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Toxicon 45 (2005) 807–815

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Antimicrobial activity of myotoxic phospholipases A_2 from crotalid snake venoms and synthetic peptide variants derived from their C-terminal region☆

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Received 28 June 2004; revised 6 September 2004; accepted 21 September 2004

Available online 5 November 2004

Abstract

A short peptide derived from the C-terminal region of *Bothrops asper* myotoxin II, a Lys49 phospholipase A_2 (PLA_2), was previously found to reproduce the bactericidal activity of its parent molecule. In this study, a panel of eight PLA_2 myotoxins purified from crotalid snake venoms, including both Lys49 and Asp49-type isoforms, were all found to express bactericidal activity, indicating that this may be a common action of the group IIA PLA_2 protein family. A series of 10 synthetic peptide variants, based on the original C-terminal sequence 115–129 of myotoxin II and its triple Tyr→Trp substituted peptide p115-W3, were characterized. In vitro assays for bactericidal, cytolytic and anti-endotoxic activities of these peptides suggest a general correlation between the number of tryptophan substitutions introduced and microbicidal potency, both against Gram-negative (*Salmonella typhimurium*) and Gram-positive (*Staphylococcus aureus*) bacteria. Peptide variants with high bactericidal activity also tended to be more cytolytic towards skeletal muscle C2C12 myoblasts, thus limiting their potential in vivo use. However, the peptide variant pEM-2 (KKWRWWLKALAKK) showed reduced toxicity towards muscle cells, while retaining high bactericidal potency. This peptide also showed the highest endotoxin-neutralizing activity in vitro, and was shown to functionally interact with lipopolysaccharide (LPS) using a chimeric bacteria model. The bactericidal and anti-endotoxic properties of pEM-2, combined with its relatively low toxicity towards eukaryotic cells, highlight it as a promising candidate for further evaluation of its antimicrobial potential in vivo.

Keywords: Antimicrobial; Peptide; Phospholipase A2; Cationic; Snake venom

1. Introduction

Secreted phospholipases A_2 ($sPLA_2$; EC 3.1.1.4) constitute a diverse group of enzymes that are widespread in nature, being particularly abundant in animal venoms. In addition to their catalytic activity, hydrolyzing the *sn*-2 ester bond of glycerophospholipids, $sPLA_2$s display an array of

☆ INSERM patent submitted. (No. 04165A10).

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0041-0101/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2004.09.012

biological actions, which may be either dependent or independent of catalysis (Kini, 1997; Lambeau and Lazdunski, 1999; Kudo and Murakami, 2002). The group IIA $sPLA_2$ of mammalian origin has been shown to exert a potent bactericidal activity of physiological relevance, and is now regarded as a component of the acute-phase response (Weinrauch et al., 1996; Buckland and Wilton, 2000; Koduri et al., 2002). The bactericidal activity of this protein involves both the recognition of anionic sites, and the enzymatic degradation of phospholipids in the target membranes, preferentially of Gram-positive species. On the other hand, killing of Gram-negative bacteria by this enzyme requires a synergistic action with bactericidal/permeability-increasing protein, but is equally dependent on enzymatic membrane degradation (Elsbach et al., 1979; Koduri et al., 2002).

In contrast to the mammalian group IIA $sPLA_2$, two homologous myotoxic $sPLA_2$s isolated from the venom of the snake *Bothrops asper*, also classified within group IIA, were shown to directly kill both Gram-positive and Gram-negative bacteria (Páramo et al., 1998). One of them, *B. asper* myotoxin II, is a catalytically-inactive Lys49 PLA_2 (Lomonte and Gutiérrez, 1989; reviewed by Lomonte et al., 2003), and therefore this demonstrated the first catalytic-independent bactericidal mechanism exerted by a $sPLA_2$ homologue (Páramo et al., 1998). Furthermore, bactericidal action was reproduced by a short sequence of the protein, corresponding to residues 115–129 of its cytolytic C-terminal region (Lomonte et al., 1994), when tested as a free synthetic peptide (Páramo et al., 1998).

