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Bactericidal activity of Lys49 and Asp49 myotoxic phospholipases A_2 from *Bothrops asper* snake venom

Synthetic Lys49 myotoxin II-(115–129)-peptide identifies its bactericidal region

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Mammalian group-II phospholipases A_2 (PLA_2) of inflammatory fluids display bactericidal properties, which are dependent on their enzymatic activity. This study shows that myotoxins II (Lys49) and III (Asp49), two group-II PLA_2 isoforms from the venom of *Bothrops asper*, are lethal to a broad spectrum of bacteria. Since the catalytically inactive Lys49 myotoxin II isoform has similar bactericidal effects to its catalytically active Asp49 counterpart, a bactericidal mechanism that is independent of an intrinsic PLA_2 activity is demonstrated. Moreover, a synthetic 13-residue peptide of myotoxin II, comprising residues 115−129 (common numbering system) near the C-terminal loop, reproduced the bactericidal effect of the intact protein. Following exposure to the peptide or the protein, accelerated uptake of the hydrophobic probe *N*-phenyl-*N*-naphthylamine was observed in susceptible but not in resistant bacteria, indicating that the lethal effect was initiated on the bacterial membrane. The outer membrane, isolated lipopolysaccharide (LPS), and lipid A of susceptible bacteria showed higher binding to the myotoxin II-(115−129)-peptide than the corresponding moieties of resistant strains. Bacterial LPS chimeras indicated that LPS is a relevant target for myotoxin II-(115−129)-peptide. When heterologous LPS of the resistant strain was present in the context of susceptible bacteria, the chimera became resistant, and *vice versa*. Myotoxin II represents a group-II PLA_2 with a direct bactericidal effect that is independent of an intrinsic enzymatic activity, but adscribed to the presence of a short cluster of basic/hydrophobic amino acids near its C-terminal loop.

Keywords: phospholipase A_2; myotoxin; bactericidal; synthetic peptide; snake venom.

Secreted forms of phospholipase A_2 (PLA_2) have been classified into three groups, on the basis of their primary structures: group I includes mammalian pancreatic and Elapidae snake venom PLA_2; group II is formed by the mammalian non-pancreatic and the Viperidae snake venom PLA_2; and PLA_2 from bee venom and lizards are classified as group III [1]. Many of the venom PLA_2 have acquired toxic activities, including neurotoxic, myotoxic, cytotoxic, anticoagulant and inflammatogenic effects [2], and an accelerated genetic evolution process has been demonstrated for some of them [3]. The discovery of natural group-II PLA_2 variants in the venoms of viperids [4, 5] that lack or have extremely low catalytic activity but which still display toxicity comparable to that of their catalytically active counterparts has been particularly valuable in demonstrating that these proteins possess membrane-perturbing activities that are not related to an intrinsic enzymatic mechanism. One of these variants is myotoxin II, a catalytically-inactive PLA_2 isoform isolated from the venom of *Bothrops asper*, which induces local myonecrosis and edema in mice [6], and is cytotoxic to a variety of cell types *in vitro* [7]. The lack of PLA_2 activity of myotoxin II is attributed to critical amino acid substitutions, including the replacement of the conserved aspartate by lysine at position 49 (sequence numbering according to [8]), together with changes in residues forming the Ca^{2+}-binding loop [9−11].

Using liposomes as a model target, a Ca^{2+}-independent membrane-disrupting mechanism, which differs from that of catalytically active PLA_2 isoforms, has been shown for myotoxin II [12, 13]. Evidence suggests that its membrane-perturbing activity is related, at least in part, to the presence of a combination of basic and hydrophobic amino acids near the C-terminal end of the molecule, comprising residues 115−129 [14]. A 13-residue synthetic peptide corresponding to this region caused cytolysis of endothelial cells *in vitro* [14], although it did not induce local myotoxicity in mice. On the other hand, cleavage of the N-terminal octapeptide of myotoxin II affected its membrane-destabilizing activities, suggesting that this region may be relevant for toxicity [15, 16].

Previous studies have described that some catalytically active group-II PLA_2 play a role in the degradation of gram-negative bacteria, acting independently [17] or in concert with other host-defense systems, such as bactericidal/permeability-increasing factor and complement [18, 19]. Using site-directed

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Abbreviations. PLA_2, phospholipase A_2; LPS, lipopolysaccharide; CFU, colony-forming units; Ph-NH-Nap, *N*-phenyl-*N*-naphthylamine.

Enzyme. Phospholipase A_2 (EC 3.1.1.4).

Table 1. Bactericidal activity of myotoxin II-(115–129)-peptide relative to that of polymyxin B sulfate. S-LPS, smooth-type LPS; R-LPS, rough-type LPS. CDC, Centers for Disease Control.

