Biochemical characterization and pharmacological properties of a phospholipase A₂ myotoxin inhibitor from the plasma of the snake *Bothrops asper*

Sergio LIZANO*†¹, Bruno LOMONTE*, Jay W. FOX‡, and José María GUTIÉRREZ*

*Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica, †Departamento de Bioquímica, Escuela de Medicina, Universidad de Costa Rica, San José, Costa Rica, and ‡Biomolecular Research Facility, University of Virginia Health Science Center, Box 441, Jordan Hall, Charlottesville, VA 22908, U.S.A.

A protein that neutralizes the biological activities of basic phospholipase A₂ (PLA₂) myotoxin isoforms from the venom of the snake *Bothrops asper* was isolated from its blood by affinity chromatography with Sepharose-immobilized myotoxins. Biochemical characterization of this *B. asper* myotoxin inhibitor protein (BaMIP) indicated a subunit molecular mass of 23–25 kDa, an isoelectric point of 4, and glycosylation. Gel-filtration studies revealed a molecular mass of 120 kDa, suggesting that BaMIP possesses an oligomeric structure composed of five 23–25 kDa subunits. Functional studies indicated that BaMIP inhibits the PLA₂ activity of *B. asper* basic myotoxins I and III, as well as the myotoxicity and edema-

forming activity *in vivo* and cytolytic activity *in vitro* towards cultured endothelial cells, of all four myotoxin isoforms (I–IV) tested. Sequence analysis of the first 63 amino acid residues from the N-terminus of BaMIP indicated more than 65 % sequence similarity to the PLA₂ inhibitors isolated from the blood of the crotalid snakes *Trimeresurus flavoviridis* and *Agkistrodon blomhoffii siniticus*. These inhibitors also share sequences similar to the carbohydrate-recognition domains of human and rabbit cellular PLA₂ receptors, suggesting a common domain evolution among snake plasma PLA₂ inhibitors and mammalian PLA₂ receptors. Despite this similarity, this is the first description of a natural anti-myotoxic factor from snake blood.

Introduction

Phospholipases A₂ (PLA₂s; EC 3.1.1.4) catalyse the Ca²⁺-dependent hydrolysis of *sn*-2 linkages in phosphoglycerides. In addition to their hydrolytic activity, PLA₂s are associated with a variety of pharmacological effects. In envenomations caused by snake bites, venom PLA₂ activity is related to a series of pharmacological as well as toxic properties ranging from muscle tissue damage, neurological effects and anticoagulant activity to inflammation. The venom of the pit viper *Bothrops asper* contains factors such as basic PLA₂ myotoxins (15 kDa) that contribute to the characteristic onset of tissue damage that takes place at the site of venom injection [1–3]. Basic PLA₂ myotoxin isoforms have been isolated from *B. asper* venom and are numbered I, II, III and IV [4–7]. These toxins are group II PLA₂, although only myotoxins I and III possess enzymic activity. Myotoxin II, one of the catalytically inactive isoforms, contains a lysine residue at position 49 (Lys⁴⁹) instead of the typical aspartic residue (Asp⁴⁹) required for the Ca²⁺-binding properties essential for PLA₂ activity [8]. None the less, all isoforms are myotoxic *in vivo* and exhibit toxicity towards cultured endothelial cells *in vitro* [3]. The mechanisms responsible for the cell damage caused by these proteins are still unclear, but it has been suggested that specific cell-receptor binding and membrane-destabilizing effects might be involved [3].

Neutralizing substances can be helpful in understanding the mechanisms whereby venom substances exert their toxic effects. In *B. asper* myotoxins, specific neutralizing antibodies [9,10] as well as particular substances such as heparin, which exhibits anti-cytotoxic activity [11,12], have been employed. In recent years, a

growing number of natural anti-toxic factors present in the blood of snakes and other mammals have been described that neutralize specific snake venom toxins, such as haemorrhagins [13,14], neurotoxins [15,16] and PLA₂ activity [17,18]. These proteins are not immunoglobulins but rather oligomeric α -globulins and albumins that possess a pI of 4–5 and a molecular mass range of 50–72 kDa, with the exception of certain anti-haemorrhagic factors with molecular masses between 780 and 880 kDa [14,19]. Studies on the mechanism of action of some antitoxins such as the crotoxin neutralizing factor from *Crotalus durissus terrificus* [16,20,21], as well as several anti-haemorrhagins [14,22], indicate that these molecules inactivate toxins by forming soluble complexes. However, no specific plasma-based inhibitors of myotoxicity have been reported.

In this study we report the biochemical characterization and pharmacological properties of a 120 kDa myotoxin inhibitor protein (BaMIP) isolated from plasma of *B. asper* that binds to basic PLA₂ myotoxin isoforms derived from its own venom. This is the first description of a specific inhibitor of myotoxicity from the blood of snakes. The potential applications of this protein in the study of the mechanism of action of myotoxins and possibly in anti-venom therapy are discussed.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise noted. CNBr-activated Sepharose 4B was pur-

Abbreviations used: PLA₂, phospholipase A₂; BaMIP, *Bothrops asper* myotoxin inhibitor protein; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; CRD, carbohydrate recognition domain.

