pressure. The hypotensive responses were dose-dependent and tachyphylatic. The acute desensitization observed after repeated injections of small doses of the protein may indicate the existence of a small pool of substrate, available to the phospholipase when i.v. injected. The hypotensive response was no longer observed when BthA-I-PLA_2 was modified after reaction with BPB. The lack of effects after the blockade of the catalytic site with BPB suggests that the hypotensive response to BthA-I-PLA_2 is due to its phospholipase activity.

BthA-I-PLA_2 was chemically modified on the histidine-48 residue by *p*-bromophenacyl bromide on the basis of homology with the data of Zhao *et al*. [50]. This amino acid

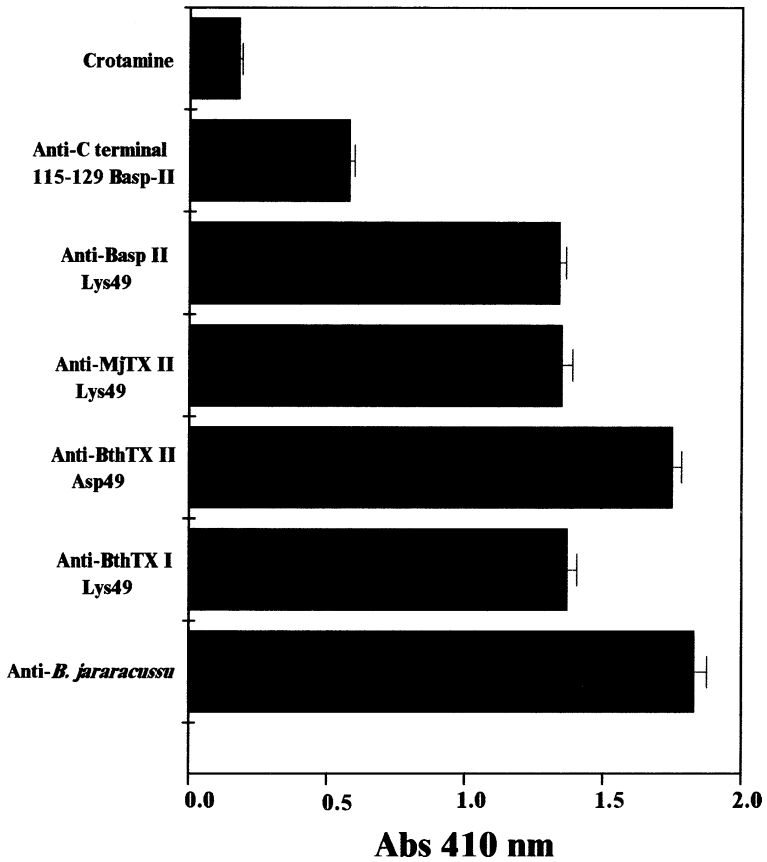

Fig. 5. Cross-reactivity of BthA-I-PLA_2 with anti-*B. jararacussu* venom antibodies, anti-Asp49 (*B. jararacussu* BthTX-II) or anti-Lys49 (*B. jararacussu* BthTX-I, *B. moojeni* MjTX-II, *B. asper* Basp-II, and *B. asper* C-terminus peptide 115–129 from Basp-II) myotoxins. Negative control: crotamine. Results are reported as means ± SD (N = 3 animals/assay).

residue is known to be crucial for the enzymatic and pharmacological activities of PLA_2s [12,14,17–20,43,48,50,51]. Modified BthA-I-PLA_2 failed to inhibit PRP aggregation as well as the hypotensive response and no phospholipase or edema activities were observed, suggesting that the enzymatic activity is important for these pharmacological effects. For some PLA_2s, there is a relationship between their pharmacological effect and enzymatic activity [14,41], while for others, no correlation is observed [51]. According to Evans and Kini [9], BthA-I-PLA_2, whose platelet aggregation inhibitory activity was dependent on catalytic activity, is better placed in class B1.

Anti-*B. jararacussu* and anti-BthTX-II antibodies recognize BthA-I-PLA_2, while anti-Lys49 PLA_2 (*B. asper* Basp-II, *B. jararacussu* BthTX-I, and *B. moojeni* MjTX-II) antibodies showed less cross-reactivity (Fig. 5). In addition, the anti-C-terminus peptide (115–129) from Basp-II weakly recognized BthA-I-PLA_2, suggesting that the C-terminus of the acidic PLA_2 is structurally different from those of basic PLA_2s. This C-terminal region from basic PLA_2 myotoxins is known to bind heparin, and it has been suggested to be the segment responsible for the cytotoxic and myotoxic effects of *B. asper* myotoxin-II [52,53]. Recently, Fuly *et al.* [49] showed that the myotoxic effect induced by the acidic PLA_2 from *Lachesis muta* venom is not inhibited by heparin, in agreement with the idea that the C-terminal regions of acidic and basic PLA_2s are structurally different.

Further interaction studies of PLA_2 with platelets and/or with isolated blood components will help to elucidate the reaction mechanism of inhibition and provide information on the amino acid residues involved in the interaction with platelets. Due to their fundamental role in hemostasis and thrombosis, inhibitors of platelet aggregation are of interest in atherosclerosis and in the regulation of tumor growth in cancer [54]. PLA_2 enzymes are also useful as tools in the study of molecular mechanisms of platelet aggregation and are potential models for the development of drugs that inhibit platelet aggregation.

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