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Research Article

Toxic Effects of Two New Phospholipases A_2 Isolated from *Bothrops Pauloensis* Snake Venom - ∂

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ABSTRACT

Bothropic venoms have several substances necessary to serpents' survival, among them Phospholipase A_2 (PLA_2) enzymes. The hydrolysis of sn-2 from membrane phospholipids and the release of lysophospholipids and fatty acids induces in the substrate a wide variety of pharmacological effects. Bp-12 (13,789.56 Da molecular mass) and Bp-13 (14,035.62 Da molecular mass) toxins isolated by chromatographic system in reverse phase HPLC on µ-Bondapack C18 column from *Bothrops pauloensis* snake venom belong to PLA_2 family (Lys-49 and Asp-49, respectively). Electrophysiological studies in mouse Phrenic Nerve-Diaphragm Preparation (PND) showed a progressive sarcolemma depolarization, reaching values from -83.9 ± 1 mV (control) until -20.2 ± 1 mV (Bp-12) and -45.0 ± 1.5 mV (Bp-13) after 90 min (n = 3-6; $P < 0.05$). Histological analysis of mice PND and *in vivo*, rat paw edema formation and mice serum Creatine Kinase (CK) release were approaches to study the toxins' toxicity. The PLA_2s Bp-12 and Bp-13 (50 µg/mL) caused ~ 30% of damage (27.3 ± 1.1% and 30.6 ± 5% of respectively ($p < 0.05$); the pathological states of fibers included fiber edema, hypercontration of sarcomeres and myofibrils clumping and condensation. After 60 minutes the CK levels were 827 ± 92.4 U/L for Bp-12 and 1440 ± 129 U/L for Bp-13 (n = 5-6) compared to control (71.4 ± 14.1 U/L, $p < 0.05$). The induction of edema of paw was toxins concentration-dependent for Bp-12 and Bp-13, respectively [2.5 µg/paw (0.44 ± 0.02 mL and 0.45 ± 0.07 mL), 5 µg/paw (0.59 ± 0.03 and 0.47 ± 0.04 mL) and 10 µg/paw (0.67 ± 0.07 and 0.45 ± 0.06 mL)] . The results lead us to conclude that Bp-12 and Bp-13 are myotoxins for mammalian and might contribute to the pharmacological and pathological effects of the crude venom.

Keywords: *Bothrops Pauloensis*; Lys 49; Asp 49; Neurotoxicity; Myotoxicity; Edematogenic Effect; Membrane Resting Potential

INTRODUCTION

Snake venom Phospholipases A_2 (PLA_2s) constitute an important group of molecules that display several pharmacological activities [1-4]. They are included in two broad categories, D49 and K49: D49 with an aspartate amino acid at position 49 and, consequently, calcium-dependent catalytic activity, and K49, with a lysine amino acid at position 49 and, consequently, catalytically inactive.

Envenomation caused by *Bothrops* snakes bites is often characterized by a complex series of local pathophysiological and systemic hemodynamic and hemostatic disturbances [5,6]. Some of their isolated components were shown to affect the dynamics of neuromuscular junction physiology *in vitro* [7-14].

In this work, we unravel some of the effects of two myotoxins isolated from *Bothrops pauloensis* snake venom which had been characterized biologically as a Lys49 PLA_2 (Bp-12) and Asp49 PLA_2 (Bp-13). The purpose was to assess the relative contribution of each toxin for the *in vitro* toxicity for the whole venom.

MATERIALS AND METHODS

Animals

Male Swiss mice (18-25g) and male Wistar rats (160-180g) were obtained from the Multidisciplinary Center for Biological Investigation at the State University of Campinas (CEMIB-UNICAMP). The animals were housed in a temperature-controlled room (25 ± 3°C) with a 12 hour light/dark cycle and access to water and food *ad libitum*. The experimental protocols were approved by the institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, Protocols 939-2 and 1266-1) and were done in accordance with the ethical guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL).

Toxins

Bp-12 and Bp-13 toxins were isolated from *Bothrops pauloensis* snake venom (*Batatais Serpentarium*, São Paulo State, Brazil) as described by [12] and [15]. All chemicals and reagents used in this research were of analytical or sequencing grade.

Intracellular recordings

Male Swiss mice were sacrificed by halothane inhalation and the hemidiaphragm along with its phrenic-nerve was removed and mounted as previously described by Bulbring for rats [16].