¹ To whom correspondence should be addressed at the Instituto Clodomiro Picado.

chased from Pharmacia (Uppsala, Sweden). *B. asper* blood was obtained from specimens kept at the serpentarium of the Instituto Clodomiro Picado, with 0.38 % sodium citrate as anti-coagulant. Specimens were gathered from the Pacific region of Costa Rica. Plasma was separated by centrifugation and stored at -20°C . Purified myotoxins I, II, III and IV were isolated from crude venom of *B. asper* by ion-exchange chromatography with carboxymethyl-Sephadex (Pharmacia) as previously described [4–7].

Affinity chromatography procedures

For the isolation of a specific myotoxin-binding protein, a mixture of purified myotoxins I, II, III and IV (50 mg of each) were coupled to 10 ml of swollen CNBr-activated Sepharose 4B as described by the manufacturer. The matrix was packed in a Bio-Rad Econopak column (Bio-Rad, Hercules, CA, U.S.A.) and equilibrated with 0.1 M sodium phosphate buffer (PB), pH 7.2. Plasma from *B. asper* (10 ml) was diluted with 5 vol. of PB and passed through the Sepharose 4B–myotoxin column at a rate of 2 ml/min. Flow-through absorbance was monitored on an ISCO UV/visible detector system at 280 nm. The sample was recirculated for 2 h, followed by extensive washing with PB. Bound material was eluted with 0.1 M glycine/HCl, pH 2.8, and the peak was collected in a reservoir containing 2 ml of 0.5 M Tris/HCl, pH 8.0, to neutralize the acid pH. The eluted fraction (approx. 10–20 ml) was dialysed extensively with PB+0.04 M NaCl (PBS) at 4°C . The dialysed fraction was concentrated by centrifugation with Centriprep cells (Amicon, MA, U.S.A.) with a molecular mass cut-off of 10 kDa, in accordance with manufacturer's instructions. Concentrated material was stored at -70°C in 1 ml aliquots.

Molecular mass and isoelectric point determinations

The fraction collected after elution from the Sepharose–myotoxin column was analysed by SDS/PAGE and isoelectric focusing. SDS/PAGE was performed with 12 % (w/v) polyacrylamide gels on the Miniprotean electrophoresis cell (Bio-Rad) by the method of Laemmli [23]. Isoelectric focusing was performed on a horizontal Flat Bed Apparatus FBE 3000 (Pharmacia) with 1 % (w/v) agarose gels (8 cm width $\times$ 14 cm depth $\times$ 0.5 cm height) containing ampholytes of pH range 3–9 (Pharmacia) as described by the manufacturer [24]. Electrophoresis was performed with a constant-power supply (ECPS 3000/150, Pharmacia) equipped with a volt-hour (Vh) integrator. The gels were prefocused before sample application for 250 Vh at a constant power setting (1000 V, 150 mA, 15 W), and sample runs were performed for an additional 750 Vh under the same power conditions. SDS/PAGE and isoelectric focusing gels were stained with Coomassie Blue R-250.

FPLC and molecular mass determination

Analytical gel filtration to determine the molecular mass of BaMIP under non-denaturing conditions was performed on an FPLC system (Pharmacia), with a pre-packed Superdex 200 (HR 10/30) column and a molecular mass calibration standards kit (Pharmacia), in accordance with the manufacturer's instructions. A flow rate of 0.4 ml/min was chosen for all experiments. PBS was used as eluent. The molecular mass of BaMIP was estimated relative to the calibration curve derived from the protein standards. K_{av} was determined by the following equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is elution volume, V_0 is void volume, and V_t is total column volume. Linear regression analysis was

performed on Sigma Plot Scientific Graph System version 1.02 (Jandel Scientific). Experiments were performed in triplicate.

N-terminal sequencing and amino acid composition analysis of BaMIP

N-terminal sequencing of BaMIP was performed with a Perkin-Elmer Procise amino acid sequencer in accordance with the manufacturer's instructions. Analysis of the amino acid composition was performed by pre-column derivatization with phenyl isothiocyanate, and phenyl isothiocyanato-amino acids were separated by reverse-phase HPLC on a C_{18} column, with a Beckman Gold HPLC system. BaMIP was also derivatized with divinylpyridine to alkylate the cysteine residues for determination.

Production of antiserum to BaMIP

Antiserum to BaMIP was prepared by the immunization of rabbits under the following scheme: primary injection with 300 μg of BaMIP mixed with complete Freund's adjuvant, followed by 300 μg three weeks later with incomplete Freund's adjuvant, and three subsequent booster doses of 300 μg mixed with sodium alginate every 3 months. Antibody titres were determined by ELISA [25] with Immulon 2 flat-bottomed 96-well microtitre plates coated with BaMIP diluted in 0.1 M Tris/0.15 M NaCl (pH 9.0) at a concentration of 0.3 $\mu\text{g}/100\text{ }\mu\text{l}$ per well. Plates were blocked with PBS containing 5 % (v/v) fetal bovine serum for 1 h and washed three times with PBS containing 0.05 % Tween-20 (PBS-T20). Serial 3-fold dilutions of plasma from rabbits immunized with BaMIP as well as from a non-immunized control were made starting from a 1:200 dilution, and 100 μl was added to duplicate microtitre wells and incubated for 1 h at room temperature. Wells were washed three times with PBS-T20, after which goat anti-(rabbit IgG)–peroxidase (1:2000 diluted in PBS-T20) was added and incubated for a further 1 h. After three washes with PBS-T20, colour was developed with 0.012 % (v/v) H_2O_2 and 2 mg/ml *o*-phenylenediamine in 0.1 M sodium citrate, pH 5.0. Absorbance was measured on a Dynatech MR5000 microplate reader (Dynatech Labs, Chantilly, VA, U.S.A.) and the titre was defined as the last dilution giving an A_{490} of 0.2.