Bacteria	Peptide or polymyxin	Reduction of CFU by		Source
		polymyxin B	peptide	
	μg	%		
B. abortus 2308, S-LPS, virulent, biotype 1	>80	29	16	National University
B. abortus 45/20, R-LPS, attenuated	>80	30	51	National University
B. abortus S19, S-LPS, vaccine, biotype 1	>80	17	21	[50]
E. coli 29648, S-LPS, sensitivity studies	40	100	74	ATCC
E. coli K-12, W-1485, serotype Ra	20	100	96	ATCC
Listeria monocytogenes WS-2247	90	100	44	ATCC
Pasteurella multocida 12945, type A	60	98	36	ATCC
Salmonella montevideo SH94, S-LPS, serogrup C1	20	100	30	Karolinska Institute
Salmonella typhi 6539, S-LPS	20	100	66	ATCC
Shigella sonnei 25931, S-LPS	20	100	95	ATCC
S. aureus Cowan-1	80	99	98	ATCC
Streptococcus pyogenes 19615, *β.* h.	70	91	41	ATCC
V. cholerae O1, Inaba, R-LPS, Tor	20	100	99	CDC, Atlanta GA
V. cholerae O1, Ogawa, S-LPS, Tor	20	100	100	National University

mutagenesis, the structural determinants of action of these PLA_2 on bacteria have been mapped to a cluster of basic amino acid residues in their N-terminal helix [20, 21]. Recently, a direct bactericidal effect of the human non-pancreatic group-II PLA_2 towards *Staphyloccocus aureus*, which correlated with its catalytic activity, was described [22]. In the present work, we describe and characterize the bactericidal effect of a group-II Lys49 PLA_2, myotoxin II, on gram-negative and gram-positive bacteria, and show that synthetic myotoxin II-(115−129)-peptide reproduces the effects of the intact protein, providing evidence for a bactericidal mechanism not related to catalytic activity in a PLA_2 molecule.

MATERIALS AND METHODS

Myotoxic PLA_2 and peptides. Myotoxin II was isolated from the venom of *B. asper* from Costa Rica as described [6], appearing as a single band on SDS/PAGE and cathodic native PAGE [23, 24], and having undetectable PLA_2 activity in an indirect hemolytic gel assay [25]. In some experiments, myotoxin III, an Asp49 catalytically active isoform [26], purified by the same procedure as for myotoxin II, was utilized for comparison. Two cationic peptides, corresponding to residues 60−71 (KKDRYSYSWKDK) and 105−117 (KKYRYYLKPLCKK) of myotoxin II (residues 69−80 and 115−129 in the common numbering system, respectively), with native endings, were synthesized by Chiron Mimotopes using Fmoc strategy [27]. Their purity was at least 95%, as assessed by HPLC on a LiCrosphere 100RP-18 column or by mass spectrometry. The sequences were based on data from Francis et al. [11]. For some experiments, myotoxin II-(115−129)-peptide was conjugated to diphtheria toxoid as a carrier, at a peptide/carrier ratio of 3.2:1 (Chiron Mimotopes). Polymyxin B sulfate, poly(L-lysine), poly(L-ornithine), cecropins A and P1, mellitin, magainin-2-amide, magainin 2b, lysozyme and lactoferrin were purchased from Sigma Chemical Co. Lactoferricin B was kindly provided by W. Belamy (Morinaga Dairy Co., Higashihara, Japan), cationic protein 18A, bactenecins 5 and 7 were donated by R. Gennaro and D. Romeo (Department of Biophysics and Chemistry of Macromolecules, University of Trieste, Italy), and defensin NP-2 by R. I. Lehrer (Department of Medicine, University of California).

Bacterial strains and growth conditions. The bacterial strains utilized are described in Table 1. Culture conditions of smooth *Brucella abortus* S19, rough *B. abortus* 45/20, *Salmonella montevideo* SH94 and smooth *Escherichia coli* ATCC 29648 have been described [28]. All other bacteria were grown in blood agar (Difco Laboratories) following established protocols [29]. Bacteria were harvested and suspended in 0.01 M sodium phosphate, pH 7.4 (buffer A) containing 1% peptone (Difco), and immediately subjected to bactericidal assays.

Bactericidal-activity assays. All the bactericidal assays were carried out in buffer A containing 1% peptone, except the assay for lactoferricin B, which was performed in buffer A without peptone. Bacteria were harvested from fresh agar plates and their concentrations were adjusted to 4×10^6 colony-forming units (CFU)/ml. 100 μl, containing 4×10^5 CFU, were incubated for 20 min at 37 °C with different concentrations of the proteins or peptides. The live bacteria were counted on trypticase soy agar (Difco) plates. Assays were performed in quadruplicate. To maximize the use of myotoxin II-(115−129)-peptide, its relative bactericidal activity was compared with that of polymyxin B, by testing both agents at the same concentration, which resulted in a 90−100% reduction in CFU for polymyxin. Minimal bactericidal concentrations were estimated as described [30]. The Students' *t*-test and variance analysis were utilized for statistical comparisons.

