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Original Paper

Pharmacological and partial biochemical characterization of Bmaj-9 isolated from *Bothrops marajoensis* snake venom

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Abstract: Bmaj-9, a basic PLA_2 (13679.33 Da), was isolated from *Bothrops marajoensis* snake venom through only one chromatographic step in reversed phase HPLC on µ-Bondapak C-18 column. The amino acid composition showed that Bmaj-9 had a high content of Lys, His, and Arg, typical of a basic PLA_2. The sequence of Bmaj-9 contains 124 amino acid residues with a *pI* value of 8.55, such as DLWQWGQMIL KETGKLPFSY YTAYGCYCGW GGRGGKPKAD TDRCCFVHDC, revealing a high homology with Asp49 PLA_2 from other snake venoms. It also exhibited a pronounced phospholipase A_2 activity when compared with crude venom. In chick biventer cervicis preparations, the time for 50% and 100% neuromuscular paralysis was respectively (in minutes): 110 ± 10 (1 µg/mL); 40 ± 6 and 90 ± 2 (5 µg/mL); 30 ± 3 and 70 ± 5 (10 µg/mL); 42 ± 1 and 60 ± 2 (20 µg/mL), with no effect on the contractures elicited by either exogenous ACh (110 µM) or KCl (20 mM). Bmaj-9 (10 µg/mL) neither interfered with the muscular response to direct electrical stimulation in curarized preparations nor significantly altered the release of CK at 0, 15, 30 and 60 minutes incubations (27.4 ± 5, 74.2 ± 8, 161.0 ± 21 and 353.0 ± 47, respectively). The histological analysis showed that, even causing blockade at the maximum dosage (5 µg/mL), the toxin does not induce significant morphological alterations such as necrosis or infiltration of inflammatory cells. These results identified Bmaj-9 as a new member of the basic Asp49 PLA_2 family able to interact with the motor nerve terminal membrane, thereby inducing a presynaptic neuromuscular blockade.

Key words: *Bothrops marajoensis*, snake venom, Asp49 PLA_2, neuromuscular blockade.

INTRODUCTION

The epidemiology of snakebites in Brazil has shown that the genus *Bothrops* accounts for the majority of accidents (1). The high incidence of snakebites has been attributed to the great number of species (over 37), their wide geographic distribution and aggressiveness, as well as a highly efficient venomous apparatus. Studies dealing with the characterization of the biochemical composition and pharmacological actions of *Bothrops* venoms are important from the medical point of view and as a way to understand the physiopathology of envenomations.

Undoubtedly, the study of venoms and toxins has been greatly facilitated by the development of high performance liquid chromatography (HPLC), automated Edman microsequencing, mass spectrometry and other high-end techniques (2).Phospholipase A_2 (PLA_2) is one of the most extensively studied family of snake venom proteins (3). They mostly exist as monomers. However, several of them interact with other PLA_2 (or PLA_2-like molecules) or with other proteins to form complexes either through covalent or noncovalent interactions. Functionally, these PLA_2 complexes exhibit presynaptic neurotoxicity being responsible for the major activity of

bothropic venoms, assembling references on the action and molecular structure of such toxins among the most cited in the scientific literature on animal toxinology in recent years (4, 5).

Combined with the accompanying variety of biophysical properties, the complexity of this venom makes the search and characterization of individual venom components a daunting challenge.

In vitro studies have shown that some *Bothrops* venoms and their major toxins are typically myotoxic in rodents; however, neurotoxic effects have been also revealed in mouse and chick. The neurotoxicity was observed by the blockade of neuromuscular transmission, either presynaptically, in a dose range similar to that of elapid venoms, or postsynaptically (6-14).

Both the myotoxic and neurotoxic effects of *Bothrops* venoms have been associated with their high PLA_2 content. Secreted PLA_2s (PLA_2, EC 3.1.1.4) can be small basic Ca^{2+}-dependent proteins (14 to 18 kDa) usually containing 5 to 8 disulfide bonds and possessing a His/Asp pair required for catalysis (2). In addition to their possible role in prey digestion, snake venom PLA_2s exhibit a wide spectrum of pharmacological effects by interfering with normal physiological processes. PLA_2 effects include neurotoxic, myotoxic, anticoagulant and antiplatelet activities.

Bothrops marajoensis, an endemic snake from Marajó Island and Amazon River delta, Brazil, has been scarcely studied regarding the effects of its crude venom or its PLA_2 pharmacological action on neuromuscular junctions (15-17). The aim of this study is to analyze the Bmaj-9 action on the neuromuscular junction and its pharmacological and biochemical aspects.

MATERIAL AND METHODS

Venom and Reagents

Bothrops marajoensis snake venom was donated (lyophilized) from Batatais Serpentarium, Batatais, state of São Paulo, Brazil. All chemicals and reagents used in this research were of analytical or sequencing grade.

Animals

Male Hy-Line W36 chicks (4-8 days old) were obtained from the Globo Aves Agrovicola Ltd. (Brazil). The animals were kept at room temperature (25 ± 3°C) in a 12-hour light/dark cycle, with free access to food and water. All experiments were approved by the Institutional Committee for Ethics in Animal Use (CEUA/IB/Unicamp, protocol number 1027-1) and were in accordance with the guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA).