Determination of binding of BaMIP to myotoxin isoforms

The ability of BaMIP to bind different myotoxin isoforms was assessed by ELISA, employing the rabbit polyclonal anti-BaMIP as detector. Purified myotoxin isoforms, I, II, III and IV were immobilized on Immulon 2 microtitre plates as described above, at a concentration of 0.4 $\mu\text{g}/100\text{ }\mu\text{l}$ per well. Different concentrations of BaMIP diluted in PBS/3 % (v/v) fetal bovine serum FBS were added and incubated for 1 h at room temperature. Wells were washed three times with PBS-T20, after which anti-BaMIP was added at 1:5000 dilution in PBS/3 % fetal bovine serum and incubated for a further 1 h. Wells were washed three times with PBS-T20, after which goat anti-(rabbit IgG)–peroxidase (1:2000) was added, incubated for a further 1 h and developed as described above.

In addition, binding to the different myotoxins was demonstrated by Western blot analysis. Purified *B. asper* myotoxins I–IV were run on SDS/PAGE [15 % (w/v) gel] and blotted on nitrocellulose on a Trans-Blot electrotransfer device (Bio-Rad) in accordance with the manufacturer's instructions. The nitrocellulose membrane was then blocked with PBS containing 2 % (w/v) BSA for 1 h at room temperature, followed by incubation

with BaMIP at a concentration of 0.1 mg/ml in PBS–2% BSA for a further 1 h. The membrane was washed three times with PBS–2% BSA, after which rabbit polyclonal anti-BaMIP serum diluted 1:5000 in PBS–2% BSA was added and incubated for 1 h. The membrane was washed three times and for 1 h with goat anti-(rabbit IgG)–peroxidase diluted 1:5000 in PBS–2% BSA. After incubation the membranes were washed twice with PBS–2% BSA and once with 0.02 M Tris, pH 7.5, followed by the addition of H₂O₂ and 4-chloro-1-naphthol for colour development [25].

Inhibition of PLA₂ and anticoagulant activities of myotoxins I and III

To determine the ability of BaMIP to inhibit the PLA₂ activity of enzymically active myotoxins I and III, these toxins were preincubated with various concentrations of BaMIP. PLA₂ activity was assayed by measuring the release of fatty acids from an egg yolk substrate solution incubated with myotoxin or BaMIP/myotoxin mixtures [26,27]. In addition, the effect of BaMIP on PLA₂ activity of these myotoxins was determined by the neutralization of radial haemolysis on agarose–erythrocyte–egg yolk gels [28]. For each assay the amount of myotoxin required for a significant PLA₂ activity signal was established by constructing a calibration curve, and corresponded to 1.25 and 10 µg for the radial haemolysis and Dole tests respectively. All reactions involving mixtures of BaMIP and myotoxin were preincubated at 37 °C for 30 min before each assay. The molar ratio of inhibition was defined by the smallest amount of BaMIP required to neutralize the PLA₂ activity of myotoxin III.

Inhibition of the anticoagulant effect of myotoxins I and III by BaMIP was determined by the method of Gutiérrez et al. [27]. The amount of myotoxin used in this assay was 2 µg.

Inhibition of cytotoxicity in cultured endothelial cells *in vitro*

The ability of BaMIP to neutralize the cytotoxic effects of the myotoxins was tested on murine capillary endothelial cells (tEnd) [29] as described by Lomonte et al. [30]. For these assays, cytotoxicity was evaluated on the basis of the release of lactate dehydrogenase (LDH) from the cells as well as microscopic examination of the integrity of the cellular monolayer. LDH activity was assayed with a test kit (Sigma 500-C) in accordance with the manufacturer's instructions. The ability of BaMIP to neutralize the toxicity of myotoxins I, II, III and IV was tested. A quantity of 200 µg of myotoxin in 150 µl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) fetal calf serum was defined as the dose necessary to produce a recognizable cytotoxic effect. Preincubations of myotoxin with BaMIP were performed at 37 °C for 30 min before the addition of the mixture to a 5 day monolayer culture of tEnd cells grown in DMEM/1% fetal calf serum in sterile 96-well microplates. Cells were incubated for 3 h at 37 °C, after which the supernatants were separated and assayed for LDH activity. The cells were fixed and stained with Toluidine Blue dye. Positive toxicity controls included 0.1% (v/v) Triton X-100, as well as 20 µg of myotoxin in the absence of BaMIP. The amount of LDH released by Triton X-100 was defined as 100% cytotoxicity, and results were reported as the relative percentage of cytotoxicity. Negative controls consisted of cells incubated with 150 µl of DMEM/1% fetal calf serum alone, or with BaMIP in the absence of myotoxin. All reactions were performed in triplicate.

