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Biochemical and pharmacological characterization of PhTX-I a new myotoxic phospholipase A_2 isolated from *Porthidium hyoprora* snake venom

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ABSTRACT

This paper reports the biochemical and pharmacological characterization of a new myotoxic PLA_2 (EC 3.1.1.4) called PhTX-I, purified from *Porthidium hyoprora* venom by one step analytical chromatography reverse phase HPLC. The homogeneity of the PhTX-I fraction and its molecular mass were initially evaluated by SDS–PAGE and confirmed by MALDI-TOF spectrometry, indicating a molecular mass of 14.249 Da and constituted of a single polipeptidic chain. Amino acid sequence was determined by "de novo sequencing," in tandem mass spectrometry, belonging to D49-PLA_2 enzyme class and exhibiting high identity (44–90%) with other myotoxics PLA_2 from snake venoms. The enzymatic investigation showed maximal activity at pH 8 and 35–45 °C. This activity was dependent on Ca^{2+}, other cations (Mg^{2+}, Mn^{2+}, Cd^{2+} and Zn^{2+}) reduced notably the enzymatic activity, suggesting that the arrangement of the catalytic site presents an exclusive structure for Ca^{2+}. Ex vivo, whole venom and PhTX-I PLA_2 caused blockade of the neuromuscular transmission in young chick biventer cervicis preparations similar to other isolated snake venom toxins from the *Bothrops* genus. In vivo, both induced local myotoxicity and systemic interleukin-6 response upon intramuscular injection, additionally, induced moderate footpad edema. In vitro, both induced low cytotoxicity in skeletal muscle myoblasts, however PhTX-I PLA_2 was able to lyse myotubes.

1. Introduction

Snake venoms comprise a complex pool of proteins (more than 90% of the dry weight), organic compounds with low molecular mass and inorganic compounds. Among the toxic compounds are included proteins such as desintegrins and bradykinin-potentiating peptides and enzymes such as metalloproteases, serinoproteases and phospholipases, etc. Among these bioactive proteins, myotoxins phospholipases A_2 (PLA_2 E.C 3.1.1.4) are the main components (Serrano et al., 2005).

PLA_2 are calcium-dependent enzymes and catalyzes the selective hydrolysis of the sn-2 acyl ester bond in 1,2-diacyl-sn-glycero-3 phospholipids, resulting in the formation of free fatty acids, such as arachidonic acid and lysophospholipids (Burke and Dennis, 2009). These lipids are also substrates for intracellular biochemical pathways that generate potent autocrine and paracrine lipid mediators such as the eicosanoids and platelet activating factor (Brash, 2001; Jensen et al., 2005; Jenkins et al., 2009).

The superfamily of PLA_2 has been classified into 15 groups (Schaloske and Dennis, 2006). Based on the amino acid sequences and cell location analysis, snake venom phospholipases A_2 belong to group I (elapid snakes) or II (viperid and crotalid snakes) species. Snake venom PLA_2 are interesting proteins, containing about 120–130 amino acids, that are cross-linked by seven disulfide bonds. In addition to their catalytic activity, this PLA_2 displays an array of biological actions which may be either dependent or independent of catalysis, including: myotoxicity, neurotoxicity, hypotensive, anticoagulant, platelet-aggregation inhibition and edema inducing activities (Kini, 2003; Magro et al., 2004; Tsai et al., 2004).

The biology and the biochemistry of a lot of PLA_2 from bothropic and crotalic venoms have been documented; however, there is scantiness of information about the phospholipases A_2 of *Porthidium hyoprora* snake venom, a poisonous snake that belongs to the family Viperidae, widely spread in the Amazonian area of Brazil, Ecuador, Peru and Colombia, (Campbell and Lamar, 2004). Studies on clinical and epidemiological features of *Bothrops*, *Porthidium* and *Botriechis* snakebites showed that edematogenic, hemorrhagic and necrosis-inducing activities of *P. hyoprora* venom were similar to *Bothrops* species (Campos et al., 1999; Otero et al., 2002). Some clinicians prefer to use the term "bothropic envenomation" because the clinical effects of those venoms are generally similar. Thus, pharmacological studies involving PLA_2 purified from this venom are required, since they are the main venom components responsible for muscular necrosis and inflammatory responses in snakebites (Gutiérrez and Lomonte, 1997; Kini, 2003).

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This paper reports the isolation, biochemical and pharmacological characterization including *ex vivo* neurotoxicity on young chicken biventer cervicis preparations, inflammatory activities in the footpad of mice, *in vivo* myotoxicity and *in vitro* cytotoxicity of a novel PLA_2 named PhTX-I from *P. hyoprora* venom.

2. Material and methods

2.1. Venom and reagents

The venom and the solvents (HPLC grade), 4-nitro-3-(octanoyloxy) benzoic acid, sequence grade bovine pancreatic trypsin and other reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Reverse Phase HPLC (RP-HPLC)

The PhTX-I PLA_2 from *P. hyoprora* venom was purified by reverse phase HPLC according to method described by Ponce-Soto et al. (2007) using 500 mg of whole venom. Briefly, 5 mg of whole venom was dissolved in 200 μL of buffer A (0.1% TFA) and centrifuged at 4500 *g*, the supernatant was then applied to a μ-Bondapack C18 column (0.78 × 30 cm; Waters 991-PDA system), previously equilibrated with buffer A for 15 min. The elution of the protein was then conducted using a linear gradient (0–100%, v/v) of buffer B (66.5% acetronitrile in buffer A) at a constant flow rate of 1.0 mL/min. The chromatographic run was monitored at 280 nm of absorbance and after elution the fraction was lyophilized and stored at −40 °C.

2.3. Electrophoresis

Tricine SDS–PAGE in a discontinuous gel and buffer system was used to estimate the molecular mass of the PhTX-I PLA_2, under reducing and non-reducing conditions (Schagger and Von Jagow, 1987). The molecular mass markers used were (in kDa): phosphorylase B — 94, albumin — 67, ovalbumin — 43, carbonic anhydrase — 30, soybean trypsin inhibitor — 20 and lysozyme — 14.

2.4. Amino acid analysis

Amino acid analysis was performed on a Pico-Tag Analyzer (Waters Systems) as described by Heinrikson and Meredith (1984). PhTX-I PLA_2, sample (30 μg) was hydrolyzed at 105 °C for 24 h, in 6 M HCl (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolyzates were reacted with 20 μL of derivatization solution (ethanol:triethylamine:water:phenylisothiocyanate, 7:1:1:1, v/v) for 1 h at room temperature, after which the PTC-amino acids were identified and quantified by HPLC, by comparing their retention times and peak areas with those from a standard amino acid mixture.

2.5. Reduction and alkylation

Purified lyophilized protein PhTX-I PLA_2 from RP-HPLC was resuspended in 8 M urea containing 10 mM DTT at pH 8.0 and the disulfide bridges were then reduced by incubation at 37 °C for 2 h. Iodoacetamide was used for alkylating the free thiols of cysteine residues, based on previous experiments, a 30% molar excess of iodoacetamide relative to the total number of thiols was eventually chosen and the mixture was incubated for 1.5 h at 37 °C in the dark. The reaction was stopped by injecting the mixture onto a RP-HPLC column followed by lyophilization of the collected peak.

2.6. Enzymatic hydrolysis

The purified protein PhTX-I PLA_2 was hydrolysed with sequencing grade bovine pancreatic trypsin in 0.4% ammonium bicarbonate, pH 8.5, for 4 h at 37 °C, at an enzyme:substrate ratio of 1:100 (w/w). The reaction was ceased by lyophilization.

2.7. Mass spectrometry

The molecular mass of intact native and alkylated PhTX-I PLA_2 were analyzed by MALDI-TOF mass spectrometry using a Voyager-DE PRO MALDI-TOF apparatus (Applied Biosystems, Foster City, CA, USA) equipped with a pulsed nitrogen laser (337 nm, pulse with 4 ns). 1 μL of sample in 0.1% TFA was mixed with 2 μL of the matrix sinapinic acid (3, 5-dimethoxy-4-hydroxycinnamic acid). The matrix was prepared with 30% acetonitrile and 0.1% TFA and its mass analyzed under the following conditions: accelerate voltage 25 kV, the laser fixed in 2890 μJ/com2, delay 300 ns, and linear analysis mode (Ponce-Soto et al., 2006).