Membrane Resting Potential (RP) of phrenic nerve-diaphragm preparations was measured through a high input impedance electrometer (World Precision 750, Sarasota, FL, USA) by using conventional microelectrode techniques [17]. The dissected muscle was mounted in an aerated chamber (95% O_2 - 5 % CO_2) with Tyrode solution (composition in mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1; *p*H 7.4, 25-28°C), with or without Bp-12 and Bp-13 (50 µg/mL) addition. The glass microelectrode filled with 3 M KCl (resistance 10 – 20 MΩ) was inserted into a muscle fiber at an endplate region. The membrane RP was recorded on an oscilloscope (Tektronix, Beaverton, OR, USA). The RP recordings were taken at the end-plate regions in the absence (control) or presence of toxins.

Myonecrosis evaluation: morphological and biochemical assays

Myotoxicity was assessed in hemidiaphragm muscle from mouse Phrenic Nerve-Diaphragm preparations (PND), after 120 min incubation in Tyrode solution (control) and Bp-12 or Bp-13 (50 µg/mL) toxins. Following the incubation period, the muscles were washed and then transferred to 4% paraformaldehyde fixative for 24 h, washed three times in saline solution, dehydrated in ethanol series (70%, 80%, 90% and 100% x 3; 30 min each bath), embedded and included in historesin (Leica Nublock/Heidelberg, Germany). Sections (2 µm thick) were obtained using a Leica RM 2035 microtome and stained with Toluidine Blue (TB) for light microscopy (Olympus BX51 light microscope, Japan) analysis. Muscle images were captured using a digital imaging system (Image Pro Plus 6.0, Mídia Cybernetics Inc., USA).

The percentage of damaged fibers was counted in three non-overlapping, non-adjacent sections per animal in a total of 15 sections per toxin- and Tyrode-incubated preparations (n = 5 mice/Bp-12/Bp-13/Tyrode). Percentage of muscle damage was assessed by multiplying the number of damaged fibers by 100 and dividing it by the total number of fibers present in the whole sectional muscle area. Damaged fibers were qualified according to the pathologic state and normal fibers were those with polygonal morphology and normal myofibrils distribution/organization.

Myonecrosis was also assayed by measuring the CK serum activity in mice after time-points of gastrocnemius Intra-Muscular (i.m) injection of Phosphate Buffered Saline (PBS) (control - 50 µL), Bp-12 and Bp-13 (50 µg/50 µL) toxins. After 30 minutes, 1, 3, 6, 9 and 12 hours of i.m. injection, the mice were deeply anesthetized and blood samples were collected from the caudal vein into heparinized

capillary tubes. Immediately after sampling, the blood was centrifuged (4°C, 3000 rpm/10 min) for serum separation and colorimetric determination of CK activity using CK-NAC kit (Bioclin/Quibasa, Belo Horizonte, MG, Brazil). The serum CK activity was expressed as international units/liter (U/L), with 1 unit of activity causing the phosphorylation of 1 nmol of creatine (substrate) per minute, at 37°C.

Measurements of rat paw edema

Male Wistar rats (120-180 g) were used for measuring the hind paw edema induced by a single sub-plantar injection (0.1 mL) of the Bp-12 or Bp-13 [dissolved in 0.9 % (w/v) sterile saline]. The paw volume was measured 15, 30, 60, 120 and 180 minutes after injection of dose range of toxins (2.5, 5 and 10 µg/paw) using a hydroplethysmometer (model 7150, Ugo Basile, Varese, Italy). The results were expressed as the increase in paw volume (mL) calculated by subtracting the basal volume measured in the contralateral paw. In some cases, the area under the time-course curve (AUC, mL min) was calculated using the trapezoidal rule, which works by approximating the region under the graph of the function f(x) as a trapezoid and calculate its area.

Statistical analysis

Results were reported as mean ± S.E.M. and the statistical comparison were obtained using one-way ANOVA followed by the Tukey and Kruskal-Wallis *post hoc* test. The Dunn's multiple test was also performed and several experimental groups were compared to the control group ($p < 0.05$ indicated statistical significance).

RESULTS

Electrophysiology

The measurement of the membrane Resting Potential (RP) of PND incubated with Lys49 PLA_2 Bp-12 or Asp49 PLA_2 Bp-13 (50 µg/mL) showed a progressive sarcolemma depolarization, reaching values of -20 ± 1 mV (Bp-12) and -45 ± 1.5 mV (Bp-13) after 90 minutes (n = 3-6) which differed significantly from the obtained for control -84 ± 1 mV at the same condition ($p < 0.05$, Figure 1).