Considering its small mass (13 amino acid residues), its direct permeabilizing action upon bacterial membranes, its ability to interact with lipopolysaccharide and lipid A (Páramo et al., 1998), and its lack of hemolytic activity (Lomonte et al., 1999a), peptide 115–129 (p115–129) called our interest as a possible lead compound with antimicrobial and antiendotoxic potential. An initial modification of its sequence, by introducing a triple Tyr→Trp substitution, resulted in a marked increase of bactericidal potency, but also drastically enhanced its toxicity towards eukaryotic cells, both in culture and in vivo (Lomonte et al., 1999a). Based on the Tyr→Trp modified sequence 115–129, a series of new synthetic peptide variants were prepared and characterized in the present study. Their bactericidal and cytolytic properties were evaluated, with the aim of identifying a suitable peptide with antimicrobial potential. In addition to these peptide variants, a panel of eight PLA_2 myotoxins, isolated from various crotalid snake species, was utilized to investigate if bactericidal activity is a common feature of this protein family. Since the C-terminal region of PLA_2 myotoxins displays significant sequence diversity among these proteins (Lomonte et al., 2003; Liu et al., 2003), this information might provide additional clues to guide the development and optimization of bioactive cationic peptides.

2. Materials and methods

2.1. Isolation of PLA_2 myotoxins

Crude venoms were fractionated by cation-exchange chromatography on CM-Sephadex C-25, in order to isolate the following myotoxic PLA_2 s: *Bothrops asper* myotoxins I (Gutiérrez et al., 1984), II (Lomonte and Gutiérrez, 1989), III (Kaiser et al., 1990), and IV (Díaz et al., 1995); *Bothriechis schlegelii* myotoxin I (Angulo et al., 1997); *Cerrophidion godmani* myotoxins I and II (Díaz et al., 1992); and *Atropoides nummifer* myotoxin I (Gutiérrez et al., 1986).

2.2. Peptide synthesis

Peptides were derived from the original sequence 115–129 (KKYRYYLKPLCKK) of *B. asper* myotoxin II (Páramo et al., 1998), and its triple Tyr→Trp substituted peptide (p115-W3; Lomonte et al., 1999a). They were synthesized by Fmoc [N-(9 fluoreny)-methoxycarbonyl] chemistry (Fields and Noble, 1990), and their amino acid sequences are listed in Table 1. Peptides were finally purified by reverse-phase high-performance liquid chromatography (RP-HPLC) and obtained with at least 95% purity. Their observed molecular masses, as determined by mass spectrometry, corresponded to expected theoretical values. Dry peptides were stored at −20 °C, and redissolved in pyrogen-free saline solution (0.15 M NaCl) immediately before use.

2.3. Bacteria and lipopolysaccharides

Bacteria were maintained at −70 °C in trypticase soy broth (TSB) containing 10% (v/v) glycerol. Frozen stocks were thawed and cultured for 4 h (*Salmonella typhimurium* and *Staphylococcus aureus*) or overnight (*Brucella abortus*) at 37 °C in TSB. Purified lipopolysaccharide (LPS) from *Escherichia coli* O111:H4 and *S. typhimurium* were kindly provided by Dr. Andrej Weintraub (Karolinska Institute, Sweden). Lyophilized LPS was diluted in endotoxin-free saline and dispersed by mild sonication, immediately before use.

2.4. Bactericidal assay

Bactericidal activity was determined as described by Páramo et al. (1998). Briefly, log-phase bacteria were obtained from TSB cultures, and their concentration adjusted to 4×10^6 colony-forming units (CFU)/ml, in 0.01 M phosphate buffer, pH 7.4, containing 1% peptone (PPB), by reading turbidity at 540 nm (*S. typhimurium* and *S. aureus*) or 420 nm (*B. abortus*), respectively. Bacterial suspensions (100 μl) were incubated with 10 μl of varying concentrations of peptides, or PPB alone, for 20 min at 37 °C. Finally, they were plated on tripticase soy or blood agar, and viable bacteria were counted after 24 h of growth at 37 °C.

Table 1
Amino acid sequences of ten synthetic peptide variants derived from the original C-terminal sequence 115–129 of *Bothrops asper* myotoxin II (p115–129), and its triple Tyr→Trp substituted peptide (p115-W3)