ESI-CID-MS/MS analyses were performed using a quadrupole-time of flight (Q-TOF) hybrid mass spectrometer Q-TOF Ultima from Micromass (Manchester, UK) equipped with a nano Zspray source operating in a positive ion mode. The ionization conditions of usage included a capillary voltage of 2.3 kV, a cone voltage and RF1 lens of 30 V and 100 V, respectively, and a collision energy of 10 eV. The source temperature was 70 °C and the cone gas was N_2 at a flow of 80 l/h; nebulizing gas was not used to obtain the sprays. Argon was used for collisional cooling and for fragmentation of ions in the collision cell. External calibration with sodium iodide was made over a mass range from 50 to 3000 m/z. All spectra were acquired with the TOF analyser in "Vmode" (TOF kV = 9.1) and the MCP voltage set at 2150 V (Damico et al., 2005).

2.8. De novo sequencing of tryptic peptides

Alkylated tryptic peptides from PhTX-I PLA_2 were fractionated by RP-HPLC, collected manually, lyophilized and re-suspended in 80% H_2O and 20% acetonitrile in 0.1% TFA. Each peptide was introduced separately into the mass spectrometer source with a syringe pump at a flow-rate of 500 nl/min^{-1}. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 400–2000 m/z, in order to select the ion of interest, subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode). Different collision energies were used, depending on the mass and charge state of the ions. The resulting product-ion spectra were acquired in the TOF analyser and deconvoluted using the MassLynx-MaxEnt 3 algorithm. Singly charged spectra were processed manually using the PepSeq application included in MassLynx.

2.9. PLA_2 activity

PLA_2 activity was measured using the assay described by Cho and Kezdy (1991) and Holzer and Mackessy (1996) modified for 96-well plates. The standard assay mixture contained 200 μL of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 μL of substrate 4-nitro-3-(octanoyloxy) benzoic acid (3 mM), 20 μL of water and 20 μL of PhTX-I PLA_2 (1 mg/mL) in a final volume of 260 μL. After adding PhTX-I PLA_2, respectively (20 μg), the mixture was incubated for up to 40 min at 37 °C, with the reading of absorbance at intervals of 10 min. The enzyme activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the increase of absorbance after 20 min. The pH and optima temperature of the PLA_2 were determined by incubating the enzyme in four buffers of different pH values (4–10) and in Tris–HCl buffer, pH 8.0, at different temperatures, respectively. The effect of substrate concentration (40, 20, 10, 5, 2.5, 1.0, 0.5, 0.3, 0.2 and 0.1 mM) on enzyme activity was determined by measuring the increase of absorbance after 20 min of incubation in Tris–HCl buffer, pH 8.0, at 37 °C. All assays were done in triplicate and the absorbances

at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA).

2.10. Chick biventer cervicis muscle preparation (BCp)

Animals were anesthetized with halothane and sacrificed by exsanguination. The biventer cervicis muscles were removed and mounted under a tension of 0.5 g, in a 5 mL organ bath (Automatic organ multiple-bath LE01 Letica Scientific Instruments. Barcelona, Spain) at 37 °C containing aerated (95% O_2–5% CO_2) Krebs solution (pH 7.5) of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.0 and glucose 11.65. Contracture to exogenously applied acetylcholine (ACh; 110 μM for 60 s) and KCl (20 mM for 130 s) was obtained in the absence of field stimulation, before and after of addition of a single dose of whole venom (20, 50 μg/mL) or PhTX-I PLA_2 (0.35, 0.7, 1.4 and 3.5 μM). A bipolar platinum ring electrode was placed around the tendon, which runs the nerve trunk supplying the muscle. Indirect stimulation was performed with a (MAIN BOX LE 12404 Panlab s.l. Powerlab AD Instruments Barcelona, Spain) stimulator (0.1 Hz, 0.2 ms, 3–4 V). Muscle contractions and contractures were isometrically recorded by force-displacement transducers (Model MLT0201 Force transducer 5 mg–25 g Panlab s.l. AD Instruments Pty Ltd. Spain) connected to a PowerLab/4SP (OUAD Bridge AD Instruments, Barcelona, Spain).

2.11. Myotoxic activity

Groups of five Swiss mice (18–20 g) received an intramuscular (i.m.) injection of variable amounts of whole venom or PhTX-I PLA_2 (10 and 20 μg), in 100 μL of PBS, in the gastrocnemius. A control group received 100 μL of PBS (0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2). At different intervals of time (1, 3, 6, 9 and 24 h) blood was collected from the tail into heparinized capillary tubes, and the plasma creatine kinase (CK; EC 2.7.3.2) activity was determined by a kinetic assay (Sigma 47-UV). Activity was expressed in U/l, one unit defined as the phosphorylation of 1 μmol of creatine/min at 25 °C.

2.12. Cytotoxic activity

Cytotoxic activity was assayed on murine skeletal muscle C2C12 myoblasts and myotubes (ATCC CRL-1772). Variable amounts of whole venom and PhTX-I PLA_2 (5, 10, 20 and 40 μg) were diluted in assay medium (Dulbecco's Modified Eagle's Medium supplemented with 1% fetal calf serum) and added to cells in 96-well plates, in 150 μL. Controls for 0 and 100% toxicity consisted of assay medium, and 0.1% Triton X-100, respectively. After 3 h at 37 °C, a supernatant aliquot was collected for determination of lactic dehydrogenase (LDH; EC 1.1.1.27) activity released from damaged cells, using a kinetic assay (Wiener LDH-P UV). Experiments were carried out in triplicate.

2.13. Edema-forming activity

The ability of whole venom and PhTX-I PLA_2 to induce edema was studied in groups of five Swiss mice (18–20 g). Fifty microliters of phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) with toxins (0.5, 1 and 2 μg/paw) were injected in the subplantar region of the right footpad. The left footpad received 50 μL of PBS, as a control. The paw volume was evaluated plethysmographically (Model 7140 Plethysmometer Ugo Basile, USA), immediately before the injection (basal) and at selected intervals of time (0.5, 1, 3, 6, 9 and 24 h). Edema-forming activity was expressed as the percentage of increase in volume of the right foot pad in comparison to the left foot pad (control). The formula for calculation of the percentage of edema in toxins injected paw was $\%\text{edema} = [(Tx \times 100)/To) - 100]$, where Tx is the edema (volume) measured at each time interval and To is the volume of the paw (intact, zero time before toxins injection). The percentage of edema calculated was subtracted from the matched values at each time point in the saline injected hind paw (control).

2.14. Quantification of Interleukin-6

Groups of five Swiss mice (18–20 g) received an injection of 10 μg of whole venom and PhTX-I PLA_2 at the tibial muscle. Blood samples were collected into heparinized capillaries in 1, 3, 6 and 9 h after injection. Briefly, 96-well plates were coated with 100 μL of the first capture monoclonal antibody anti-IL-6 and incubated for 2 h at 37 °C. Abs at 450 nm was recorded and concentrations of IL-6 were estimated from standard curves prepared with recombinant IL-6.

2.15. Statistical analyses

Results were reported as mean ± SEM. The significance of differences among means was assessed by analysis of variance followed by Dunnett's test, when several experimental groups were compared with the control group. Differences were considered statistically significant if $p < 0.05$.

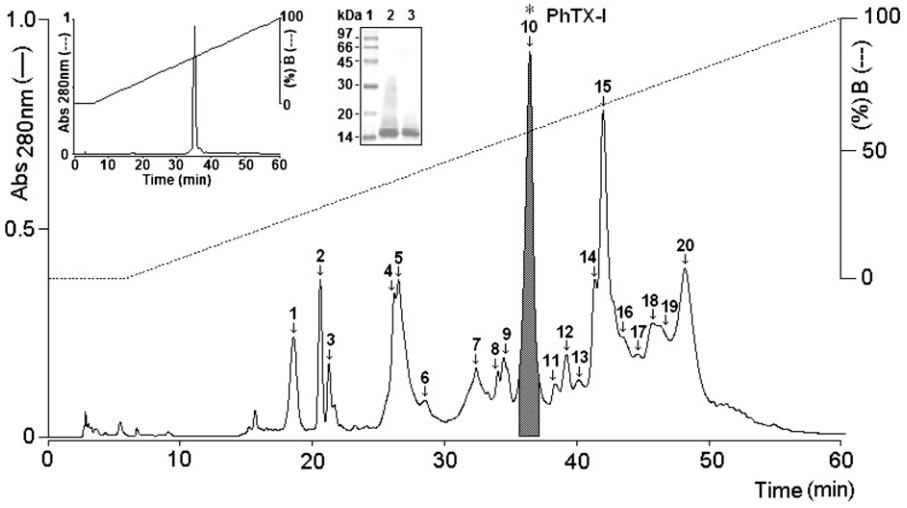

Fig. 1. Elution profile of *P. hyoprora* venom by RP-HPLC on a μ-Bondapack C18 column. *Fraction 10 (PhTX-I PLA_2) contained PLA_2 and myotoxic activity. Insert: re-chromatography on RP-HPLC of PhTX-I. Electrophoretic profile in Tricine SDS–PAGE (1) molecular mass markers; (2) PhTX-I not reduced; (3) PhTX-I reduced with DTT (1 M).