Myonecrosis evaluation- Histology

Figure 2 shows cross-section of mouse hemidiaphragm muscle fibers after 120 min of the incubation with Tyrode solution (control) or PLA_2 Bp-12 or PLA_2 Bp-13 toxins. Control muscle fibers displayed a normal polygonal cross profile shape, even distribution of myofibrils and intact sarcolemma; no damaged fiber was observed (Figure 2A). Preparations incubated with (50 µg/mL) of Lys49 PLA_2 Bp-12 or Asp49 PLA_2 Bp-13 showed 27.3 ± 1.1% and 30.6 ± 5.1% of fibers with different pathological states, respectively (Figures 2B and 2C, respectively; $p < 0.05$ compared to control – 1.1 ± 0.2). Figure 2B and 2C illustrates altered fibers whose pathological states include rounded polygonal profile typical of osmotic disturbance and sarcolemma impairment, sarcoplasmic area devoid of myofibrils intermingled with fibrils densely clumped and hypercontracted.

Myonecrosis evaluation – Creatine kinase

Creatine Kinase (CK) release was evaluated to substantiate the myonecrosis observed in histological section. Figure 3 shows that after 60 minutes of incubation the CK serum levels were 71.4 ± 14 U/L for control mice gastrocnemius injected with sterile saline solution (0.9%) and 827 ± 92.4 U/L for Lys49 PLA_2 Bp-12 and 1440 ± 129 U/L for Asp49 PLA_2 Bp-13 (n = 5-6; $p < 0.05$).

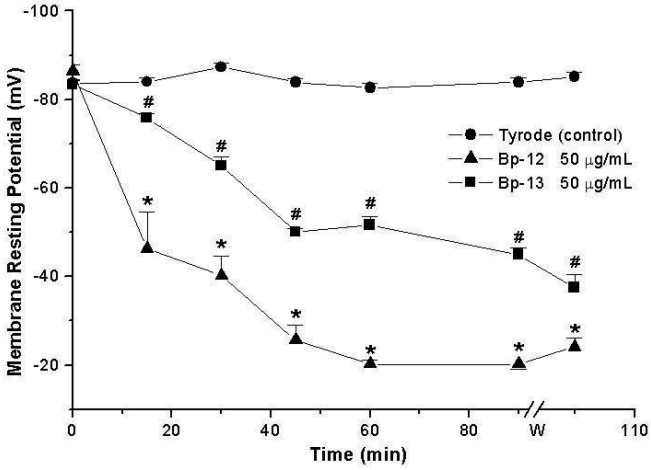

Figure 1: Membrane Resting Potential (RP) of mice hemidiaphragm muscle preparations (PND) after 90 minutes of Tyrode (control), Bp-12 and Bp-13 (50 µg/mL, respectively) toxins incubation. Note the progressive sarcolemma depolarization caused by Bp-12 (-20.2 ± 1 mV) and Bp-13 (-45.0 ± 1.5 mV). SEM of 3-6 experiments. * and # $p < 0.05$ compared to control (-83.9 ± 1 mV). W = washes.

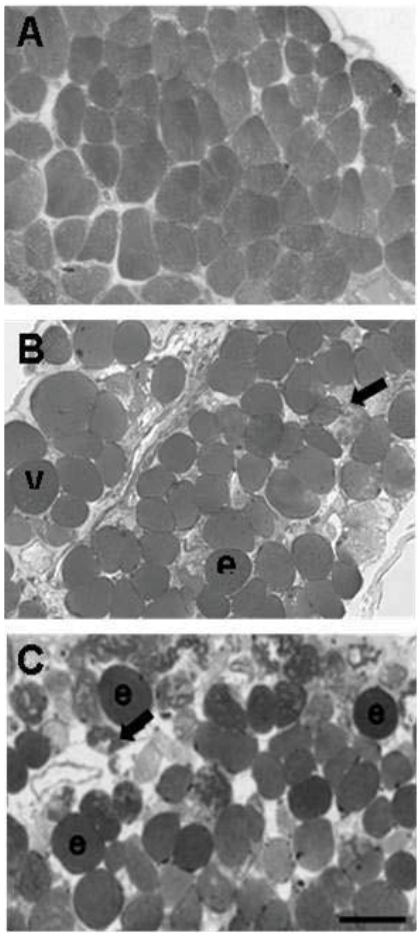

Figure 2: Light micrographs showing transverse section of diaphragm muscle after 120 minutes of Tyrode (A) (control), Bp-12 (B) and Bp-13 (C) (50 µg/mL, respectively) toxins incubation. (A) Note the normal polygonal fibers aspect and intact sarcolemma. (B) Bp-12 and (C) Bp-13 toxins presented 27.3 ± 1.1% and 30.6 ± 5.1% of muscle damage, respectively ($p<0.05$ compared to control – 1.1 ± 0.2%). Observe the hypercontracted (arrow), vacuolated (v) and edematous fibers (e). Bar = 50 µm.