Numbering *	**115**	**116**	**117**	**118**	**119**	**120**	**121**	**122**	**123**	**124**	**125**	**128**	**129**
p115-129 (original) **	**K**	**K**	**Y**	**R**	**Y**	**Y**	**L**	**K**	**P**	**L**	**C**	**K**	**K**
p115-W3 (variant) **	**K**	**K**	**W**	**R**	**W**	**W**	**L**	**K**	**P**	**L**	**C**	**K**	**K**
pEM-1	K	K	W	R	W	W	L	K	P	L	A	K	K
pEM-2	K	K	W	R	W	W	L	K	A	L	A	K	K
pEM-3	K	K	Y	R	W	Y	L	K	P	L	C	K	K
pEM-4	K	K	Y	R	Y	W	L	K	P	L	C	K	K
pEM-5	K	K	W	R	Y	Y	L	K	P	L	C	K	K
pEM-6	K	K	W	R	W	Y	L	-	P	L	C	K	K
pEM-7	K	K	Y	R	W	W	L	K	P	L	C	K	K
pEM-8	K	K	W	R	Y	W	L	K	P	L	C	K	K
pEM-9	K	K	W	R	W	W	L	W	W	R	W	K	K
pEM-10	K	K	A	L	A	K	L	K	A	L	A	K	K

* Numbering according to Renetseder et al. (1985). ** Previously characterized by Páramo et al. (1998) and Lomonte et al. (1999a), respectively; in pEM-1 through pEM-10, differences from the original p115–129 sequence are highlighted by a gray background.

2.5. Cytolytic activity

The cytolytic effect of peptides was determined on the murine skeletal muscle cell line C2C12 (ATCC CRL-1772), as described (Lomonte et al., 1999b). In brief, cells were grown to near-confluence in 96-well plates, in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 15% fetal calf serum (FCS), in an atmosphere with 7% CO_2, at 37 °C. Immediately before the experiments, growth medium was aspirated, and replaced with 150 μl of assay medium (DMEM with 1% FCS), containing various peptide amounts (12, 25, 50, and 100 μg). After 3 h at 37 °C, the activity of lactic dehydrogenase (LDH) released from damaged cells was determined colorimetrically (Sigma 500C) in supernatant samples. Controls for 0% and 100% toxicity values consisted of assay medium, and 0.1% Triton X-100 in assay medium, respectively.

2.6. Limulus amoebocyte lysate (LAL) assay

In vitro neutralization of LPS activity was assessed by the LAL chromogenic assay (BioWhittaker, Walkersville, MD), according to the manufacturer's instructions. *E. coli* LPS was solubilized at a concentration of 5 endotoxin units (EU)/ml by sonication in endotoxin-free water. Peptides were prepared at concentrations of 12.5, 25, 50, 100 and 200 μg/ml. Fifty microlitre of endotoxin were mixed with 50 μl of the different peptide solutions, in a 96-well plate, and incubated at 37 °C for 20 min. Then, 100 μl of LAL reagent were added to each well, and incubated for 10 min at 37 °C. Finally, 100 μl of substrate (Ac-Ile-Glu-Ala-Arg-pNA colorless peptide) was added to the wells, mixed, and incubated at 37 °C for 6 min. Reactions were stopped with 100 μl of 25% (v/v) acetic acid. Final absorbances were determined at 405 nm, on a Labsystems RC microplate reader. Results were expressed as inhibition percentages, considering the control endotoxin activity values as 100%.

2.7. Preparation of bacterial LPS chimeras

Coating of live rough *B. abortus* 45/20 with *S. typhimurium* LPS was carried out as described (Martínez de Tejada et al., 1995), with minor modifications. LPS (20 mg/ml) was sonicated for 20 s at maximum frequency (Model 450 Sonifier, Branson Ultrasonics) in PPB, and sterilized by filtration through 0.22 μm membranes (Millipore Corp.). Log-phase *B. abortus* 45/20 (200 μl), harvested in PPB, were adjusted to 4×10^7 CFU/ml and mixed with an equal volume of *S. typhimurium* LPS, at various concentrations. Mixtures were sonicated briefly and incubated overnight at 37 °C. Unbound LPS was removed by washing repeatedly in PPB at $14{,}000\times g$ for 15 min, at room temperature. Bacterial pellets were resuspended in 200 μl of PPB, and used immediately for bactericidal assays.

2.8. Statistical analyses

All assays were performed in triplicate, and at least two independent assays were performed for all data collection. Values were expressed as mean ± SD, and statistically compared using ANOVA or unpaired Student's *t*-test.

3. Results

3.1. Bactericidal effect of myotoxic phospholipases A_2

All of the eight purified myotoxins, including both 'Asp49' and 'Lys49' PLA_2 variants, displayed direct bactericidal activity (Fig. 1). Minor variations in their bactericidal potencies were observed, although within the same order of magnitude, requiring concentrations of nearly 250 μg/ml to achieve complete bacterial killing under the conditions described (Fig. 1).