Table 1
Parameters of PhTX-I PLA_2 purification.

Step	Volume (mL)	Protein (mg/mL)	Activity (U/mL)	Total activity (U.T.)	Specific activity (U/mg)	Recovery (%)	Purification
Crude venom	20	0.9154	274.5	5490	299.87	100	1
(HPLC) PhTX-I	1	0.0018	735	735	408,333.3	13.39	1361.7

3. Results

3.1. Purification and biochemical characterization of the PhTX-I PLA_2

Fractionation of *P. hyoprora* venom by RP-HPLC on a μ-Bondapack C18 column resulted in twenty peaks (1–20) (Fig. 1). The 20 peaks were screened for myotoxic and PLA_2 activities. Peaks 10, 11 and 12 caused local myotoxicity at concentrations ranging from 5 to 20 μg/mL in the mouse gastrocnemius muscle. In addition, peak 10, named PhTX-I (*P. hyoprora* toxin 1) showed high PLA_2 activity (14,5 nmoles/min/mg) and was selected for biochemical and pharmacological characterization. This rapid procedure showed high yield, with high purity levels (Table 1). The purity of this peak was confirmed by rechromatography on an analytical RP-HPLC μ-Bondapack C18 column, showing the presence of only one peak and by Tricine SDS-PAGE, that revealed the presence of one electrophoretic band with *Mr* around 15 kDa (Fig. 1, inset), in the absence and presence of DTT (1 M).

MALDI-TOF mass spectrometry analysis confirmed the homogeneity of the peak PhTX-I and determined the exact molecular mass of 14249.22 Da, the MH^+, MH^{2+} and $2MH^+$ species are also shown (Fig. 2). This value of molecular mass was used in calculating the molar concentrations of toxin used in the experiments described below.

The mass of the PhTX-I PLA_2 after reducing and alkylation was 15061.2 Da (Fig. 2 insert), since the Cys is the favorite site of alkylation, the mass increasing of 812 Da indicated the presence of 14 Cys modified residues. In agreement with this, the presence of 14 half-Cys residues involved in disulfide bond formation is fully conserved in this group of proteins. This suggested the occurrence of seven disulfide bonds as a typical characteristic for group II of PLA_2.

Amino acid analysis revealed the following composition of PhTX-I PLA_2: D/11, T/9, S/7, E/9, P/4, G/11, A/5, C/14, V/4, M/3, I/5, L/7, Y/11, F/5, K/7, H/3 and R/10. PhTX-I PLA_2 showed a high content of K, Y, G and P residues-typical of a basic PLA_2 protein.

3.2. Determination of the amino acid sequences of the PhTX-I PLA_2

The alkylated protein was digested with trypsin and the resulting tryptic peptides (18) were fractionated by RP-HPLC (not shown data). *De novo sequencing* by ESI-MS/MS was carried out for each peptide peak. The data obtained were processed using the Mascot MS/MS Ion Search software (www.matrixscience.com). Table 2 shows the deduced sequence and measured masses of alkylated peptides obtained for PhTX-I PLA_2. Isoleucine and leucine residues were not discriminated in any of the sequences reported since they were indistinguishable in low energy CID spectra. Because of the external calibration applied to all spectra, it was also not possible to resolve the 0.036 Da difference between glutamine and lysine residues, except for the lysine that was deduced based on the cleavage and missed cleavage of the enzyme. Each de novo sequenced peptide of the PhTX-I PLA_2 was submitted to the NCBI database, using the protein search program BLAST-p with the search being restricted to the sequenced proteins from the basic protein with phospholipase A_2 activity family.

The primary structure of the PhTX-I PLA_2 (Fig. 3) was determined by comparing the sequences of the overlapping alkylated tryptics 18 peptides purified and sequencing from the above digests. The tandem mass spectra shown in Fig. 4, relative to the peptide 8 of the digest, having the sequence C C F V H D C C R, allows to classify the protein as D49-PLA_2.

The results of the primary structures show that PhTX-I PLA_2 is composed of 123 amino acid residues and shares the conserved sequence domains common to D49 PLA_2 group, including the 14 cysteines, the calcium-binding site located on (Y)27, (G)29, (G)31, (G)32, the catalytic network common formed by (H)48, (D)49, (Y)52 and (D)89. The PhTX-I PLA_2 revealed the presence of some important amino acid replacements (S)1→(D)1; (Q)4→(E)4; (Q)7→(K)7; (R)34→(G)34; (R)70→(W)70; I(86)→(L)86; (D)118→(A)118. A comparative analysis of the sequence of PhTX-I PLA_2 with other

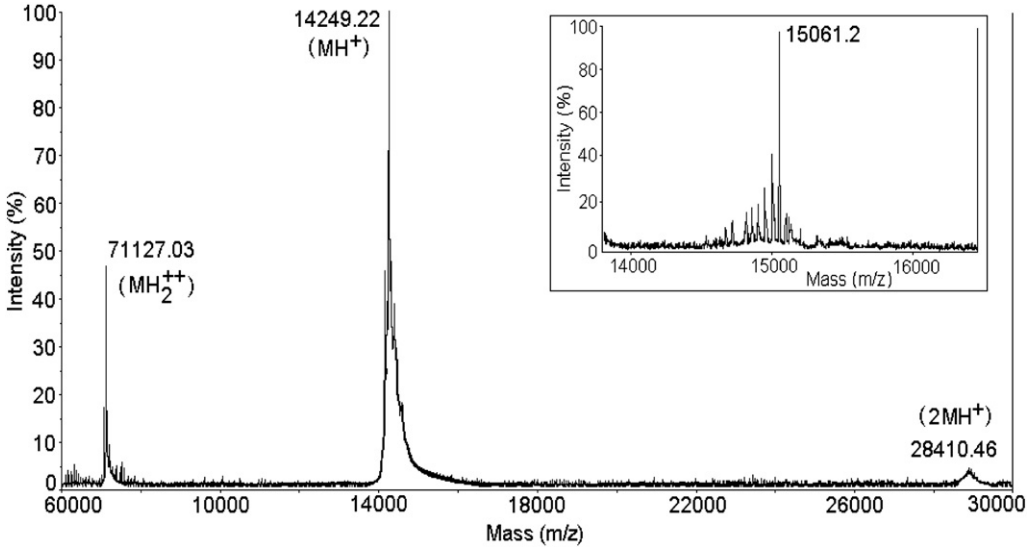

Fig. 2. Mass determination of the native PhTX-I PLA_2 by MALDI-TOF mass spectrometry. The MH^+, MH_2^{++} and $2MH^+$ species are shown in the mass spectrum. Insert MALDI-TOF mass spectrum, showing multiple alkylation channels of alkylated PhTX-I PLA_2 isolated from *P. hyoprora*.

Table 2
Measured molecular masses and deduced amino acid sequences obtained by ESI-MS/MS based on the alkylated tryptic peptides of PhTX-I PLA_2. The peptides were separated by RP-HPLC and sequenced by mass spectrometry. *C* = alkylated cysteine, lysine residues shown in bold were deduced on the cleavage and missed cleavage by trypsin. All molecular masses are reported as monoisotopic.