Isolation and Purification by Reversed Phase HPLC (RP-HPLC)

Ten milligrams of the whole venom of *B. marajoensis* were dissolved in 250 µL of 0.1% (v/v) trifluoroacetic acid (TFA, solvent A) and the resulting solution was clarified by centrifugation prior to loading the supernatant onto a µ-Bondapak C-18 column (0.78 cm x 30 cm; Waters 991-PDA system, USA). Proteins were eluted using a linear gradient (0-100% v/v) of acetonitrile in 0.1% (v/v) TFA (solvent B), at a flow rate of 1 mL/minute. The elution profile was monitored at 280 nm and fractions were manually collected, lyophilized and stored at –20°C. The purified protein was referred to as Bmaj-9.

SDS-PAGE

The fractions obtained by RP-HPLC were individually submitted to tricine SDS-PAGE in a discontinuous gel and buffer system to estimate the molecular mass of the Bmaj-9 PLA_2 (18). The samples were boiled for ten minutes in a sample buffer containing 2.5% (m/v) SDS before electrophoresis. After the run, the gels were stained with Coomassie brilliant blue G [0.2% (m/v)] in methanol-acetic acid-water (4:1:6, v/v) and maintained in the same solution without dye. The molecular mass markers used were (in kDa): phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14 kDa) (Bio Agency, UK). For protein determination, the micro-biuret method by Itzhaki and Gill (19) was employed.

MALDI-TOF Mass Spectrometric Analysis

The molecular mass of the Bmaj-9 fraction was determined by a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, USA). Thus, 1 µL of Bmaj-9 in 0.1% TFA was mixed with 2 µL of matrix sinapinic acid (3.5-dimethoxy-4-hydroxycinnamic acid) prepared using 30% acetonitrile and 0.1% TFA. The equipment conditions were as follows: 25 kV acceleration

voltage, laser fixed in 2.890 μJ/com², delay of 300 ns and linear analysis mode (20).

Amino Acid Sequence

The amino acid sequence was conducted using a Pico-Tag amino acid analyzer (Waters Systems, USA) (21). The purified Bmaj-9 was hydrolyzed at 105°C for 24 hours in 6.0 M HCl (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolysates were reacted with 20 μL of derivatization solution (ethanol:triethylamine:water;phenylisothiocyanate, 7:1:1:1, v/v) for one hour, at room temperature. PTC-amino acids were identified and quantified by HPLC, comparing their retention times and peak areas with those from a standard amino acid mixture.

Phospholipase A_2 Activity

PLA_2 activity was determined at pH 8.0, which was adapted for 96-well plates in the present experiment (22-24). The standard assay mixture used contained 200 μL of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl), 20 μL of substrate (4-nitro-3-octanoyloxy-benzoic acid) and 20 μL of water. To measure PLA_2 activity, 20 μL of Bmaj-9 was added to the mixture to achieve a final volume of 260 μL. After adding Bmaj-9, the final sample was incubated at 37°C for 40 minutes, and the absorbance at 425 nm was recorded at ten-minute intervals using a SpectraMax 340 multiwell plate reader (Molecular Devices, USA). Enzyme activity, expressed as velocity of reaction (V), was calculated based on the increase in absorbance after 20 minutes. The assays were performed in triplicate.

Chick Biventer Cervicis Muscle Preparation (BC)

Male chicks (n = 6 for each Bmaj-9 concentration) were killed with halothane, and their biventer cervicis muscles were removed and mounted under a constant tension of 1 g in a 5 mL organ-bath containing warmed (37°C), aerated (95% O_2 + 5% CO_2) Krebs solution [(mM, pH 7.5): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.8, KH_2PO_4 1.17, $MgSO_4.7H_2O$ 1.17, glucose 11.65 and $NaHCO_3$ 25] (25). A bipolar platinum ring electrode was placed around the muscle tendon, within which runs the motor nerve trunk supplying the muscle. Field stimulation was done using a Grass S48 stimulator (Grass Instrument Co., USA) with 0.1 Hz, 0.2 ms, 4-6 V. Muscle contractions and contractures were recorded isometrically via a force-displacement transducer (Load Cell BG – 10GM, USA) coupled to a physiograph (Gould model RS 3400, USA).

The muscle responsiveness to exogenously applied acetylcholine (ACh, 110 μM for 60 s) and KCl (20 mM for 180 s) was recorded in the absence of field stimulation both prior to addition of toxin (Bmaj-9) and at the end of the experiment. The BC preparation was allowed stabilizing for at least 20 minutes before addition of ACh or KCl. A range of Bmaj-9 concentrations (1, 5, 10 and 20 μg/mL) was added to the incubation bath and compared with control BC preparations incubated with Krebs solution. Experiments using 10 μg/mL of Bmaj-9 were done in curarized preparations (incubation with *d*-tubocurarine, *d*-Tc, 5 μg/mL; 0.1 Hz, 2 ms, 8-20 V).

Creatine Kinase Activity (CK)

Aliquots of organ bath solution (50 μL) were collected at time 0 and after 15, 30, 60, 90 and 120 minutes of incubation of BC with toxin (10 μg/mL, n = 6 per time period). The samples were stored at 4°C and CK activity was assayed within 4 hours after the experiment, using a commercial kit (CK-NAK, Bioclin, Quibasa, Química Básica Ltd., Brazil, batch 0107). For each aliquot collected, the corresponding 50 μL were replaced with fresh Krebs solution. CK activity was also assayed in control preparations without Bmaj-9. Enzyme activity was expressed in international units per liter (IU/L), with one unit of activity corresponding to phosphorylation of 1 μmol of creatine/min at 25°C.