Inhibition of myotoxicity *in vivo*

Myotoxic activity *in vivo* was determined by measuring the plasma levels of creatine kinase in groups of four Swiss–Webster mice (18–20 g body weight) injected intramuscularly in the gastrocnemius muscle with 0.1 ml of myotoxin (75 µg) either alone or preincubated with various concentrations of BaMIP. Negative control groups were injected with 0.1 ml of PBS. Blood samples were collected by cutting the tip of the tail 3 h after injection. Creatine kinase activity was determined by using the Sigma kit 520. Activity was represented in units/ml (one creatine kinase unit is defined as the amount of enzyme required to phosphorylate 1 nmol of creatine/min at 25 °C). In addition, samples of muscle tissue were collected 24 h after injection and processed for histological analysis, as described by Gutiérrez et al. [27].

Inhibition of myotoxin-induced oedema in mice

Basic PLA₂ myotoxins induce oedema, which is part of the local effects caused by *B. asper* venom [31]. The inhibition of this effect by BaMIP was assessed by the injection of myotoxin or mixtures of BaMIP/myotoxin in the right foot pad of mice, by the method of Yamakawa et al. [32]. The left foot pad of each mouse was injected with PBS as a negative control. The degree of oedema induced by each myotoxin or BaMIP/myotoxin mixture was expressed as the percentage increase in weight of the right foot pad relative to the weight of the foot pad injected with PBS.

RESULTS

Isolation and biochemical characterization of BaMIP

A myotoxin-binding protein factor, BaMIP was isolated from pooled plasma of *B. asper* specimens by affinity chromatography with purified myotoxin II immobilized on Sepharose. SDS/PAGE analysis under denaturing conditions (2-mercaptoethanol) showed a band migrating at approx. 25 kDa (Figure 1). On

Figure 1 SDS/PAGE analysis of BaMIP

The affinity-purified fraction of BaMIP and crude *B. asper* plasma were separated by SDS/PAGE on discontinuous 12% (w/v) polyacrylamide gels under reducing conditions (2-mercaptoethanol) and stained with Coomassie R-250. Lane a, molecular mass markers (molecular masses shown at the left); lane b, crude *B. asper* plasma; lane c, affinity-purified BaMIP fraction recovered from Sepharose 4B–myotoxin on elution with 0.1 M glycine, pH 3.0.

Figure 2 Molecular mass determination of BaMIP by gel filtration
Selectivity curve derived from the analytical gel filtration of BaMIP and standard molecular mass markers. (For calculation of K_{av} , see the Materials and methods section.)

analysis of the protein by FPLC gel filtration on Superdex 200 HR 10/30 column under non-denaturing conditions, an approximate molecular mass of 120 kDa was determined on the basis of a selectivity curve with standard markers (Figure 2). The curve had a correlation coefficient (r^2) of 0.94. Other biochemical analyses included isoelectric focusing, in which the protein focused at an estimated pI of 4.0, and the periodic acid Schiff assay, which indicated a positive test for glycosylation (results not shown).

The N-terminal sequence comprising the first 63 amino acid residues BaMIP was determined (Table 1). This segment of BaMIP shares sequence similarity to other natural inhibitor proteins from snake plasma, namely the PLA₂ inhibitor isolated from *Agkistrodon blomhoffii siniticus* [17] (68% sequence similarity) and subunits A and B of the PLA₂ inhibitor from *Trimeresurus flavoviridis* [33] (78% and 68% sequence similarity respectively). Similarity with these inhibitors was also reflected by amino acid composition analysis of the entire molecule (results not shown), in which the molar fractions of each amino acid residue in BaMIP (tryptophan not determined) were generally similar to the compositions of both the *T. flavoviridis* and *A. blomhoffii siniticus* [17] inhibitors.

Moreover, amino acid residues 49, 50, 51, 53 and 57 from BaMIP overlap with the region of the *A. blomhoffii siniticus* anti-PLA₂, which bears similarity to the carbohydrate recognition domains (CRDs) of the rabbit and human cellular PLA₂ receptors [17]. In contrast, the N-terminus of BaMIP did not share sequence

Figure 3 Binding of BaMIP to myotoxins I, II, III and IV by ELISA
ELISA showing the binding of increasing concentrations of BaMIP to each myotoxin immobilized on microplates. Myotoxin was bound at a concentration of 0.4 ng/100 μ l per well. Polyclonal rabbit anti-BaMIP was used at a 1:5000 dilution in PBS, followed by incubation with anti-(rabbit IgG)-peroxidase conjugate (diluted 1:500 in PBS) and colour development with peroxidase substrate (see the Materials and methods section).

similarity to other characterized PLA₂ inhibitors from snake plasma, such as the crotoxin neutralizing factor from *Crotalus durissus terrificus* [21] or the inhibitor isolated from the Thailand cobra *Naja naja kaouthia* [34].

Binding studies

BaMIP binds to the different *B. asper* myotoxin isoforms, as demonstrated by Western blot analysis and ELISA. On Western blot, interactions of myotoxins I–IV with BaMIP were evidenced by the appearance of the 15 kDa myotoxin bands on development with BaMIP as described in the Materials and methods section (results not shown). ELISA binding studies employing each myotoxin immobilized on microtitre plates and incubated with increasing amounts of BaMIP suggested that the binding strengths of the inhibitor to the different isoforms are similar (Figure 3), with the possible exception of myotoxin II, which exhibited a lower binding signal than the rest on addition of anti-(rabbit IgG)-peroxidase and peroxidase substrate.