HPLC fraction	Measured mass (Da)	Amino acid sequence
1	1312.38	ENI/LGTYNK/QK/QYR
2	1547.52	GAYGCYCGWGGRG**K**
3	1763.75	DPCK/QEI/LCECDK/QAAAV
4	503.18	MI/LI/L**K**
5	2102.91	KI/LTGCPK/QTNDRYSYSW**K**
6	671.28	GK/QPK/QD**K**
7	918.39	MI/LI/LK/QETG**K**
8	1384.54	CCFVHDCCR
9	2042.64	DK/QTDDRCCFVHDCCR
10	797.19	NPFPYY
11	1840.82	AAAVCFRENI/LGTYNK/Q**K**
12	884.41	GRGK/QPK/QD**K**
13	1204.55	SI/LCK/QK/QADK/QPC
14	893.32	DI/LWEFG**K**
15	1883.76	ETGK/QNPFPYYGAYGCY
16	1650.77	K/QYRYHI/LRSI/LCK/Q**K**
17	1160.44	I/LTGCPK/QTNDR
18	2793.06	TNDRYSYSWK/QDI/LTI/LVCGEDDPC**K**

myotoxic D49-PLA_2 belonging to Viperidae family showed similarity of 44.3–90.9% (Fig. 5).

3.3. Activity measurements of the PhTX-I PLA_2

The PLA_2 activity of *P. hyoprora* venom and PhTX-I PLA_2 was examined using the substrate 4-nitro-3-(octanoyloxy) benzoic acid. The PLA_2 specific activity of the purified protein (14.69 nmoles/min/mg) was higher than that of the whole venom (5.49 nmoles/min/mg) (Fig. 6A). Under the conditions used, PhTX-I PLA_2 showed a discrete sigmoidal behavior, mainly at low concentrations (Fig. 6B). V*max* and K*m* were calculated to be 11.76 nmoles/min/mg and 1.96 mM, respectively. Maximum enzyme activity occurred at 40 °C (Fig. 6C) and the pH optimum was pH 8.0 (Fig. 6D). The PhTX-I PLA_2 showed a strict dependence on calcium ions (10 mM) for full activity. The addition of Mg^{2+}, Mn^{2+}, Cd^{2+} and Zn^{2+} (10 mM) in the absence or presence of low Ca^{2+} concentration (1 mM) significantly decreases the enzyme activity (Fig. 6E).

3.4. Pharmacological activities of the P. hyoprora venom and PhTX-I PLA_2

The chick biventer cervicis nerve–muscle preparation was used as a convenient test for the pharmacological activity of *P. hyoprora* venom and PhTX-I PLA_2. Whole venom concentrations of 20 and 50 μg/mL as well as 0.35, 0.7, 1.4 and 3.5 μM of PhTX-I PLA_2 were tested on indirectly stimulated preparations. Both caused a slow, progressive decrease in the twitch responses to nerve stimulation (Fig. 7A–D). The time required by venom to cause 50% twitch tension blockade was 19.7 ± 3.8 min (50 μg/mL), 74.6 ± 5.9 min (20 μg/mL). The time required by PhTX-I PLA_2 to cause 50% blockade was about 15.8 ± 1.5, 22.5 ± 2.1, 33.8 ± 3.6 min at the concentration tested (3.5, 1.4, 0.7 μM respectively). Lower concentration (0.35 μM) was not as effective and did not reach 50% within 120 min. (Fig. 7B). Whole venom (20 and 50 μg/mL) and higher concentrations of PhTX-I PLA_2 (1.4 and 3.5 μM) decreased the contractures induced by acetylcholine and KCl after 120 min. In lower concentrations (0.35 and 0.7 μM), contractures to exogenous acetylcholine and KCl were still seen after PhTX-I PLA_2-induced blockade (Fig. 7E–F).

As shown in Fig. 8A and B, whole venom and PhTX-I PLA_2, respectively, induced a conspicuous local myotoxic effect, evidenced by dose-dependent elevation of plasma CK. The highest activity was found with 20 μg/mouse of PhTX-I PLA_2 (Fig. 8A) with activity similar to that seen for the whole venom (20 μg) (Fig. 8B). The time-course analysis evidenced rapid activity, which reached a maximum increase 3–6 h after i.m. injection, and returned to normal by 24 h.

Doses of 5 and 10 μg/150 μL of whole venom and PhTX-I PLA_2 show a slight cytolytic effect when were tested against C2C12 skeletal muscle myoblastos, (Fig. 8C). The toxin PhTX-I lysed myotubes at doses from 5 to 40 μg/well, since the whole venom induced the same effect at doses 10–40 μg/well (Fig. 8D).

The subplantar injection of either *P. hyoprora* venom (1–2 μg/paw) or PhTX-I (0.5–1 μg/paw) induced moderated paw oedema. The paw oedema induced by crude venom and PhTX-I had a similar time-course profile as characterized by a rapid onset (maximal responses obtained at 30 min post-injection) and returned to normal levels after 24 h (Fig. 8E). To further analyze and compare the mechanism of the inflammatory events induced by *P. hyoprora* venom and purified PhTX-I PLA_2 (Fig. 8F), the concentrations of IL-6 in the plasma were measured. Both, whole venom and PhTX-I PLA_2 (10 μg), caused a marked increased in IL-6 levels, reaching maximum increase 3 h after the injection when compared to the control. Six hours after the injection, there was a decrease of the IL-6 levels.

4. Discussion

PLA_2 are among the most abundant components of snake venoms, showing a wide range of activities even presenting conserved structures (Kini, 2003; Magro et al., 2004; Tsai et al., 2004). Understanding the structural basis for their diverse toxic activities, including neurotoxicity and myotoxicity, is still a challenging task. In this work, the first biochemical and toxinological characterization of the new PLA_2 from the venom of *P. hyoprora* here named PhTX-I, is reported.

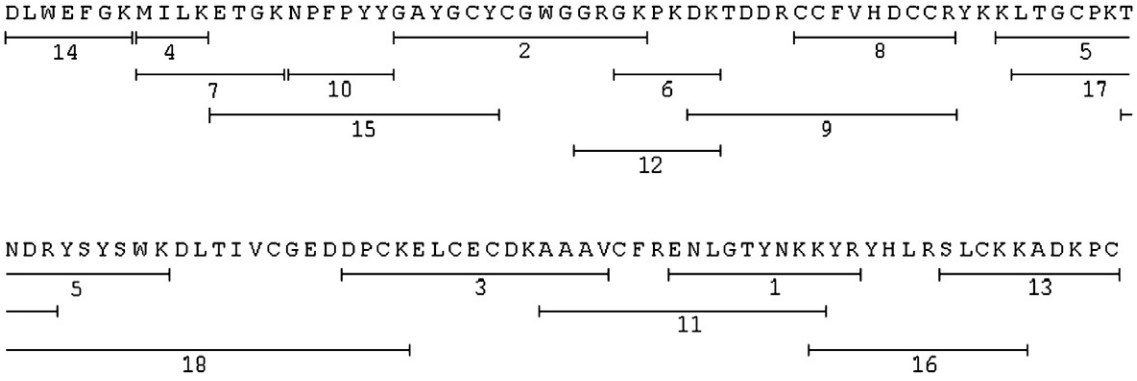

Fig. 3. Determination of the complete amino acid sequence of PhTX-I PLA_2, deduced by overlapping of the sequence of peptides obtained by tryptic digesting of the alkylated protein. The peptides were separated by RP-HPLC.