Histopathological and Morphometric Analysis

The Bmaj-9 (1 and 10 μg/mL) myotoxic activity was assessed in biventer cervicis muscles, after 120 minutes of incubation, and compared with muscles incubated in Krebs solution for the same period. After, the muscles were maintained in Bouin's fixative for 24 hours, washed three times in aqueous ammonia solution, dehydrated in increasing ethanol concentrations (70%, 90% and 3 x 100%) and embedded in historesin (Leica Nublock, Germany). Sections (2-μm thick) were obtained using a Leica RM 2035 microtome (Germany) and stained with toluidine blue for light microscopy (Olympus BX51 light microscope, Japan) analysis. Muscle images were

captured using a digital imaging system (Image Pro Plus 6.0, Media Cybernetics Inc., USA). Three non-overlapping and non-adjacent sections were taken from each muscle. The percentage of Bmaj-9 damaged muscle fibers was assessed by multiplying the number of injured fibers (vacuolated, swollen, and heavily stained fibers) by 100 and dividing it by the total number of fibers present in the whole sectional muscle area. The results were compared with Krebs control (n = 5 per treatment). Normal fibers were considered those with both polygonal profile and myofibers evenly distributed inside the fascicle.

Statistical Analysis

All data were reported as mean (± S.E.M.) of the number of animals used in each experiment. Statistical comparison of data used repeated measures ANOVA followed by the Tukey's and Kruskal-Wallis post-*hoc* tests. Dunn's multiple test was also performed, and all experimental groups were compared with control group, and $p < 0.05$ indicated statistical significance.

RESULTS

Biochemical Characterization of BMAJ-9

The elution profile of *B. marajoensis* crude venom, after purification in one chromatographic step, displayed 18 peaks named according to their position from Bmaj-1 to Bmaj-18 (Figure 1). Peak 9, Bmaj-9, resulted in one small peak with a retention time of 36 minutes, eluting at 55% of solvent B. SDS-PAGE gel displayed one major protein band, indicating that Bmaj-9 fraction was obtained with high homogeneity. The Bmaj-9 molecular mass, ~14 kDa (Figure 1), was confirmed by MALDI-TOF mass spectrometry (13.67933 Da) (Figure 2). Bmaj-9 PLA_2 enzymatic activity was detectable in 4-nitro-3-octanoyloxy-benzoic acid substrate, being 7.9 ± 0.5 nmol/min/mg compared with 2.3 ± 1.1 nmol/min/mg for *B. marajoensis* crude venom, which indicated a high catalytic activity (Figure 3). Amino acid composition determined by HPLC was: Asp/8, Glu/9, Ser/6, Gly/13, His/2, Arg/11, Thr/8, Ala/7, Pro/6, Tyr/9, Val/3, Met/1, Cys/14, Ile/5, Leu/6, Phe/6 and Lys/10. The higher content of Lys, Hys and Arg residues, and the positive PLA_2 activity characterized this fraction as a typical basic PLA_2 protein.

The N-terminal sequence of Bmaj-9 was DLWQWGQMIL KETGKLPFSY YTAYGCYCGW GGRGGKPKAD TDRCCFVHDC… An alignment of this sequence with homologous snake venom PLA_2