3.2. Bactericidal activity of p115–129 variants

With the exception of pEM-10, the different peptide variants displayed significant bactericidal activity, both against *S. typhimurium* (Fig. 2) and *S. aureus* (Fig. 3). In general, peptides tended to be more active against the latter, as observed at lower concentrations (5 and 10 μg/ml). Peptide variants containing a double Tyr→Trp substitution, such as pEM-7 and pEM-8, showed a higher microbicidal potency against both types of bacteria, in comparison to those possessing single Tyr→Trp substitutions (pEM-3, pEM-4, pEM-5). However, pEM-6, also having two tryptophans, but lacking a lysine (Lys122), had a weaker bactericidal effect than pEM-7 and pEM-8. On the other hand, the triple Tyr→Trp substituted peptide pEM-2, and the construct pEM-9 (which mirrors the N-terminal half of pEM-2, and containing a total of six tryptophan residues) showed the highest activities against both bacteria, especially against *S. typhimurium*. Their bactericidal potencies were similar to that of the previously characterized p115-W3 (Lomonte et al., 1999a; and data not shown). The variant pEM-1, despite possessing three tryptophan residues, but conserving the original proline of p115–129 (Pro123), killed bacteria at a lower rate than pEM-2. Finally, pEM-10 (which duplicates the C-terminal half of pEM-2 in a mirror fashion), having six cationic residues, but devoid of tryptophan, was virtually inactive against both types of bacteria.

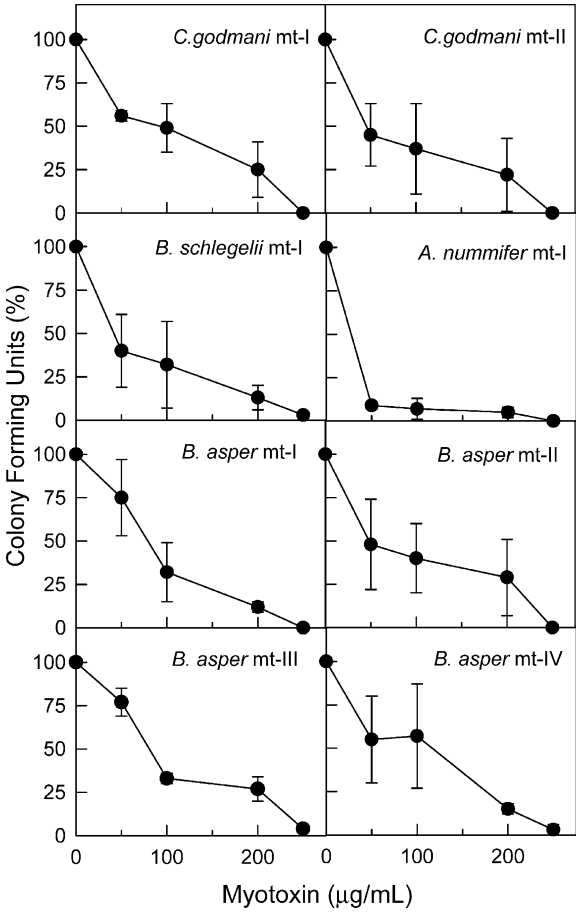

Fig. 1. Bactericidal activity of myotoxic phospholipases A_2 isolated from snake venoms. Different amounts of myotoxins, dissolved in 10 μl, were incubated at 37 °C for 20 min with 100 μl of *Salmonella typhimurium* (4×10^6 CFU/ml), and then viable counts of the bacteria were performed, after 24 h, under the conditions described in Section 2. CFU, colony-forming units. Each point represents mean ± SD of triplicate counts.

3.3. Cytolytic activity of p115–129 variants

Toxicity of the peptide variants towards C2C12 myoblasts is summarized in Fig. 4. In general, the cytolytic action of peptides paralleled their bactericidal effects, but required considerably higher concentrations. The construct pEM-9 (containing six tryptophan residues) showed the highest toxicity against myoblasts, with nearly complete lysis induced at 333 μg/ml. Among the highly bactericidal variants, pEM-2 showed the lowest cytotoxic effect, below 10% at a concentration of 333 μg/ml (Fig. 4). In similarity with bactericidal assays, peptide pEM-10 was also virtually inactive upon the cultured cells.