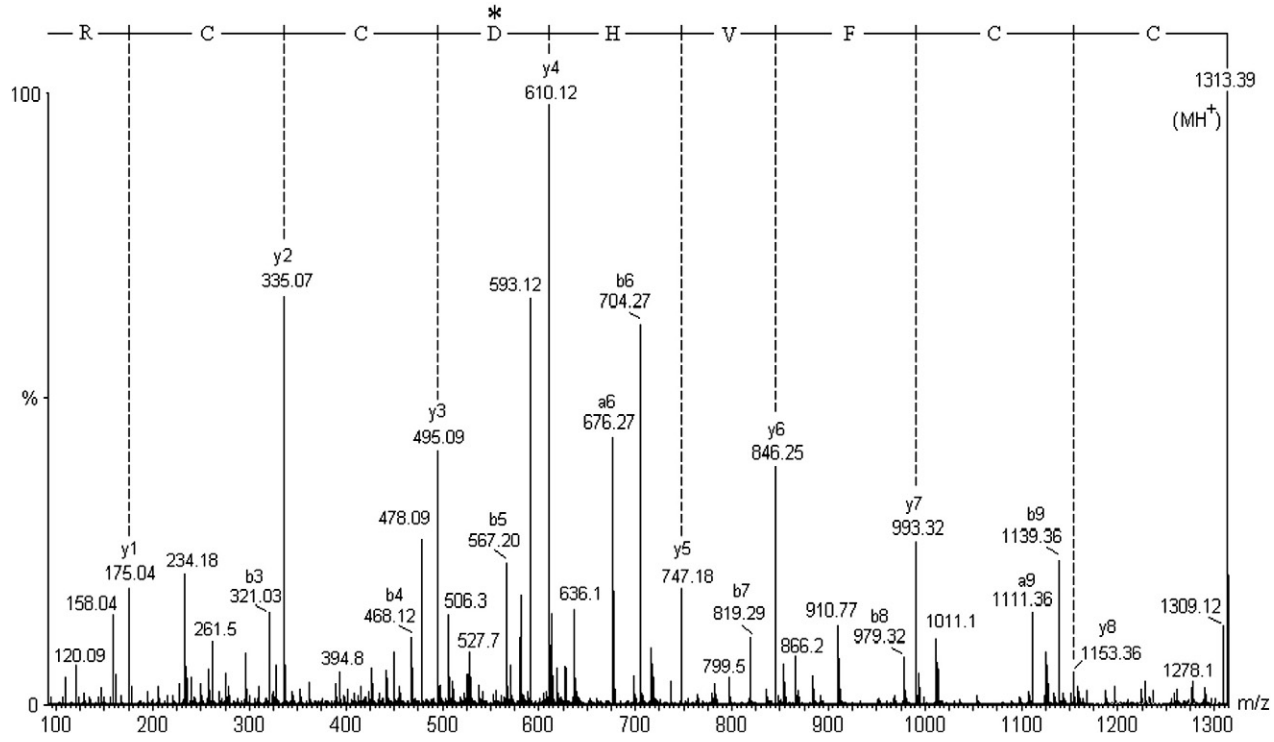

Fig. 4. ESI-QTOF-MS/MS spectrum of the tryptic peptide of 1312.38 Da. Series of *y* fragment ions of 9-residue-long tryptic peptide eluted in fraction 8 of the RP-HPLC of the alkylated PhTX-I PLA_2 containing the aspartic acid residue at position 49 in the amino acid sequence.

The work group has been optimizing and standardized the methodology of purification of PLA_2 of snake venom in a single step chromatographic in a column μ-Bondapack C-18 coupled to a system of reverse phase HPLC, as a result of the proposed method, several toxins have been efficiently purified (Calgarotto et al., 2008; Randazzo-Moura et al., 2008; Huancahuire-Vega et al., 2009; Ponce-Soto et al., 2010; Romero-Vargas et al., 2010). The purification procedure herein described was efficient for the obtainment of the new PhTX-I PLA_2 toxin from *P. hyoprora*

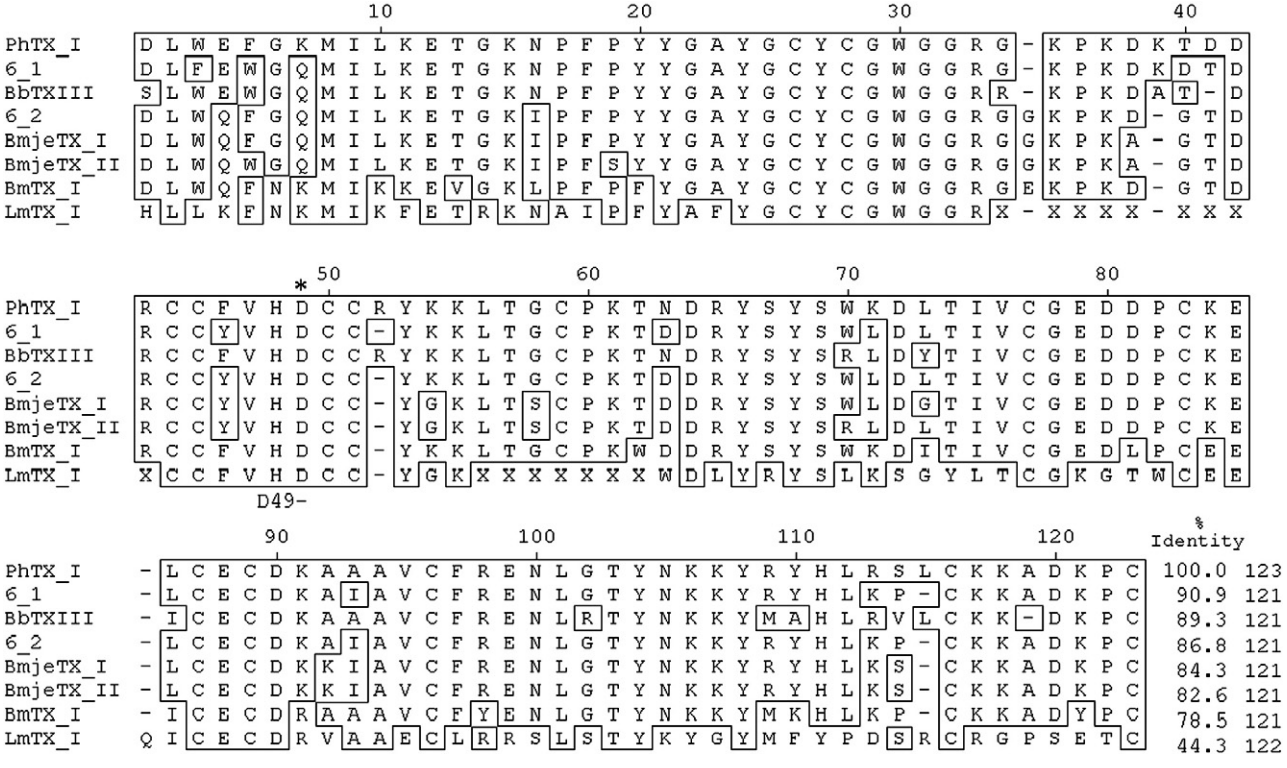

Fig. 5. Alignment of the deduced amino acid sequence of the PhTX-I PLA_2 with D49-PLA_2. Brazilitoxin III (BbTX-III) from *Bothrops brazili* (Huancahuire-Vega et al., 2009); 6_1 and 6_2 from *Bothrops jararacussu* (Ponce-Soto et al., 2006); BmTX-I from *Bothrops moojeni* (Calgarotto et al., 2008); BmjeTX-I and BmjeTX-II from *Bothrops marajoensis* (Ponce-Soto et al., 2010); LmTX-I from *Lachesis muta muta* (Damico et al., 2005).