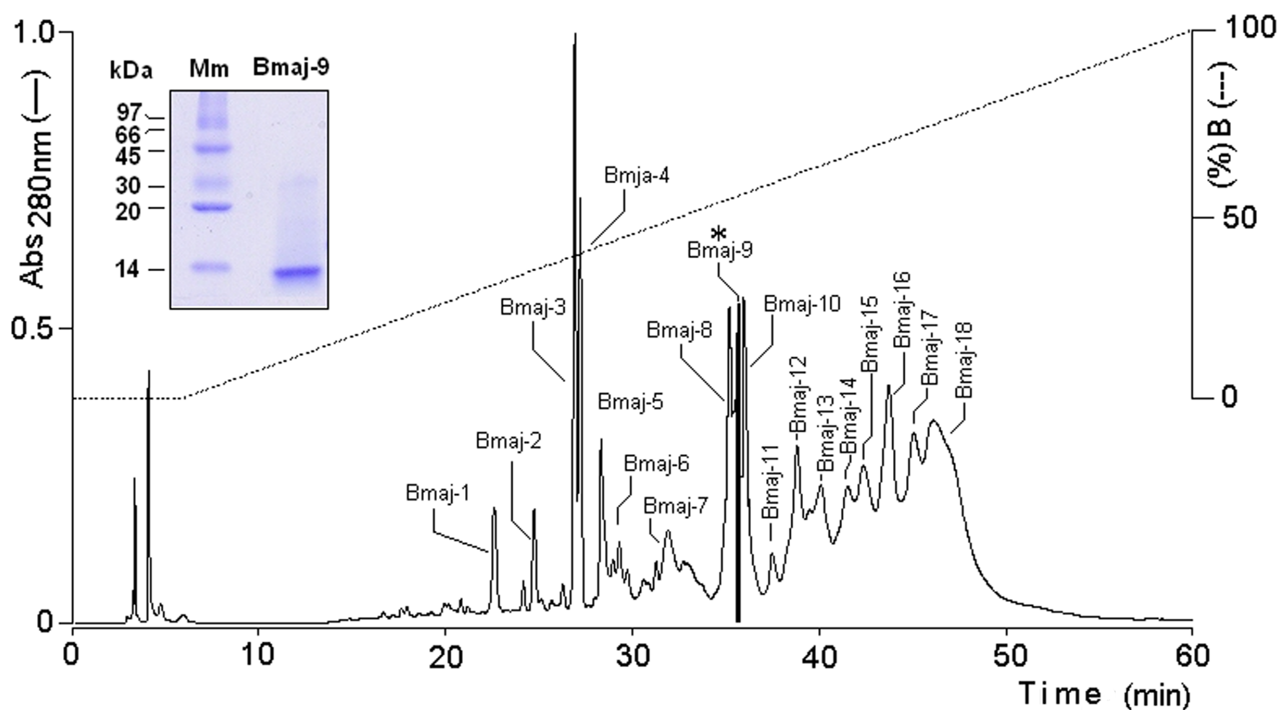

Figure 1. RP-HPLC chromatography of *B. marajoensis* crude venom. The elution profile (280 nm, 55% solvent B; retention time of 36 minutes) displays Bmaj-9 (*) as the main fraction obtained. Insert: electrophoretic profile in SDS-PAGE (12.5% gel).

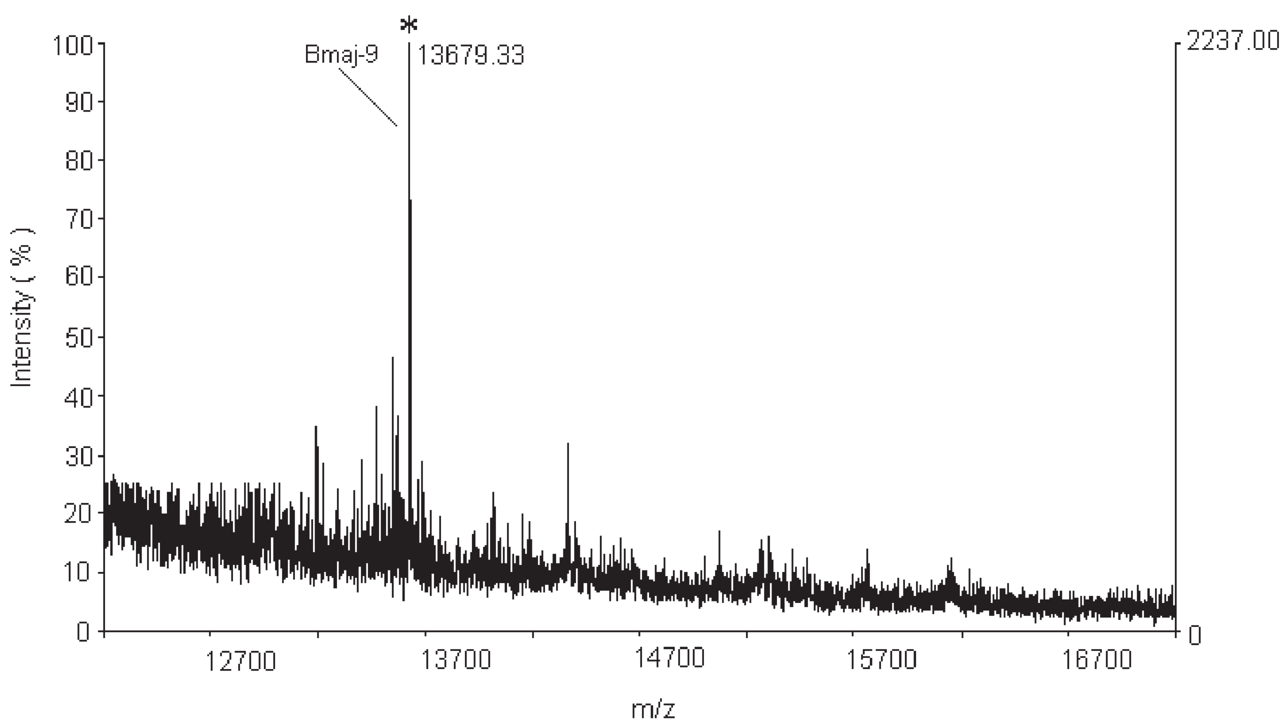

Figure 2. Bmaj-9 mass (13679.33 Da) determined by MALDI-TOF mass spectrometry.

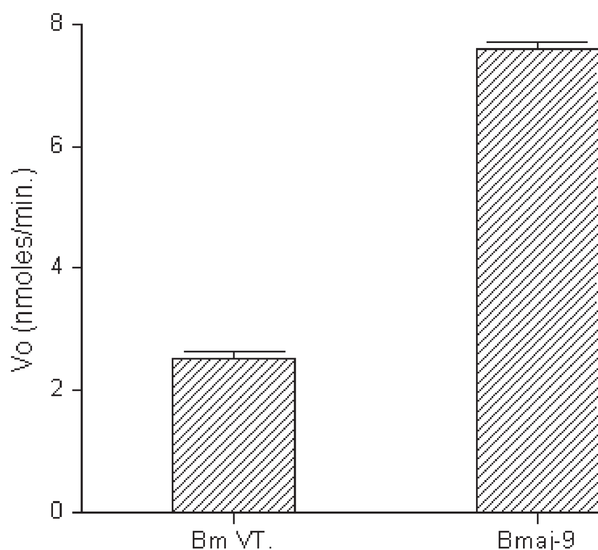

Figure 3. The graphic represents Bmaj-9 PLA_2 enzymatic activity in comparison to *B. marajoensis* crude venom, indicating a high catalytic activity of the toxin.

from *Bothrops* venom is shown in Figure 4. This result revealed that Bmaj-9 is an Asp49 (D49).