3.4. LPS–peptide interaction

The ability of peptides to inhibit the LAL reaction was utilized as an indirect measure of their interaction with bacterial LPS. As summarized in Fig. 5, peptides displayed endotoxin inhibitory activity, within the range of 50–60% in comparison to polymyxin B, a control antiendotoxic compound. The highest antiendotoxin activity was obtained with pEM-2, with 85% inhibition (Fig. 5). The functional interaction between pEM-2 and LPS was further evaluated using bacterial LPS-chimeras. As shown in Fig. 6, the incorporation of LPS derived from the pEM-2-sensitive bacteria *S. typhimurium*, into *B. abortus*, a bacterial species known to be resistant to cationic peptides (Freer et al., 1996), caused a significant increase in its susceptibility to the bactericidal action of pEM-2.

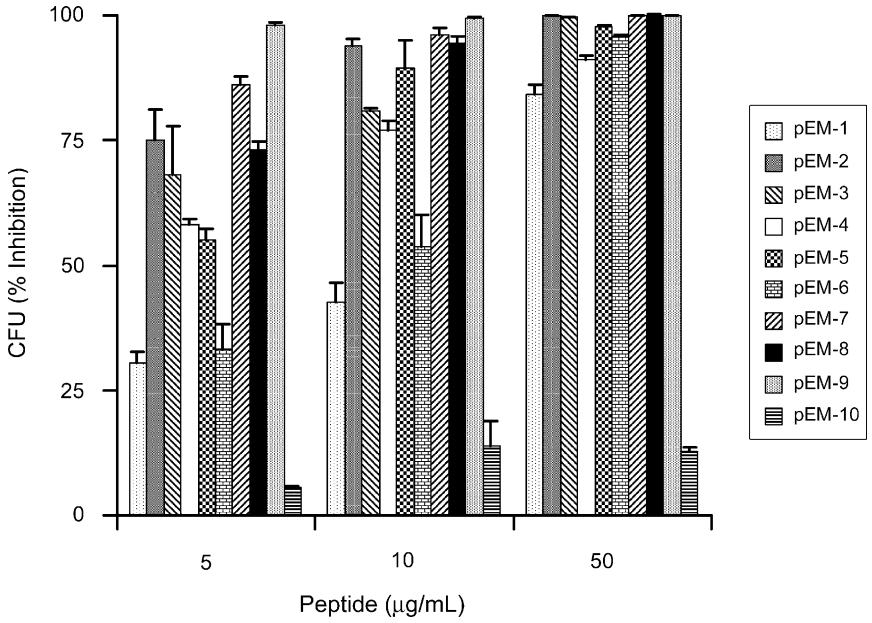

Fig. 2. Bactericidal activity of *B. asper* myotoxin II-derived synthetic peptide variants against *Staphylococcus aureus* ATCC 23923. Different amounts of peptides, dissolved in 10 μl, were incubated at 37 °C for 20 min with 100 μl of bacteria (4×10^6 CFU/ml), and then viable counts were performed, after 24 h, under the conditions described in Section 2. CFU, colony-forming units. Bars represent mean ± SD of triplicate viable counts.

4. Discussion

The antibacterial effect of PLA_2 s derived from snake venoms was first reported for myotoxins of *B. asper*, demonstrating a bactericidal mechanism independent from catalysis in the case of myotoxin II (Páramo et al., 1998). Recently, two other myotoxic Lys49 PLA_2s from snake venoms have also been reported to express direct