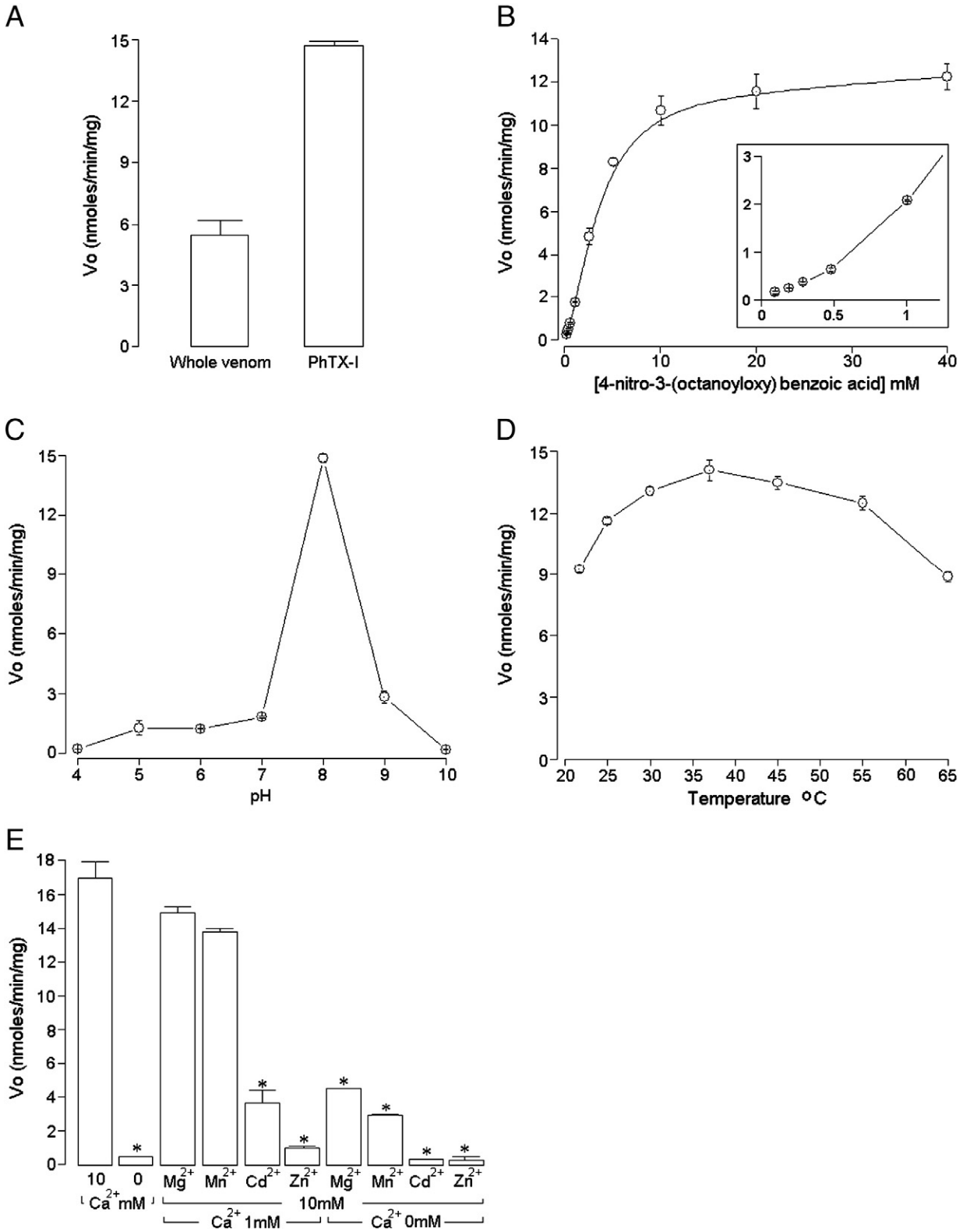

Fig. 6. (A) PLA_2 activity of *P. hyoprora* venom and PhTX-I PLA_2 (B) Effect of substrate concentration on the PLA_2 activity of PhTX-I. Insert: details in low substrate concentrations; (C) Effect of pH on the PLA_2 activity of PhTX-I; (D) Effect of temperature on the PLA_2 activity of PhTX-I; (E) Influence of ions (10 mM each) on PLA_2 activity of PhTX-I in the absence or presence of 1 mM Ca^{2+}. The results of all experiments are the mean ± SEM of three determinations ($p<0.05$).

snake venom since the protein was purified 1361 times with high yield, producing 8–15 mg of the toxin and high purity levels (Table 1).

A great number of PLA_2 isolated from the venoms of *Bothrops* sp are oligomers constituted by two or more subunits (Magro et al., 2009), nevertheless, Tricine SDS-PAGE showed (Fig. 1inset) that PhTX-I PLA_2, have one band with *Mr* ~15 kDa, being a protein constituted of a single polypeptidic chain. Brazilitoxin III of *Bothrops brazili* (Huancahuire-Vega et al., 2009), *bl*D-PLA_2 of *Bothrops leucurus* (Higuchi et al., 2007) and LmTX-I of *Lachesis muta muta* (Damico et al., 2005) are D49-PLA_2 that also have structure monomeric.

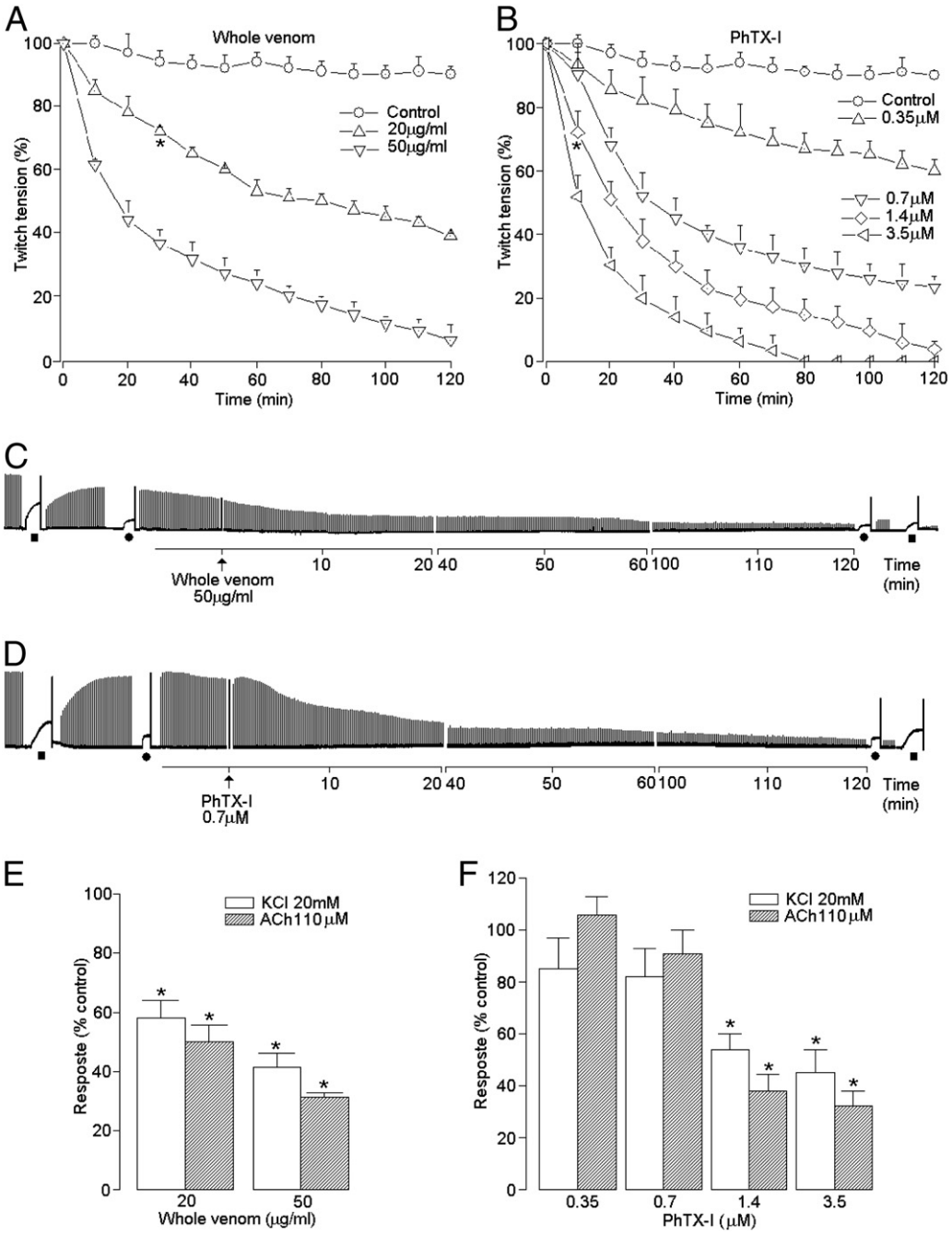

Fig. 7. Neuromuscular blockade of chick biventer cervicis preparations incubated with *P. hyoprora* venom and PhTX-I PLA_2 at 37 °C. The points are the mean ± SEM of six experiments. *$p<0.05$ compared to the twitch-tension before *P. hyoprora* venom (A) and PhTX-I PLA_2 (B) addition. Panels C and D shows a representative recording from a preparation treated with 50 μg of *P. hyoprora* venom and 0.7 μM of PhTX-I PLA_2, respectively. Tissue responses to KCl (■, 20 mM) and acetylcholine (ACh, ●, 110 μM) were obtained before and after toxins addition. Panels E and F shows the effect of *P. hyoprora* venom and PhTX-I PLA_2, respectively, on muscle contractures evoked by KCl and acetylcholine (ACh). Each point is the mean ± SEM of six experiments. *$p<0.05$ compared to the control (pre-toxin) contractures (100%).