Anti-PLA₂ activity and anticoagulant properties of BaMIP

BaMIP inhibited the PLA₂ activities of the enzymically active myotoxins I and III, as demonstrated by neutralization of the

Table 1 N-terminal amino acid sequence alignment of BaMIP with the PLA₂ inhibitors (PLIs) from *Agkistrodon blomhoffii siniticus* and *Trimeresurus flavoviridis*

Name	Residue...	5	10	15	20	25	30	35	40	45	50	55	60	Sequence similarity (%)
BaMIP		D	E	V	D	P	D	G	K	V	L	N	S	–
<i>T. flavoviridis</i> PLI-A [23]		H	E	T	D	P	D	G	Q	V	M	S	S	78
<i>T. flavoviridis</i> PLI-B [23]		H	E	T	D	S	D	G	Q	V	M	N	S	68
<i>A. blomhoffii siniticus</i> PLI [17]		H	E	T	D	P	D	G	H	V	L	N	S	68

Figure 4 Inhibition of PLA₂ activity of myotoxins I and III at different BaMIP myotoxin molar ratios

The PLA₂ activity of myotoxin or mixtures of BaMIP and myotoxin at different molar ratios were measured [26,27]. The full activity of each myotoxin alone was defined as 100% PLA₂ activity, which corresponded to 28.8 μ -equiv of NaOH/min per mg of myotoxin. BaMIP-to-myotoxin molar ratios were based on the molecular mass of BaMIP determined by FPLC analysis, relative to the reported molecular masses of the myotoxins [1–3].

Table 2 Neutralization of PLA₂ and anticoagulant activities of myotoxins I and III

PLA₂ activity was measured by radial haemolysis on agarose/erythrocyte/egg yolk gels [29]. Myotoxin (1.5 μ g) dissolved in PBS was applied alone or preincubated for 30 min at 37 °C with different amounts of BaMIP. Samples were applied to a well 4 mm in diameter and incubated overnight at 37 °C in a humid chamber. Haemolysis diameter values are reported after subtraction of the well. Coagulation times were measured as described in [28]. Myotoxin (2 μ g) or myotoxin/BaMIP mixtures contained in 0.1 ml of sample volume were added to 0.5 ml of platelet-depleted sheep plasma and incubated at 37 °C until coagulation. BaMIP alone (20 μ g) was used as a negative control in both assays. BaMIP-to-myotoxin molar ratios (B/M) were estimated on the basis of a BaMIP molecular mass of 120 kDa.

Sample	Diameter of haemolysis (mm)		Anticoagulant activity (time to coagulation) (min)	
	Myotoxin I	Myotoxin III	Myotoxin I	Myotoxin III
PBS	0	0	5–8	5–8
Myotoxin	4.5	5	∞ (> 60)	∞ (> 60)
BaMIP	0	0	5–8	5–8
B/M 1.25:1	0	0	5–8	5–8
B/M 0.62:1	1	0	5–8	5–8
B/M 0.31:1	1	0	5–8	11
B/M 0.15:1	3	1	5–8	13
B/M 0.08:1	4	5	30–60	30–60

release of fatty acids in the Dole test (Figure 4) as well as by neutralization of the anticoagulant and haemolytic properties of these toxins (Table 2). On the basis of the estimated molecular masses of BaMIP (120 kDa) and the myotoxins (15 kDa), different molar ratios of inhibitor to myotoxin were tested. For myotoxins I and III, inhibition of over 75% of its PLA₂ activity as measured by the Dole test was achieved at a BaMIP-to-myotoxin molar ratio of 0.15:1, whereas neutralization of radial haemolysis occurred at a ratio of 0.31:1.

A similar pattern of inhibition was observed when the anticoagulant properties of myotoxins I and III were measured

Figure 5 Inhibition of cytotoxicity *in vitro* of myotoxins I, II, III and IV by BaMIP

Activity was measured by the release of LDH by a monolayer of tEnd cells cultured on flat-bottomed 96-well microplates incubated with myotoxin alone or with various ratios of BaMIP to myotoxin. Full activity (100%) was defined as the amount of LDH released on incubation of the cells with myotoxin alone.

(Table 2), in which the BaMIP-to-myotoxin molar ratio of inhibition was at least 0.15:1, as indicated by a coagulation time of less than 8 min for myotoxin I, and 13 min for myotoxin III.

Inhibition of toxic effects *in vitro* and *in vivo*

The effect of BaMIP on the cytotoxicity of the different myotoxin isoforms on tEnd cells *in vitro* was assessed by measuring the release of LDH after incubation of the cells at different BaMIP-to-myotoxin ratios (Figure 5). For the PLA₂-active myotoxins I and III, more than 75% inhibition of cytotoxicity was detected at BaMIP-to-myotoxin molar ratios greater than 0.15:1, which correlates with anti-PLA₂ activity (see above). A similar ratio of inhibition was observed in the case of the enzymically inactive myotoxin IV. However, for myotoxin II, more than 75% neutralization of cytotoxicity was not accomplished until a BaMIP-to-myotoxin molar ratio of 0.6:1 was reached. Neutralization was confirmed by microscopic analysis, which indicated an intact endothelial cell monolayer at inhibitory concentrations of BaMIP compared with a total destruction of the monolayer when using myotoxin alone (results not shown).