Considering that this protein is an important molecule that acts by blocking the neuromuscular junction presynaptically, the partial sequence of PLA from *Bothrops marajoensis* snake venom in this study was submitted to Universal Protein Resource (UniProt – http://www.uniprot.org). The code is B3A0N3 and its similarities with the homologous snake venom PLA_2 shown in Figure 4 are: 92% to PLA_2 BmjeTX-II, 88% to PLA_2 BmjeTX-I, 86% to PLA_2 6-2, 78% to PLA_2 6-1 and PLA_2 BmjeTX-I.

Twitch Tension Recording of BC

Bmaj-9 (1, 5, 10 and 20 µg/mL) induced an irreversible neuromuscular blockade in BC preparation, which was time/concentration-dependent even after several washes (Figure 5 – A). According to toxin concentration, the

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                         10                  20                  30                  40         *  50    %
                                                                                                        Identity
A2_Bmaj_9      D L W Q W G Q M I L K E T G K L P F S Y Y T A Y G C Y C G W G G R G G K P K A - D T D R C C F V H D C... 100.0
A2_BmjeTX_II   D L W Q W G Q M I L K E T G K I P F S Y Y G A Y G C Y C G W G G R G G K P K A - G T D R C C Y V H D C...  92.0
A2_BmjeTX_I    D L W Q F G Q M I L K E T G K I P F P Y Y G A Y G C Y C G W G G R G G K P K A - G T D R C C Y V H D C...  88.0
A2_6_2         D L W Q F G Q M I L K E T G K I P F P Y Y G A Y G C Y C G W G G R G G K P K D - G T D R C C Y V H D C...  86.0
A2_6_1         D L F E W G Q M I L K E T G K N P F P Y Y G A Y G C Y C G W G G R G - K P K D K D T D R C C Y V H D C...  78.0
A2_BmTX_I      D L W Q F N K M I K K E V G K L P F P F Y G A Y G C Y C G W G G R G E K P K D - G T D R C C F V H D C...  78.0
                                                                                                  D49-
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Figure 4. N-terminal amino acid sequence of Bmaj-9 compared with other Asp49 PLA_2. BmjeTX-I and -II from *B. marajoensis* venom, 6-1 and 6-2 toxins from *B. jararacussu* venom and BmTX-I from *B. moojeni* venom.