The molecular masses obtained by mass spectrometry showed to be similar to that of other snake venom PLA_2 (Fig. 2) (Ponce-Soto et al., 2007, 2010; Perumal et al., 2008; Garcia et al., 2010). The amino acid composition of the PhTX-I PLA_2 toxin suggest the presence of 14 half-Cys residues, provided the basis for a common structural feature of PLA_2 in the formation of its seven disulfide bridges (Gutiérrez and Lomonte, 1995, 1997; Ponce-Soto et al., 2007; 2010) and a high content of basic and hydrophobic residues, that provides a clue point the interaction of the PLA_2 with negatively charged phospholipids of cells membranes (Murakami and Arni, 2003). Such interaction is important to explain the effect of these enzymes on different cells types, both prokaryotes and eukaryotes (Lomonte et al., 1999; Kini, 2003).

Comparison of the amino acid sequence of PhTX-I PLA_2 showed high homology with others myotoxin PLA_2 from the genus *Bothrops* (Fig. 5). One of the highly conserved regions in the amino acid sequences of PLA_2 is the Ca^{2+}-binding loop, segment from (Y)24 to (G)35, that contains four glycine residues at positions 26, 30, 32, and 33, and two cysteine residues at positions 27 and 29. The calcium ion is coordinated by three main chain oxygen atoms from residues (Y)28, (G)30, (G)32, and two carboxylate oxygen atoms of (D)49. Two generally conserved solvent water molecules complete the coordination sphere of the calcium ion forming a pentagonal bipyramidal geometry. It is believed that two disulfide bridges (C)27–(C)119 and (C)29–(C)45 ensure the correct relative orientation of the calcium

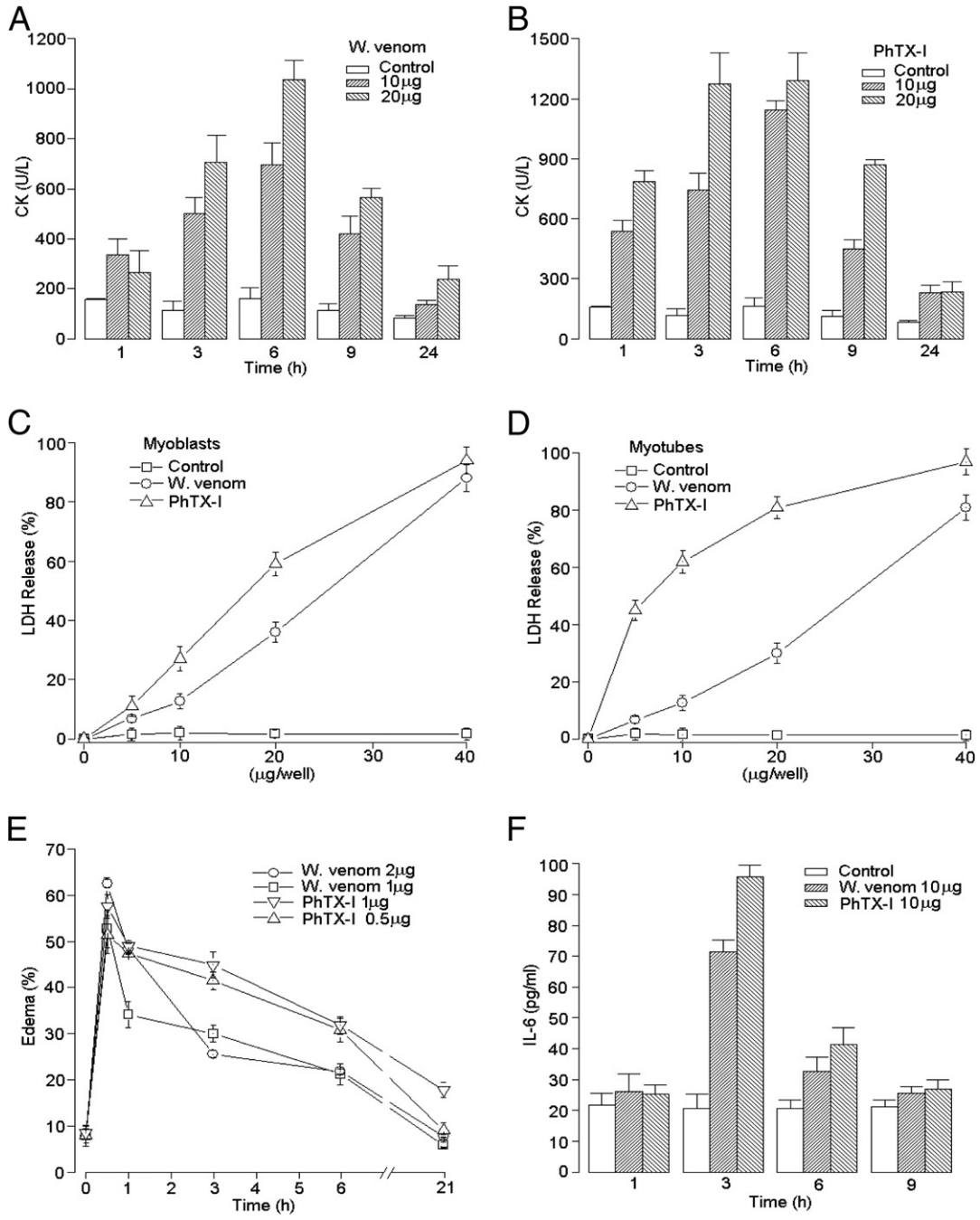

Fig. 8. (A) Time-course of the increments in plasma CK activity after intramuscular injection of *P. hyoprora* venom (10 and 20 μg) and (B) PhTX-I PLA_2 (10 and 20 μg). Controls were injected with 100 μl of PBS. At different times, blood was collected, and serum levels were measured. Values are means ± SEM of five mice at each time point. (C) In vitro cytotoxic activity of *P. hyoprora* venom and PhTX-I PLA_2 on murine C2C12 skeletal muscle myoblasts and (D) myotubes. Cell lysis was estimated by the release of lactic dehydrogenase (LDH) to supernatants, after 3 h of exposure to the toxin, in a volume of 150 μL/well. Each point represents mean ± SEM of triplicate cell cultures. (E) Edema-forming activity of *P. hyoprora* venom (1, 2 μg) and PhTX-I PLA_2 (0.5, 1 μg) in mice. Induction of edema by toxins, injected s.c. in the footpad of mice. At various time intervals the increase in footpad volume, as compared to controls, was expressed as percent edema. Each point represents the mean ± SEM of five animals. (F) Systemic interleukin-6 response induced by 10 μg of *P. hyoprora* venom and PhTX-I PLA_2 in mice. Plasma levels of IL-6 were determined at the indicated time points, by enzyme immunoassay. Each point represents ± SEM of five mice.

binding loop in relation to the amino acids of the catalytic network (Scott et al., 1990). The residues (H)48, (Y)52 and (D)99 which are responsible for catalytic activity have an ideal stereochemistry with the presence of the so called "catalytic network", a system of hydrogen bonds which involves the catalytic triad (Scott et al., 1990). Residues forming the Ca^{2+}-binding loop and the catalytic network of PhTX-I PLA_2 show a high conservation grade, reflecting the non-decreased catalytic activity.

In a similar way, the PhTX-I PLA_2 showed a complete overlap with other class II PLA_2 for the invariant hydrophobic side-chains of (L)2, (W/F)3, (I)9, (Y)22, (C)29, (C)45 and (A)93 which form the walls of the hydrophobic channel for the binding of the substrate in PLA_2. The side-chains of these residues are directed towards, and thus might interact with the phospholipid layer (Singh et al., 2001).

It is well know that the *N*-terminal region of PLA_2, which is high conservative in catalytically active PLA_2 enzymes, displays important roles for catalysis as well as for pharmacological activities (Qin et al., 2005). Several lines of evidence show that the amino acids (L)2, (Y)3, (Q)4, (K)6, (I)9 (E)12 and (T)13 of the *N*-terminal region functionally contributes to facilitate a productive-mode orientation of PLA_2 at the

membrane surface (Qin et al., 2004, 2005). The substitution of (Y)3 by (W)3 and (Q)4 by (E)4 in the PLA_2 PhTX-I suggest that these residue contribute to the conformational maintenance of hydrophobic pocket at the *N*-terminal region, since the modifications presented in this PLA_2 do not obstruct the catalytic function, permitting the lipidic substrate to continue to get access to PLA_2 catalytic site.