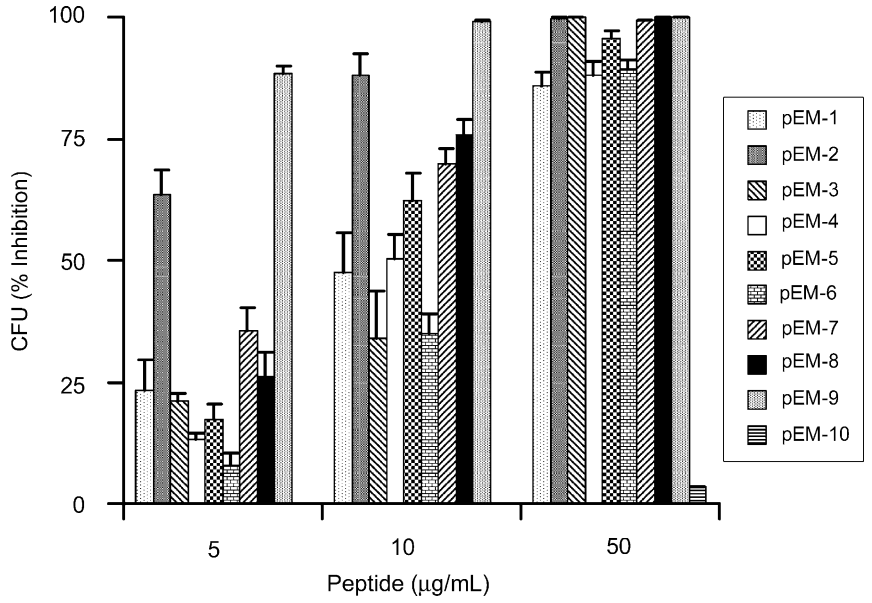

Fig. 3. Bactericidal activity of *B. asper* myotoxin II-derived synthetic peptide variants against *Salmonella typhimurium* ATCC 14028. Different amounts of peptides, dissolved in 10 μl, were incubated at 37 °C for 20 min with 100 μl of bacteria (4×10^6 CFU/ml), and then viable counts were performed, after 24 h, under the conditions described in Section 2. CFU, colony-forming units. Bars represent mean ± SD of triplicate viable counts.

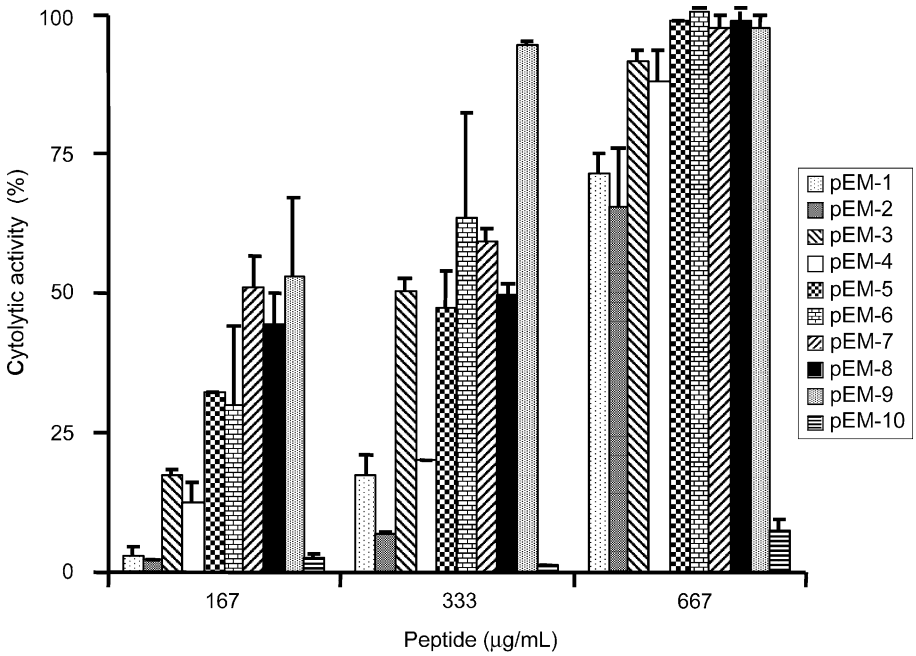

Fig. 4. Cytolytic activity of *B. asper* myotoxin II-derived synthetic peptide variants against C2C12 murine skeletal muscle myoblasts. Assay medium (150 μl) containing different amounts of peptides was added to cells and incubated for 3 h at 37 °C. Lactate dehydrogenase activity was then assayed in cell supernatants, as an indicator of cytolysis, under the conditions described in Section 2. Bars represent mean ± SD of triplicate assays.