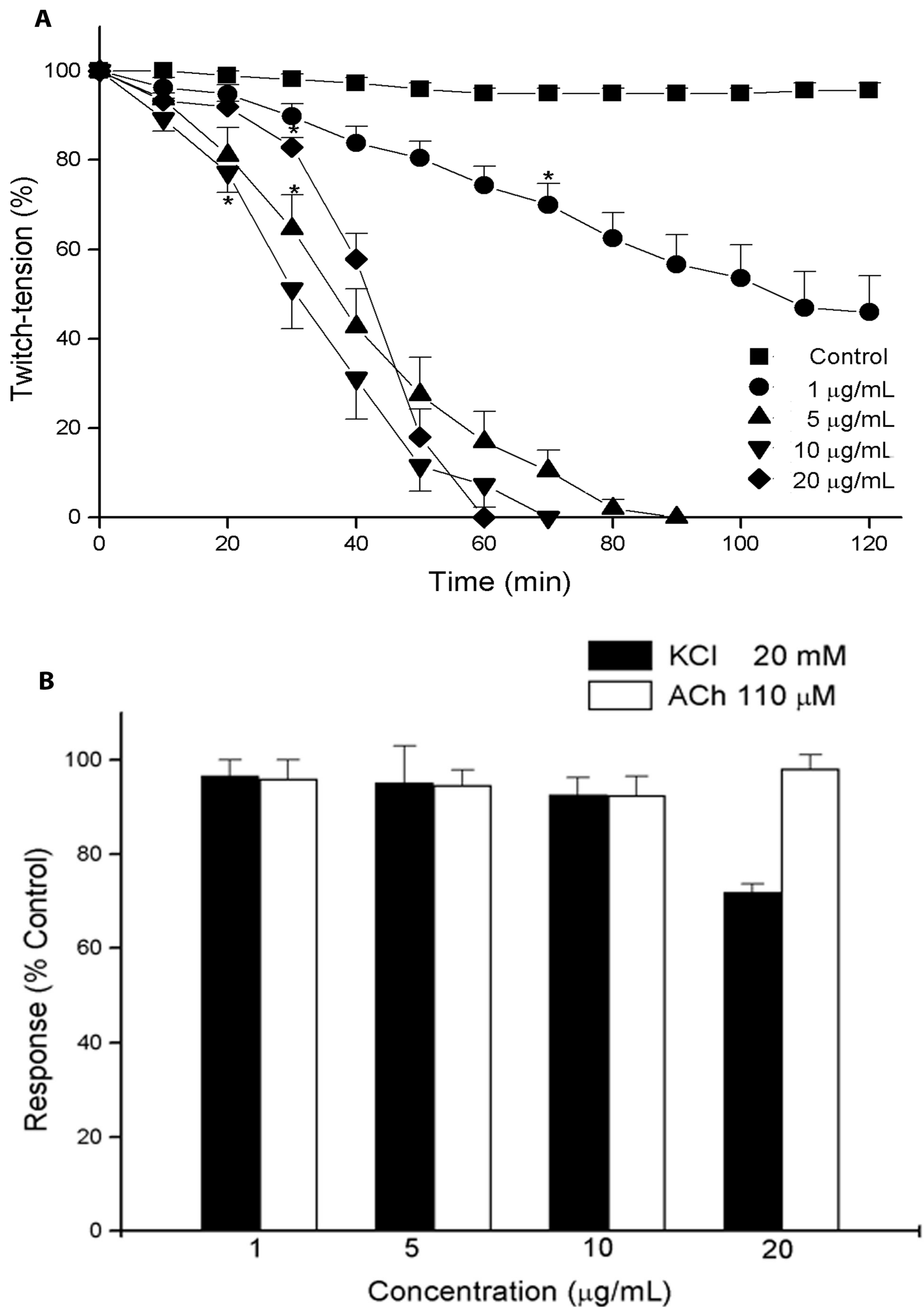

Figure 5. (**A**) Neuromuscular blockade induced by crescent concentrations (1, 5, 10 and 20 µg/mL) of Bmaj-9 in BC preparation. Note the irreversible twitch-tension neuromuscular blockade for 5, 10 and 20 µg/mL concentrations. (**B**) The graphic represents the effect on contractures to exogenous acetylcholine (ACh) and potassium (KCl). Each point is the mean ± S.E.M. (* $p < 0.05$).

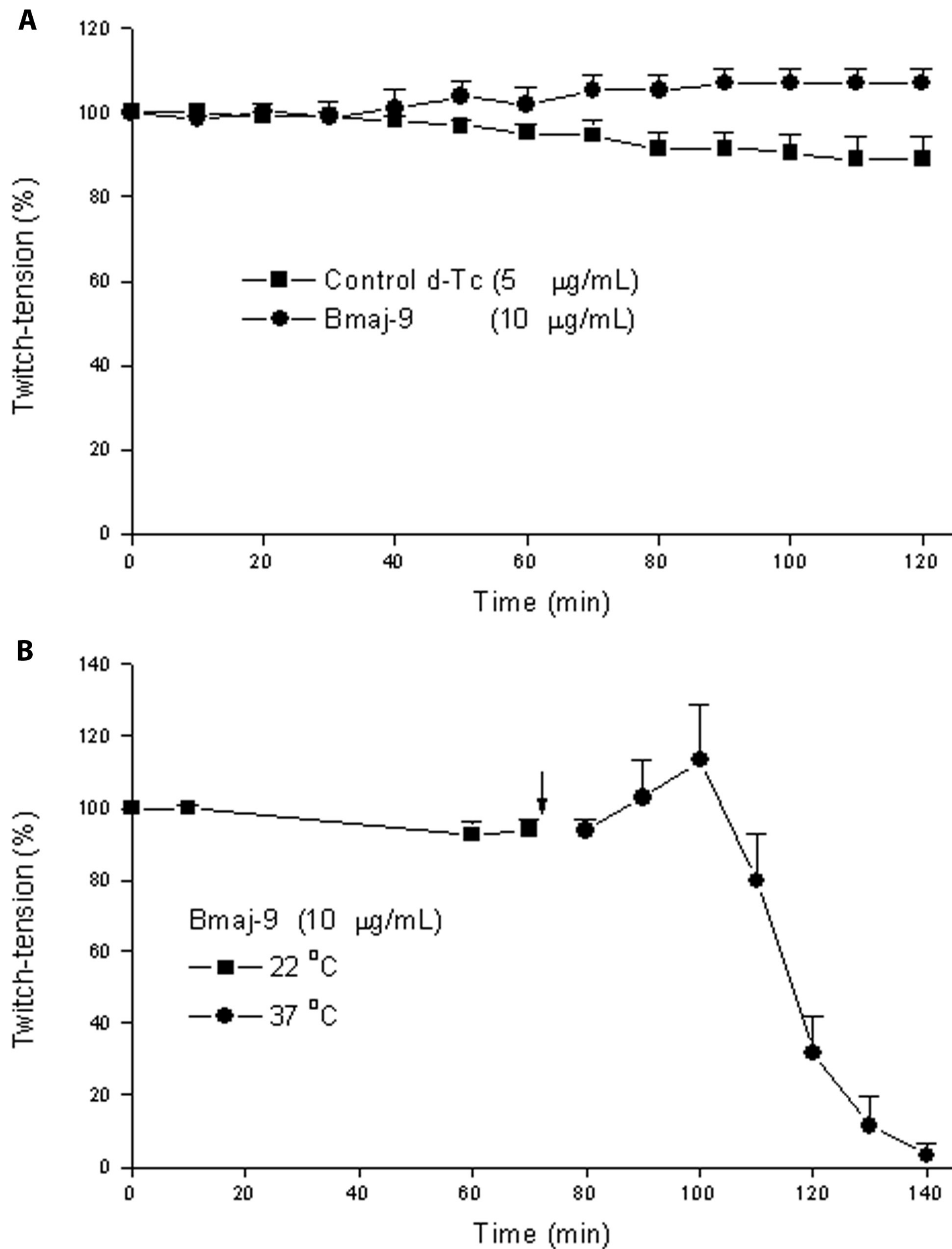

Figure 6. (**A**) Effect of Bmaj-9 (10 µg/mL) on curarized preparations. Note the absence of effect in the muscular response to direct electrical stimulation. (**B**) The characteristic neuromuscular blockade was not observed when the preparations were incubated with Bmaj-9 (10 µg/mL) at low temperature (22°C).