BaMIP also inhibited the myotoxicity *in vivo* and oedema-forming properties of the myotoxins (Table 3). As in the cytotoxicity studies *in vitro*, myotoxins I, III and IV neutralized muscle tissue damage as indicated by more than 75% decrease in blood creatine kinase levels after the injection of myotoxin preincubated with BaMIP at a BaMIP-to-myotoxin molar ratio of 0.15:1, whereas the same effect caused by myotoxin II was accomplished at a ratio greater than 0.6:1. These results were confirmed by a histological analysis of tissue sections of gastrocnemius muscle (results not shown).

The oedema induced by all four *B. asper* myotoxins was also neutralized by BaMIP (Table 3). As in the experiments *in vitro* and *in vivo*, over 80% inhibition of oedema was achieved at a BaMIP-to-myotoxin molar ratio of 0.15:1 for myotoxins I, III and IV, whereas the level of inhibition of myotoxin II was attained at a ratio of 0.6:1.

Table 3 Inhibitory effect of BaMIP on the oedema-causing properties and myotoxicity of *B. asper* PLA₂ myotoxins I, II, III and IV *in vivo*

The amount of myotoxin administered intradermally (50 µl) corresponded to the minimal oedematizing dose (1 dose equals the minimum amount of toxin capable of inducing a 30% increase in weight of the injected foot pad compared with a negative control injected with PBS): 6 µg for myotoxins I and III, and 18 µg for myotoxins II and IV. Oedema was reported as the percentage increase in weight of the right foot pad of mice relative to the left foot pad injected with PBS. Myotoxicity was assessed by measuring the amount of creatine kinase activity in blood of mice 3 h after the injection of myotoxin (75 µg) alone or BaMIP/myotoxin mixtures. Experiments were performed with groups of four mice. BaMIP alone (200 µg) was used as a negative control. Creatine kinase (CK) activities are given as units/ml of plasma. In both assays, BaMIP was preincubated with myotoxin for 30 min at 37 °C at a BaMIP-to-myotoxin molar ratio of 0.25:1 (with the exception of myotoxin II, which was incubated at a ratio of 0.6:1). * $P < 0.001$ compared with relevant myotoxin alone.

Sample	Oedema	<i>In vivo</i> myonecrosis	
	Increase in oedema (%)	Inhibition by BaMIP (%)	CK activity (units/ml)
Myotoxin I	41.9 ± 7.2		299.2 ± 60.8
Myotoxin I + BaMIP	7.7 ± 3.0*	81.6	75.8 ± 3.5*
Myotoxin II	27.5 ± 3.4		102.7 ± 29.2
Myotoxin II + BaMIP	0.5 ± 0.3*	98.2	10.0 ± 2.8*
Myotoxin III	38.3 ± 8.8		210.5 ± 54.7
Myotoxin III + BaMIP	6.7 ± 1.4*	82.5	11.4 ± 5.9*
Myotoxin IV	43.1 ± 5.8		185.3 ± 34.2
Myotoxin IV + BaMIP	6.0 ± 5.0*	86.1	49.5 ± 23.2*
BaMIP	3.8 ± 1.8		24.3 ± 12.8

DISCUSSION

In this study a novel inhibitor of *B. asper* basic PLA₂ myotoxins (BaMIP) was characterized. BaMIP has certain biochemical features (namely low isoelectric point and an oligomeric structure) that are common among blood-derived inhibitors of snake venom in general (including snake and mammalian anti-haemorrhagic and anti-neurotoxic factors) (reviewed in [19]). However, the strong amino acid sequence similarity between BaMIP and the PLA₂ inhibitors of the crotalid snakes *T. flavoviridis* [33] and *A. blomhoffii siniticus* [17], plus the fact that all these factors inhibit group II PLA₂, suggests a common evolution of a particular class of anti-PLA₂ factors among both crotalid and viperid snakes. This group seems to be distinct from a separate class of PLA₂ inhibitors from snake blood represented by the crotoxin neutralizing factor of *C. durissus terrificus* [21] and the two subunits (25 and 31 kDa) of the 90 kDa PLA₂ inhibitor from *N. naja kaouthia* [34]. The 31 kDa subunit of the *N. naja kaouthia* factor shares 59% sequence similarity with crotoxin neutralizing factor and has internal sequence repeats with a pattern of cysteine residues similar to those found in urokinase-type plasminogen activator receptor and the superfamily of Ly-6-related proteins, such as the cell surface antigens Ly-6A/E, Ly6C, ThB and CD59 [34].

A common feature of BaMIP and its related PLA₂ inhibitors is the presence of motifs similar to the CRDs of Ca²⁺-dependent (C-type) lectins, which bind mannose-containing carbohydrate residues in glycoproteins such as mannose-binding protein A and pulmonary surfactant apoprotein [17,33]. In the macrophage mannose receptor, CRDs have an important role in the endocytosis of glycoproteins and the recognition of mannose residues on the surface of micro-organisms before phagocytosis [35]. CRDs are also structural elements of PLA₂ receptors, such as the pancreatic group I PLA₂ receptors and the receptors for secretory

(group II) PLA₂ derived from rabbit, rat, bovine and human tissues [36–38]. Mutagenesis studies have established that CRDs on these receptors are involved in binding to PLA₂ [36,37]. Although no similar studies have been performed with PLA₂ inhibitors from snake blood, it is likely that the CRDs constitute at least one type of domain involved in the binding and inactivation of PLA₂-type proteins.