Paw edema formation

Paw edema induction was observed after 30 minutes of 2.5 µg/paw (0.44 ± 0.02 mL), 5 µg/paw (0.59 ± 0.03 mL) and 10 µg/paw (0.67 ± 0.07 mL) for Lys49 PLA_2 Bp-12 injection (n = 5) (Figure 4A). However, for Asp49 PLA_2 Bp-13 the paw edema was observed after 15 minutes, showing 0.45 ± 0.07 mL (2.5 µg/paw), 0.47 ± 0.01 mL (5 µg/paw) and 0.45 ± 0.06 mL (10 µg/paw) of edema (n = 5) (Figure 4B).

DISCUSSION

Classical manifestation of Bothropic envenomation is local and systemic effects, the magnitude of which could cause local and systemic myolysis, hemodynamic disturbances and renal dysfunction [18]. Although envenoming by *Bothrops* produces no neurotoxic clinical signs, venoms from several species can cause neuromuscular blockade *in vitro* and produce signs of peripheral muscular weakness in chickens and mice. Up to the present time, only *B. jararacussu* and *B. pauloensis* species were satisfactorily investigated regarding this activity, including the isolation of neurotoxic and myotoxic components [7,14,19,20,21]. These authors showed that the presynaptic effects from *B. pauloensis* venom were mainly due to the presence of phospholipases as one of its components.

PLA_2s are among the most abundant components of snake venoms, showing a wide range of activity even presenting conserved structures [22]. Understanding the structural basis for their diverse toxic activities, including neurotoxicity and myotoxicity, is still a challenging task. In this work, two new toxins, Bp-12 (Lys49) and Bp-13 (Asp49) from *B. pauloensis* snake venom were pharmacologically characterized. These toxins induced neuromuscular blockade in mouse phrenic nerve-diaphragm preparations [12,15]. The same was observed for Lys 49 myotoxin II (BnSP-7), another PLA_2 also isolated from *B. pauloensis* venom, which displayed neurotoxic activity upon chicken biventer cervicis preparations [14].

Suggested that neuwieditoxin-I and II, another Asp49 PLA_2s isolated from *B. pauloensis* venom, were probably responsible for the presynaptic neurotoxicity *in vitro*. As reported in the present research, Bp-13 can also participate of the neurotoxic venom effect [7].

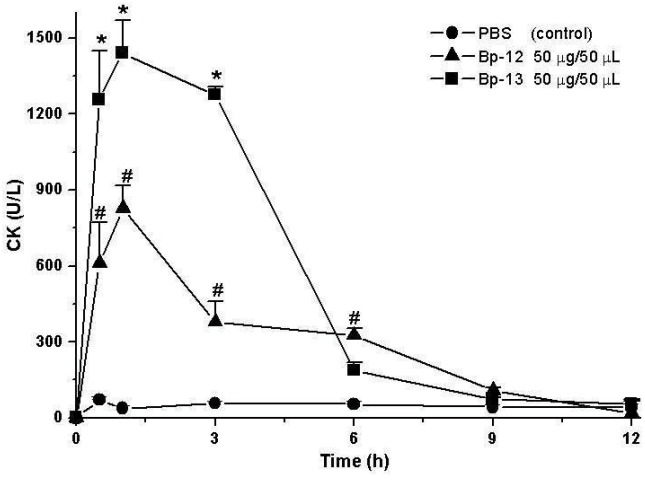

Figure 3: Creatine Kinase (CK) serum levels after 60 minutes of PBS (control), Bp-12 and Bp-13 (50 µg/50 µL, respectively) toxins injection into the mice gastrocnemius muscles. Observe the CK increase caused by Bp-12 (827.0 ± 92.4 U/L) and Bp-13 (1440.0 ± 129.0 U/L). SEM of 5-6 experiments. *and # $p < 0.05$ compared to control (71.4 ± 14.1 U/L).