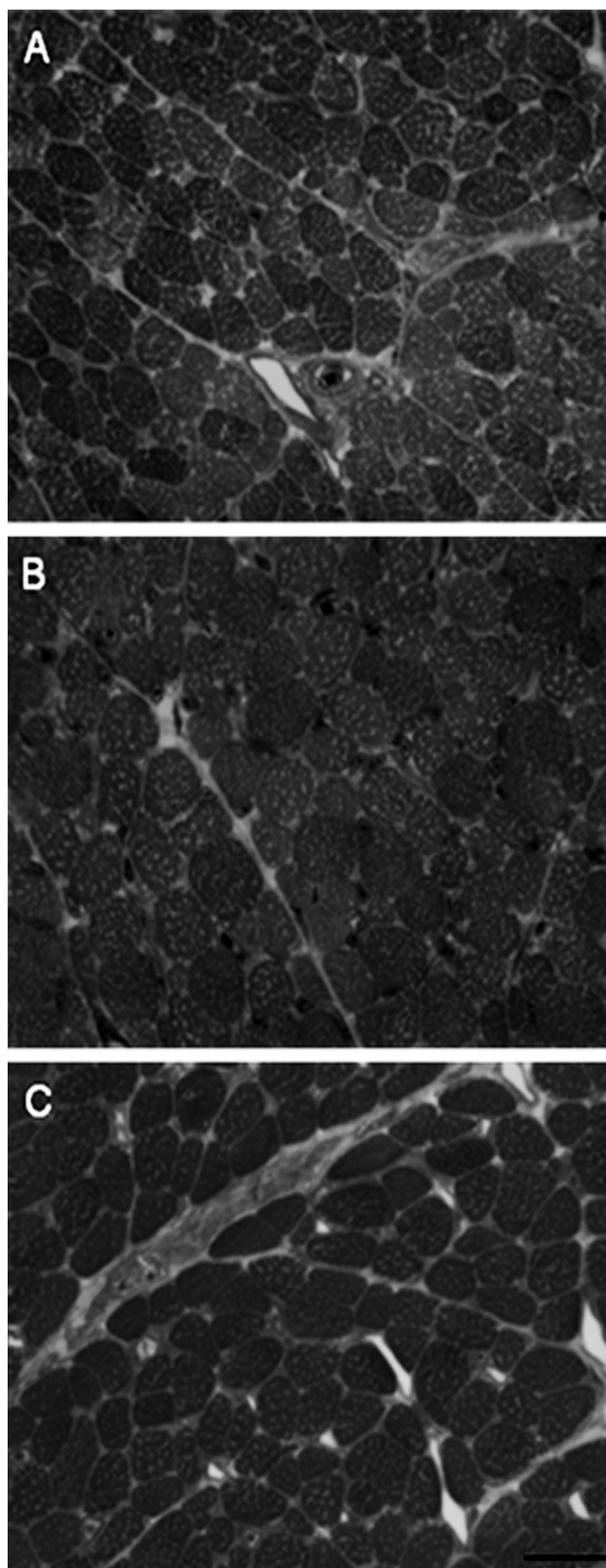

Figure 7. Morphological aspects of BC muscle after 120 minutes of incubation: (**A**) Krebs control, (**B**) 1 µg/mL and (**C**) 10 µg/mL Bmaj-9. Note the preserved polygonal cross-sectional profile of muscle fibers in all treatments. No damage was detected in muscle fibers. Bar = 50 µm.

time to achieve 50% neuromuscular paralysis after indirect electrical stimulation was: 110 ± 10 minutes (1 µg/mL), 40 ± 6 minutes (5 µg/mL), 30 ± 3 minutes (10 µg/mL) and 42 ± 1 minutes (20 µg/mL), whereas the time to reach 100% blockade was 90 ± 2 minutes (5 µg/mL), 70 ± 5 minutes (10 µg/mL) and 60 ± 2 minutes (20 µg/mL) (n = 5 per dose), all of them significantly different from control ($p < 0.05$).

Note that Bmaj-9 concentrations ranging from 5 to 20 µg/mL exhibited similar blocking potency, indicating that, at the present experimental condition, 5 µg/mL represents the minimal dose to cause total twitch tension paralysis (Figure 5 – A). The contractures induced by exogenous ACh (110 µM) or KCl (20 mM) remained unchanged regardless the toxin concentration assayed (Figure 5 – B).

Conversely, in curarized preparations directly stimulated, Bmaj-9 PLA_2 did not affect the muscular contractile response (Figure 6 – A). In addition, at a lower temperature (22°C), the toxin (10 µg/mL) did not induce the characteristic neuromuscular blockade seen at 37°C with the same concentration and time interval (Figure 6 – B).