The similarity between these snake PLA₂ inhibitors and mammalian PLA₂ receptors might also explain their physiological role as soluble anti-toxins in snake blood. Recently, it has been demonstrated that the human-derived secretory PLA₂ receptor also exists as a soluble form devoid of the trans-membrane domain and might have an important role in regulating secretory PLA₂ activity, which has pro-inflammatory properties in mammals [37]. On this evidence, snake PLA₂ inhibitors could perhaps be 'soluble' counterparts of putative cellular receptors involved in the clearance of venom PLA₂ from blood before it reaches a target cell. However, such a mechanism of natural resistance necessitates the characterization of a similar membrane receptor form.

The neutralization of the different biological activities of myotoxins I, III and IV occurred consistently within a BaMIP-to-myotoxin molar ratio of 0.15:1 to 0.31:1, which suggests that approximately four or five molecules of myotoxin are inhibited per molecule of BaMIP. Given the oligomeric structure of BaMIP, it is possible that each 25 kDa subunit might bind to and inactivate one molecule of myotoxin. However, with myotoxin II, the binding affinity might be lower, as suggested by the higher BaMIP-to-myotoxin inhibitory ratio.

The parallel relationship between anti-PLA₂ activity and the other anti-toxic effects of BaMIP towards myotoxins I and III suggest a direct correlation between enzymic activity and toxicity. This is consistent with recent studies reporting a parallel decrease in both PLA₂ and myotoxic activities of myotoxin III on covalent modification of its His⁴⁸ by alkylation with *p*-bromophenacyl bromide [39]. However, studies employing inhibitors of myotoxicity such as heparin [11] and EDTA [40] have demonstrated instead a dissociation between the enzymic and toxic properties of myotoxin III. Heparin binds to a strongly cationic peptide representing a PLA₂-independent region near the C-terminus of myotoxin II (residues 115–129), which is cytotoxic by itself [12]. None the less, the lack of correlation between enzymic activity and toxicity in these studies does not necessarily preclude the possibility that PLA₂ activity might contribute to some extent to the overall membrane damage and cell destruction induced by these toxins. Our data on the myotoxic effects of the myotoxins (Table 3) support previous observations [41] that toxicity tends to be higher for the enzymically active myotoxins (e.g. myotoxin I) than for myotoxin II.

Whether or not PLA₂ activity contributes to the toxic effects of myotoxins, it is evident from the inhibition of the biological activities of the enzymically inactive myotoxins II and IV that a PLA₂-independent mechanism of membrane damage is involved. Within this context there are at least two mechanisms by which BaMIP could neutralize these non-PLA₂ related activities. For example, BaMIP might bind to the PLA₂-related domains via its CRD-like domains and block, perhaps by steric hindrance, the interaction between the cytotoxic domain(s) and the cell membrane. Alternatively, BaMIP might also bind directly to non-PLA₂-related domains in the myotoxins, thus preventing a direct contact with the membrane. Future studies on the structure–function relationships of BaMIP might help elucidate the molecular basis of the mechanism of action of the myotoxins.

Although BaMIP inhibited the enzymic activity of myotoxins I and III, it did not inhibit the overall PLA₂ activity of crude *B.*

asper venom. Thus BaMIP might not neutralize all PLA₂ isoenzymes in the venom. Perhaps the presence of other PLA₂ inhibitors in the blood, as well as the existence of alternative mechanisms of natural resistance to venoms, might combine to neutralize the wide array of sources of PLA₂ activity in snake venom. The identification of natural anti-venom factors might become useful in the study of mechanisms of venom toxicity, and might perhaps constitute a novel alternative in the treatment of snake envenomations. Conventional polyvalent anti-venom is sometimes ineffective in neutralizing tissue necrosis and haemorrhage in *B. asper* envenomation. Supplementation of anti-venoms with factors such as antihemorrhagins and PLA₂ inhibitors might enhance the neutralization potential during therapy.

We thank Grettel Chanto, Javier Núñez and Marjorie Romero for their skilful technical support, as well as Dr. Fernando Chaves for his help in the oedema experiments, Dr. Yamileth Angulo for supplying some of the purified myotoxin material, and Rodrigo Aymerich at the serpentry of the Instituto Clodomiro Picado for providing the snake specimens. This work was supported by grant F/2485-1 from the International Foundation for Science awarded to S. L., and grant 741-96-575 from the Vicerrectoría de Investigación of the University of Costa Rica.