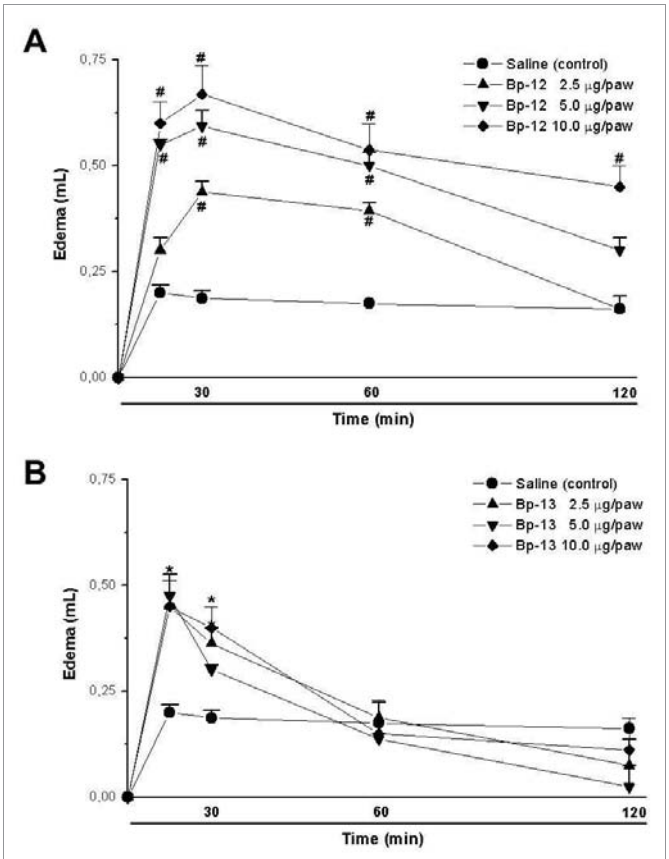

Figure 4: Effect of saline (control), Bp-12 (A) and Bp-13 (B) toxins subplantar injection into the rat paw. The edema was expressed as increase of paw volume (mL). Note that both toxins caused paw edema when compared to control values. However, the Bp-12 edema formation [2.5 µg/paw (0.44 ± 0.02 mL), 5 µg/paw (0.59 ± 0.03 mL) and 10 µg/paw (0.67 ± 0.07 mL)] (A) was observed 15 minutes later than Bp-13 injection [0.45 ± 0.07 mL (2.5 µg/paw), 0.47 ± 0.01 mL (5 µg/paw) and 0.45 ± 0.06 mL (10 µg/paw)] (B). SEM of 5 experiments. *and # $p < 0.05$ relative to saline control.

The majority of PLA_2 bothropic myotoxins, as Lys49 PLA_2 Bp-12 and Asp49 PLA_2 Bp-13, affect the membrane resting potential, in contrast to BthTX-I from *B. jararacussu* and BaTx from *B. alternatus* which at low concentrations do not change this parameter [11,23].

The morphological analysis combined with CK release was performed for a more consistent assessment of myotoxicity. Histological analysis after Bp-12 and Bp-13 diaphragm muscle exposure showed signified muscle damage, displaying hypercontracted, edematous and vacuolated fibers, which include both toxins into the myotoxic phospholipase group. Such results are in accordance to those observed for BthTX-I [11,24] and BnpTX-I and –II, Bp-PLA_2 [4,25].

In the present research, CK data confirmed the histological results, as also reported for other bothropic toxins [4,10,23-26]. In addition to morphological analysis and determination of CK serum, both Bp-12 and Bp-13 induced footpad edema as reported to BbTX-I and –II from *B. brazili* [10], BmjeTX-I and –II from *B. marajoensis* [26], as well as 6-1, 6-2 and Bj-VII from *B. jararacussu* [27]. The mechanism of edema formation by PLA_2 may be explained by phospholipid hydrolysis, probably due to liberation of precursors of a variety of eicosanoids and platelet activator factors [28].

The results presented for Bp-12 and Bp-13 did not show a marked difference between both toxins, even considering the fact that

they are Lys-49 and Asp-49, respectively, inferring that they act by different mechanisms exerted by molecular regions distinct from the enzymatic sites.

CONCLUSION

Taken together, these results characterized biologically Lys49 PLA_2 Bp-12 and Asp49 PLA_2 Bp-13 as classical myotoxins considering membrane resting depolarization, morphological and biochemical damage, and proinflammatory effects.

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