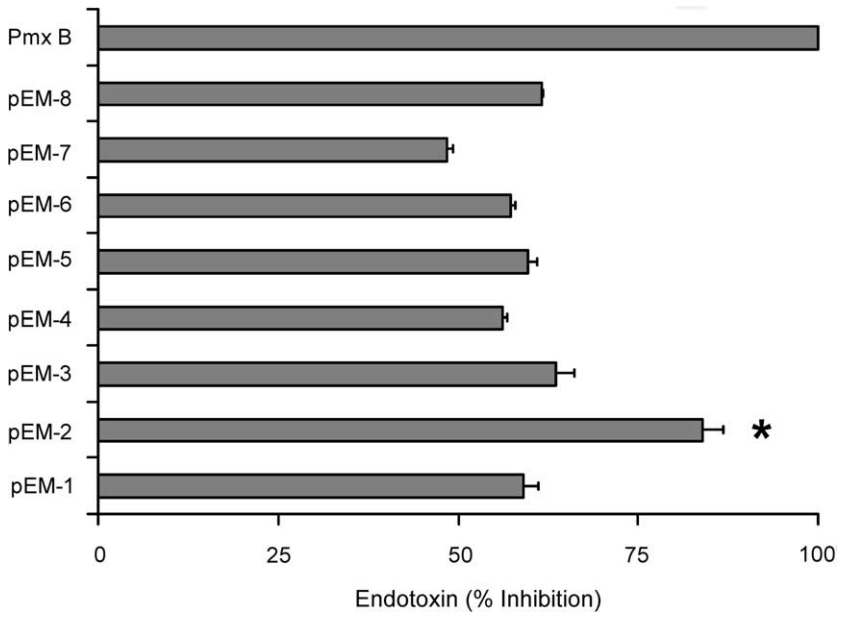

Fig. 5. Inhibition of the endotoxin activity of LPS by *B. asper* myotoxin II-derived synthetic peptide variants, using the chromogenic LAL assay. LPS from *Escherichia coli* (50 μl of five endotoxin units/ml) was incubated with the different peptides (50 μl of 200 μg/ml) for 20 min at 37 °C. LAL reactions were then performed as described in Section 2, and read colorimetrically at 405 nm. Results were expressed as inhibition percentages, considering the control endotoxin activity values as 100%. Bars represent mean ± SD of triplicate assays. Polymyxin B (Pmx B) was included as a control antiendotoxic reference. (*) Statistically significant difference ($p < 0.05$) between pEM-2 and the other synthetic peptides.

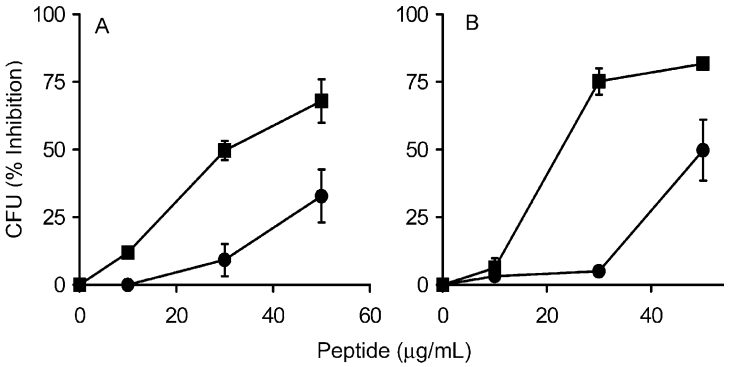

Fig. 6. Bactericidal activity of pEM-2 (A) and polymyxin B (B) on control *Brucella abortus* 45/20 (●) and chimeric *Brucella abortus* 45/20 containing *S. typhimurium* LPS (■). LPS from *S. typhimurium* was incorporated into *B. abortus* as described in Section 2, and then bactericidal activity of pEM-2 was assayed on both bacteria. CFU, colony-forming units. Each point represents mean ± SD of triplicate viable counts.

bactericidal action (Soares et al., 2000, 2001). In the present study, results obtained with a panel of eight myotoxic PLA_2s indicate that bactericidal activity may be a common effect of the group IIA PLA_2 protein family. Interestingly, both the catalytically-inactive (Lys49 PLA_2s: *C. godmani* myotoxin II, *B. schlegelii* myotoxin I, *A. nummifer* myotoxin I, *B. asper* myotoxins II and IV) and the catalytically-active (Asp49 PLA_2s: *C. godmani* myotoxin I, *B. asper* myotoxins I and III) proteins showed bactericidal activity. In the Asp49 group, however, it remains to be studied if the antimicrobial action depends on their catalytic activity, or if it is a catalytic-independent effect, as in Lys49 myotoxins.