REFERENCES

- Gutiérrez, J. M. and Lomonte, B. (1989) Mem. Instituto Butantan **51**, 211–223
- Ownby, C. L. (1990) in Handbook of Toxinology (Shier, W. T. and Mebs, D., eds.), pp. 602–654, Marcel Dekker, New York
- Gutiérrez, J. M. and Lomonte, B. (1995) Toxicon **33**, 1405–1424
- Gutiérrez, J. M., Ownby, C. L. and Odell, G. V. (1984) Toxicon **22**, 115–128
- Lomonte, B. and Gutiérrez, J. M. (1989) Toxicon **27**, 725–733
- Kaiser, I. I., Gutiérrez, J. M., Plummer, D., Aird, S. D. and Odell, G. V. (1990) Arch. Biochem. Biophys. **278**, 319–325
- Díaz, C., Lomonte, B., Zamudio, F. and Gutiérrez, J. M. (1995) Nat. Toxins **3**, 26–31
- Francis, B., Gutiérrez, J. M., Lomonte, B. and Kaiser, I. I. (1991) Arch. Biochem. Biophys. **284**, 352–359
- Lomonte, B., Gutiérrez, J. M., Carmona, E. and Rovira, M. E. (1990) Toxicon **28**, 379–384
- Lomonte, B., Gutiérrez, J. M., Ramírez, M. and Díaz, C. (1992) Toxicon **30**, 239–245
- Lomonte, B., Tarkowski, A., Bagge, U. and Hanson, L. Å. (1994) Biochem. Pharmacol. **47**, 1509–1518
- Lomonte, B., Moreno, E., Tarkowski, A., Hanson, L. Å. and Maccarana, M. (1994) J. Biol. Chem. **269**, 29867–29873
- Ovadia, M. (1978) Toxicon **16**, 661–672
- Borkow, G., Gutiérrez, J. M. and Ovadia, M. (1994) Biochim. Biophys. Acta **1201**, 482–490
- Ovadia, M., Kochva, E. and Moav, B. (1977) Biochim. Biophys. Acta **491**, 370–386
- Perales, J., Villela, C., Domont, G. B., Choumet, V., Saliou, B., Moussatché, H., Bon, C. and Faure, G. (1995) Eur. J. Biochem. **227**, 19–26
- Ohkura, N., Inoue, S., Ikeda, K. and Hayashi, K. (1993) J. Biochem. (Tokyo) **113**, 413–419
- Ohkura, N., Inoue, S., Ikeda, K. and Hayashi, K. (1994) Biochem. Biophys. Res. Commun. **200**, 784–788
- Domont, G. B., Perales, J. and Moussatché, H. (1991) Toxicon **29**, 1183–1194
- Fortes-Dias, C. L., Fonseca, B. C. B., Kochva, E. and Diniz, C. R. (1991) Toxicon **29**, 997–1008
- Fortes-Dias, C. L., Lin, Y., Ewell, J., Diniz, C. R. and Liu, T.-Y. (1994) J. Biol. Chem. **269**, 15646–15651
- Borkow, G., Gutiérrez, J. M. and Ovadia, M. (1995) Biochim. Biophys. Acta **1245**, 232–238
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Pharmacia Fine Chemicals (1982) Isoelectric Focusing: Principles and Methods. Ljungföretagen AB, Örebro, Sweden
- Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Dole, V. P. (1956) J. Clin. Invest. **35**, 150–154
- Gutiérrez, J. M., Lomonte, B., Chaves, F., Moreno, E. and Cerdas, L. (1986) Comp. Biochem. Physiol. **84C**, 159–164
- Gutiérrez, J. M., Avila, C., Rojas, E. and Cerdas, L. (1988) Toxicon **26**, 411–413
- Bussolino, F., De Rossi, M., Sica, A., Colotta, F., Wang, J. M., Bocchietto, E., Padura, I. M., Bosia, A., Dejana, E. and Mantovani, A. (1991) J. Immunol. **147**, 2122–2129
- Lomonte, B., Tarkowski, A. and Hanson, L. Å. (1994) Toxicon **32**, 1359–1369
- Gutiérrez, J. M., Cerdas, L., Arroyo, O., Rojas, E., Lomonte, B. and Gené, J. A. (1982) Acta Méd. Costarricense **25**, 255–262
- Yamakawa, M., Nozaki, M. and Hokama, Z. (1976) in Animal, Plant, and Microbial Toxins (Ohsaka, A., Hayashi, K. and Sawai, Y., eds.), pp. 97–109, Plenum Press, New York
- Inoue, S., Kogaki, H., Ikeda, K., Samejima, Y. and Omori-satoh, T. (1991) J. Biol. Chem. **266**, 1001–1007
- Ohkura, N., Inoue, S., Ikeda, K. and Hayashi, K. (1994) Biochem. Biophys. Res. Commun. **204**, 1212–1218
- Taylor, M. E., Conary, J. T., Lennartz, M. R., Stahl, P. D. and Drickamer, K. (1990) J. Biol. Chem. **265**, 12156–12162
- Ishizaki, J., Higashino, K.-I., Ohara, O. and Arita, H. (1995) in Advances in Prostaglandin, Thromboxane, and Leukotriene Research, vol. 23 (Samuelsson, B. et al., eds.), pp. 85–87, Raven Press, New York
- Ancian, P., Lambeau, G., Mattéi, M.-G. and Lazdunskii, M. (1995) J. Biol. Chem. **270**, 8963–8970
- Higashino, K.-I., Ishizaki, J., Kishino, J., Ohara, O. and Arita, H. (1994) Eur. J. Biochem. **225**, 375–382
- Díaz, C. and Gutiérrez, J. M. (1996) Toxicon, in the press
- Bultrón, E., Gutiérrez, J. M. and Thelestam, M. (1993) Toxicon **31**, 217–222
- Díaz, C., Gutiérrez, J. M., Lomonte, B. and Gené, J. A. (1991) Biochim. Biophys. Acta **1070**, 455–460