Breithaupt (1976) reported that venom PLA_2 shows classic Michaelis-Menten behavior against micellar substrates. However, the enzymatic activity of PhTX-I PLA_2, against non-micellar substrate, showed a discrete sigmoidal behavior, mainly at low concentrations (Fig. 6B), what is in agreement with the results obtained by Pereañez et al., 2009 for the PLA_2 Cdcum6 of *Crotalus durissus cumanensis* venom. Highest enzymatic activity occurs at pH 7–8 and 35–45 °C (Fig. 6C and D), like other PLA_2, PhTX-I is relatively resistant to heat (Kini, 1997; Damico et al., 2005; Calgarotto et al., 2008). Due to its ectothermic nature, the variation of the corporal temperature of the reptiles affects many physiologic processes and of development (Peterson et al., 1993). Molecules with stable structures that can maintain its functionality front to the variations of temperature of the environmental are necessary for the survival of the snakes.

PhTX-I PLA_2 showed that the Ca^{2+} ion is an obligatory co-factor for its enzymatic activity. Studies of its substitution by other divalent ions (Mg^{2+}, Mn^{2+}, Cd^{2+}, and Zn^{2+}) were not able to keep the substrate bound to the enzyme, only Ca^{2+} supports the catalytic activity (Fig. 6E). This can be explained by different coordination geometries assumed by the tetrahedral intermediate due to the presence of the Ca^{2+} ion which determine the electrophilic behavior of the catalytic site, as well as stabilizes the otherwise flexible Ca^{2+}-binding loop and appears to optimize the local protein conformation for substrate interactions (Scott et al., 1990; Yu et al., 1993). Other metals fail to productively bind to the enzyme, competitively inhibit Ca^{2+}-mediated activity, or are weakly active (Mezna et al., 1994). Interestingly Mg^{2+} and Mn^{2+} in presence of low Ca^{2+} concentrations support a significant catalytic activity.

Snakebite envenoming is a global public health problem, particularly in tropical and subtropical countries. To understand how venoms work requires quantitative data on the occurrence of individual toxins in given venom (Calvete et al., 2009). A profile of toxic activities was investigated for PhTX-I PLA_2, including ex vivo neurotoxicity and cytotoxicity, in vivo myotoxic, and edematogenic activities.

Although "bothropic envenomation" produces no neurotoxic clinical signs, venoms from several species can cause neuromuscular blockade ex vivo and produce signs of peripheral muscular weakness in chickens and mice (Oshima-Franco et al., 2004; Calgarotto et al., 2008; Randazo-Moura et al., 2008). *P. hyoprora* venom and PhTX-I PLA_2 caused concentration-dependent blockade of elicited twitch responses of the chick biventer cervicis muscle preparation at all concentrations (Fig. 7A–D). Whole venom (20 and 50 μg/mL) reduced response to ACh and KCl after incubation with the venom (Fig. 7E). Such inhibition could involve damage to nicotinic receptors by the proteas es present in this venom, as well as damage to muscle fibers (Prianti et al., 2003; Abreu et al., 2007). On the other hand, PhTX-I PLA_2 (0.35 and 0.7 μM) not interfere with the muscle contractures to exogenous ACh and KCl, indicating that the toxin had not direct action on nicotinic receptors and muscle fibers. However, using highest concentrations (1.4 and 3.5 μM) inhibition of the responses to ACh and KCl was observed (Fig. 7F). The fact that PhTX-I did not significantly affect the responses to ACh and KCl, except when high concentrations were used, suggests that PhTX-I PLA_2 presents a primordial presynaptic action. Similar results were obtained for BmjeTX-I and II (Ponce-Soto et al., 2010), BmTX-I (Calgarotto et al., 2008), LmTX-I (Damico et al., 2005).

P. hyoprora venom and PhTX-I PLA_2 increase the plasmatic CK levels after i.m. injection (Fig. 8A and B, respectively), revealing drastic local myotoxicity. This myotoxicity induced by snake venoms, including *P. hyoprora*, may result from the direct action of myotoxins on the plasma membranes of muscle cells, or indirectly, as consequence of vessel degenerations and ischemia caused by hemorrhagins or metaloproteases (Prianti et al., 2003; Abreu et al., 2007). PhTX-I PLA_2 contributes significantly to local myotoxic action in vivo. It was already demonstrated that the snake venom PLA_2 are the principal cause of local damage (Gutierrez and Ownby, 2003). Myotoxic PLA_2 affect directly the plasma membrane integrity of muscle cells, originating an influx of Ca^{2+} ions to the cytosol that starts several degenerative events with irreversible cell injures (Montecucco et al., 2008). The binding sites of myotoxins on the plasma membranes are not clearly established, although two types have been proposed: (a) negatively charged phospholipids (Diaz et al., 2001), present on membranes of several cell types, explaining the high in vitro cytotoxic action of these enzymes (Angulo and Lomonte, 2005), and (b) protein receptors, which make muscle cells more susceptible to myotoxin action (Lomonte et al., 1999).

Whole venom and PhTX-I PLA_2 did not show cytotoxic effect in skeletal muscle of myoblasts in a dosage of 5 to 10 μg/well. However an increased susceptibility of the myotubes was clearly observed using the PhTX-I PLA_2 toxin (Fig. 8C and D). This fact is probably due to the ability of this toxin in disturbing the membrane, and seems to involve the expression of a huge number of acceptors or a high acceptors site affinity for the PLA_2 plasmatic membrane. It is not possible to assert which would be the acceptors or which specific region/residue of PhTX-I PLA_2 would be involved or even which interactions would be involved in the process. However, the possible participation of intracellular processes arising after myoblast fusion, as seen in the enhanced damage to myotubes, cannot be excluded at this time (Angulo and Lomonte, 2005). High concentration of whole venom and PhTX-I PLA_2 (20, 30 and 40 μg/well) caused a significant cytotoxicity in myoblasts and myotubes, probably due to links with non-specified tubes.

Bothrops snake venoms induce local edema in humans and experimental animals (Kini, 1997). The acute inflammatory reaction is characterized by exudation of fluid and plasma proteins leading to a local edema formation. *P. hyoprora* venom and PhTX-I PLA_2 induced paw edema in mice increasing the microvascular permeability. This activity was time-dependent and it reached the maximum answer in the first 30 min (Fig. 8E). The edema formation induced by *P. hyoprora* venom was similar to that of other bothropic venoms, including *Bothrops asper* (Chaves et al., 1995, 1998; Teixeira et al., 2009), *Bothrops insularis* (Barbosa et al., 2003) and *Bothrops lanceolatus* (de Araujo et al., 2000).

PLA_2 are multifunctional proteins able to participate in inflammatory processes since they provide precursors for pro-inflammatory lipid substances such as arachidonic acid-derived mediators and PAF (Teixeira et al., 2003). Our results shown that PhTX-I PLA_2 induces increased edema formation in mouse paw in the same magnitude that the whole venom (Fig. 8E), suggesting that at least part of the inflammatory actions of crude venom is due to PhTX-I PLA_2. Other venom components, such as metalloproteinases or lectins may also contribute to the edematogenic properties of crude venom. Studies have been directed trying to understand the mechanisms involved in the inflammatory response induced by myotoxic PLA_2 from several snake venoms (Teixeira et al., 2003). However, the relationship between enzymatic activity and edema is contradictory (Vishwanath et al., 1987). It is assumed that myotoxic and edematogenic activities can be induced by different structural domains in these PLA_2, or that a partial overlapping of these domains occurs (Zuliani et al., 2005).

There was an increase of IL-6 levels after the injection PhTX-I PLA_2 and *P. hyoprora* venom (Fig. 8F). Myonecrosis might be the trigger for the systemic release of the proinflammatory cytokine IL-6 observed, in agreement with earlier findings with *B. asper* myotoxin II (Lomonte et al., 1993) and *Bothrops moojeni* BmTX-I (Calgarotto et al., 2008). IL-6 is a main signal multifunctional cytokine involved in the regulation of the immune response, hematopoiesis, and inflammation

(Akira et al., 1990) and elevations have been documented in clinical envenomations by *Bothrops* species (Avila-Aguero et al., 2001).

In conclusion, we report the first PLA_2 from *P. hyoprora* snake venom, the study show that PhTX-I, is a monomeric new D49-PLA_2 which displays the major toxic actions reported for this type of proteins, including local myotoxicity, neurotoxicity, cytotoxicity, pro-inflammatory activity and probably has a principal role in envenomings by this snake species. The elucidation of its complete primary structure, here reported, will be of value in future comparative studies attempting to identify the molecular determinants of toxic activities by other basic PLA_2 in viperid venoms.

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