CK Activity

Incubation with Bmaj-9 (10 µg/mL) at 0, 15, 30 and 60 minutes did not significantly alter the release of CK in BC (27.4 ± 5, 74.2 ± 8, 161.0 ± 21 and 353.0 ± 47) when compared with the corresponding controls (37.7 ± 12, 111.5 ± 26, 142.1 ± 23 and 248.2 ± 50). However, at 90 and 120 minutes of toxin exposure there was a mild but significant increase of CK activity compared with control (494.7 ± 50 and 812.6 ± 95 versus 331.0 ± 44 and 414.6 ± 67, respectively $p < 0.05$).

Histological Analysis

The calculation of damages caused by different Bmaj-9 concentrations (1, 10, 20 and 40 µg/mL, n = 5) of BC muscle after 120 minutes of indirect electrical stimulation showed the following negligible percentages of damaged fibers: 0.11 ± 0.1, 0.70 ± 0.6, 0.01 ± 0.01 and 3.56 ± 1.4% (data not shown), which did not differ from the Krebs solution (0.90 ± 0.41). Figure 7 shows that the muscle fiber morphology practically did not differ in the biventer cervicis muscle incubated with Krebs solution (Figure 7 – A) or with 1 and 10 µg/mL of Bmaj-9 (Figures 7 – B and C, respectively).

DISCUSSION

Snake venom PLA_2 enzymes typically exist in venoms as monomers and sometimes as complexes formed between PLA_2 enzymes, as well as with other proteins, by covalent or noncovalent interactions. Snake venoms often contain many isoforms or isoenzymes, and therefore care must be taken during purification to separate them, as isoforms or isoenzymes may induce different pharmacological effects through their interaction with protein receptors/acceptors. This specific interaction with their target protein is mediated through specific pharmacological sites on the molecular surface. Upon binding to their target protein, they induce their effects, which may be dependent or independent of enzymatic activity (26).

One chromatographic step identified a new basic toxin, coined as Bmaj-9, which was shown to be similar to other snake venom PLA_2s (20, 27-28). Bmaj-9 amino acid composition showed a high content of basic and hydrophobic residues, with 14 half-Cys. The N-terminal amino acid sequence revealed a high level of homology among Bmaj-9 from *B. marajoensis* venom and other toxins from the same venom species, such as D49 PLA_2s, BmjeTX-I and BmjeTX-II isoforms, 6-1 and 6-2 toxins from *B. jararacussu* venom and BmTX-I from *B. moojeni* venom (20, 27-28). Studies on sequence homology of Bmaj-9 showed an extremely conserved position in PLA_2 amino acids. At positions 1 and 2, amino acid sequence SL are prevalent; at position 4, amino acid Q; at positions 7-10, amino acids KMIL; at positions 12 and 13, amino acids ET; at position 21, amino acid Y; at positions 25-26 and Y28, G30, G32, D49, H48 and Y52 are directly or indirectly linked in the catalysis of Bmaj-9. In Asp49 PLA_2s there are many conserved residues that have important functions in the expression of the PLA_2 activity (29).

Bmaj-9 reveals a high sequential homology in the N-terminal region with PLA_2 from snake venoms displayed at positions 2 (L), 4 (Q), 8-10 (MIL) and 12-13 (ET), except for one substitution. Only one difference in the N-terminal sequence of Bmaj-9 consisted of replacement S1 → D1, I16 → L16, G22 → T22, G40 → D40 and Y46 → F46. The amino acid differences in the N-terminal sequence did not decrease either the catalytic or the pharmacologic activity of Bmaj-9 PLA_2.

Our findings showed that Bmaj-9 has potent neurotoxic activity, since concentrations as low as 1 and 5 μg/mL can lead to the blockade of the muscle contractile response in BC preparations. Bmaj-9 PLA_2 neuromuscular activity was higher than the exhibited by the whole venom. Although envenomations by *Bothrops* snakes produce 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 (7). Other PLA_2 isolated from other bothropic venoms have also shown neurotoxic effects (3, 17, 26, 27).

Thus, only *B. jararacussu*, *B. insularis* and *B. pauloensis* venoms were satisfactorily investigated regarding this activity, including the isolation of neurotoxic components, all of them with presynaptic effects, which were mainly due to PLA_2 (6, 30, 31).

Bmaj-9 PLA_2 from *B. marajoensis* venom induced an irreversible twitch-tension neuromuscular blockade at several toxin concentrations (1, 5, 10 or 20 μg/mL) without interfering with ACh and KCl induced contractures, hence characterizing this fraction as neurotoxic. In fact, even after twitch-tension complete paralysis (after 60 minutes of incubation with 10 μg/mL of Bmaj-9), neither muscle damage (fiber morphology, CK activity and response to direct stimulation) nor KCl response remained unchanged, hence showing absence of muscular effect. The findings indicate that Bmaj-9 could be included in the group of PLA_2 neurotoxins at least in the present experimental conditions.

The fact that the neuromuscular effect of Bmaj-9 was temperature-dependent suggests a role of the enzymatic activity in its neuromuscular action. In support to this view, a high catalytic activity was found for neuwieditoxin-I and -II from *B. neuwiedi pauloensis* venom (as per Bmaj-9, also Asp49 PLA_2s), and whose neuromuscular blockade also depends on the temperature (6).

All together, these results identify Bmaj-9, isolated from *B. marajoensis* venom, as a new member of the Asp49 PLA_2 family, which acts by blocking the neuromuscular junction in presynaptic sites.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Institutional Committee for Ethics in Animal Use (CEUA/IB/Unicamp, protocol number 1027-1). Moreover, all tests were in accordance with the guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA).

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