The effector region for bactericidal activity in myotoxin II encompasses its residues 115–129 (Páramo et al., 1998). Several modifications of this sequence were evaluated, with the aim of identifying an antimicrobial peptide with high activity against bacteria, but minimal toxicity towards eukaryotic cells. Modifications (Table 1) were based on a previously described derivative of p115–129, named p115-W3, which expresses a markedly enhanced bactericidal action due to a triple Tyr→Trp substitution (Lomonte et al., 1999a). Changes evaluated in the present study focused on the tryptophan substitutions (pEM-3 through pEM-8). Also, the substitution of cysteine (to avoid oxidative peptide dimerization; pEM-1, pEM-2) and proline (to facilitate synthesis; pEM-2) by alanine, were assessed in some variants. The variant pEM-6 lacked Lys122, a residue identified as critical for the membrane-distrupting activities of the Lys49 PLA_2 *B. jararacussu* bothropstoxin I (Chioato et al., 2002). Two additional peptides corresponded to constructs that duplicated the N-terminal (pEM-9) and C-terminal (pEM-10) halves of pEM-2, in a mirror fashion, in order to evaluate which played the most relevant role in bactericidal activity.

Results suggest a general correlation between bactericidal potency and number of tryptophan residues. Peptides with double Tyr→Trp substitutions (pEM-2, pEM-7–pEM-9), irrespective of their positions, displayed an enhanced bactericidal activity in comparison to single Trp-substituted peptides, against both Gram-negative and Gram-positive bacteria. Results obtained with pEM-10, which was harmless to both bacteria and muscle cells, demonstrate that a highly cationic content alone (provided by the C-terminal half of pEM-2), in the absence of the tryptophan residues from the N-terminal half of pEM-2, cannot support a membrane-damaging effect. Altogether, these findings are in agreement with the concept that bactericidal peptides permeabilize membranes on the basis of combined ionic and hydrophobic interactions (Kini and Evans, 1989; Besalle et al., 1993; Hancock and Scott, 2001), where tryptophan often has a critical role (Yau et al., 1998; Baker et al., 1998; Gelb et al., 1999). They also support the notion that membrane-perturbing effects of cationic peptides are not strictly dependent on a fixed, invariant sequence, but may be achieved with a variety of amino acid combinations, where specific sequences might not be as critical as the overall amphiphilic nature of the compounds (Kini and Evans, 1989; Hancock and Lehrer, 1998; Lomonte et al., 2003).

An important limitation to the potential clinical applications of cationic peptides is their relatively high toxicity (Hancock and Scott, 2001; Zasloff, 2002). Using an in vitro murine myoblast model, it was shown that the PLA_2-derived peptides have variable toxic potencies against eukaryotic cells. In general, peptides with two or more tryptophan residues induced higher damage to cells, suggesting that a high hydrophobic index promotes not only the interaction with prokaryotic membranes, but also with eukaryotic ones. The variant pEM-9, with six tryptophan residues, was particularly cytolytic, even at low concentrations. Conversely, peptides with one, or devoid of tryptophan, tended to display lower cytotoxic potencies.

Among the strongly bactericidal peptides, showing a potency similar to the highly toxic p115-W3 (Lomonte et al., 1999a), pEM-2 had the lowest cytolytic action against myoblasts, and was therefore selected for further characterization. In the LAL assays, pEM-2 also showed

the highest endotoxin-neutralizing ability, compared to the other peptide variants, a feature that could be potentially useful in the treatment of endotoxic shock. Furthermore, the interaction between pEM-2 and LPS was demonstrated to be functionally relevant, using chimeric *B. abortus* containing *S. typhimurium* LPS, which clearly modified their susceptibility to the bactericidal action of this peptide. On the basis of its combined properties, the snake venom PLA_2-derived peptide pEM-2 appears as an interesting candidate for further preclinical assessment of its bactericidal and antiendotoxic potential, in future studies.

Acknowledgements

We gratefully acknowledge the support by the NeTropica Sweden-Central America network (01-R-2003), the American Society for Microbiology-MIRCEN, Lindbergh Foundation, CR-USA Foundation, Florida, Ice and Farm Company, the Embassy of France, the Embassy of Japan, and the University of Costa Rica (VI-741-99-269). Thanks are also due to Drs P. Fourquet (CIML, Marseille, France), A. Tarkowski (University of Göteborg, Sweden), and A. Weintraub (Karolinska Institute, Sweden) for valuable collaborations. This work was performed in partial fulfillment of the requirements for the MSc degree of C. Santamaría at the University of Costa Rica.

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