Extraction and purification of bacterial molecules. Extraction and purification of smooth type lipopolysaccharide (LPS) from *B. abortus* 2308, and *E. coli* ATCC 29648 have been described elsewhere [31, 32]. *Brucella* LPS was obtained from the phenol phase of the method of Baker and Wilson [33] and digested in three consecutive steps with DNAase I, RNAase A and proteinase K. After centrifugation at $10000\times g$ the preparation was subjected to gel filtration in the presence of chaothropic agents [34]. The LPS was centrifuged, and removal of phospholipids and ornithine lipids was performed with chloroform/methanol/water (1:8:0.8, by vol.) followed by chloroform/methanol/7 M ammonia (65:25:4, by vol.). *E. coli* ATCC 29648 smooth LPS was obtained from the aqueous phase of the method described [35], sonicated, and digested with DNAase I, RNAase A and proteinase K. The preparation was extracted with phenol/water, dialyzed and recovered by centrifugation. All LPS preparations were lyophilized after extensive dialysis, and analyzed

by standard procedures [31]. Data on the chemical and physical characteristics of these LPS preparations have been described [34, 36, 37]. Lipid A and O-chain polysaccharides were obtained by mild acetic acid hydrolysis of the LPS [38] and purified as described [39]. Lipoteichoic acids from *S. aureus* Cowan-1 were extracted at 68 °C with phenol/water [40]. The aqueous phase was precipitated with 5 vol. absolute ethanol (Merck) with 10% saturated sodium acetate at −20 °C. The mixture was centrifuged, and the precipitate washed twice with cold absolute ethanol, dialyzed and lyophilized. The material was digested with RNAase, DNAase, and proteinase K, then centrifuged, extracted with hot phenol/water, precipitated with ethanol, dialyzed and lyophilized. The purity of lipoteichoic acids was evaluated by SDS/PAGE followed by silver staining [23, 41].

Transmission electron microscopy. For the electron microscopic assessment of morphological alterations, bacteria or bacterial LPS chimeras were adjusted to 10^9-10^{10} CFU/ml, and incubated with the different agents at the same proportions utilized in bactericidal assays. Samples were fixed with 4% glutaraldehyde in buffer A for 1 h at 4 °C, embedded in 3% low-gelling-temperature agarose (Sea Plaque, FMC Corp.), and stored for 12 h in fixative at 4 °C. Agarose pellets were cut into 3-mm^3 pieces, immersed in fixative for 2 h at 25 °C, washed three times for 10 min with buffer A, and fixed with 1% osmium tetraoxide (Agar Scientific Ltd) in buffer A, for 2 h at 25 °C [42]. Sections were washed three times, dehydrated in increasing concentrations of ethanol and propylene oxide, and embedded in Spurr resin (Agar Scientific). Polymerization was performed at 60 °C for 48 h, and ultrathin sections were prepared with a Sorvall MT2 ultramicrotome. The sections were placed on 5% collodion-coated 100-mesh grids, and stained with 4% uranyl acetate (Agar Scientific) for 15 min, followed by 2.6% lead citrate (Agar Scientific) for 15 min. Samples were observed under a Hitachi 1100 transmission electron microscope (Hitachi Scientific Instruments) operating at 100 kV.

Fluorimetry. The fluorescent probe *N*-phenyl-1-naphthylamine (Ph-NH-Nap; Ferosa, Scharlau S.A.) was utilized to assess alterations in outer membrane permeability of bacteria, since its quantum yield increases when transferred from a hydrophilic to hydrophobic environment [43]. Fluorimetric assays were carried out as described [28], with some modifications. Exponentially growing bacterial cells were suspended in buffer A containing 1 mM KCN, pH 7.2, adjusted to an A_{600} of 0.47, and transferred (1.5 ml) to fluorimetric cuvettes. 5 min before the addition of Ph-NH-Nap, myotoxin II-(115−129)-peptide (46 nM) or myotoxins (10 μM) were added. After stabilization of the mixture, Ph-NH-Nap (10 μM) was added and the fluorescence monitored at 20 °C in a LS-50 fluorimeter (Perkin-Elmer Ltd) set as follows: excitation 350 nm; emission 420 nm; slit width 2.5 nm. Under these conditions quenching was not observed. Boiled bacteria were used to determine maximal Ph-NH-Nap transference. Experiments were repeated three times under the same conditions, and the results expressed as relative fluorescence.

Binding of myotoxin II-(115–129)-peptide to bacterial cells and molecules. Binding of myotoxin II-(115−129)-peptide to whole bacterial cells or purified components was estimated indirectly, by comparing the diameters of inhibition halos caused by the peptide, either alone or after incubation with test substances. For whole-bacterial-cell-binding experiments, suspensions adjusted at different concentrations in buffer A were incubated with peptide (20 μg) in 50 μl, for 5 min at 37 °C, then for 20 min at 4 °C. For binding experiments with LPS, lipid A, O-polysaccharide chain and lipoteichoic acids, preparations adjusted to different concentrations in buffer A were incubated with peptide (10 μg) in 100 μl, for 20 min at 37 °C. Following incubation, all mixtures were centrifuged at 14000×*g* for 20 min at 4 °C, and the supernatants (containing the unbound peptide) were tested for bactericidal activity as follows. 5 μl supernatant were dispensed into 3-mm wells punched in 1% agar plates containing 1% peptone, 1% glucose and 0.8% yeast extract, previously inoculated with 1×10^8 CFU/ml *E. coli* ATCC 29648. After incubation of the plates for 12 h at 37 °C, diameters of the growth-inhibition halos were measured.

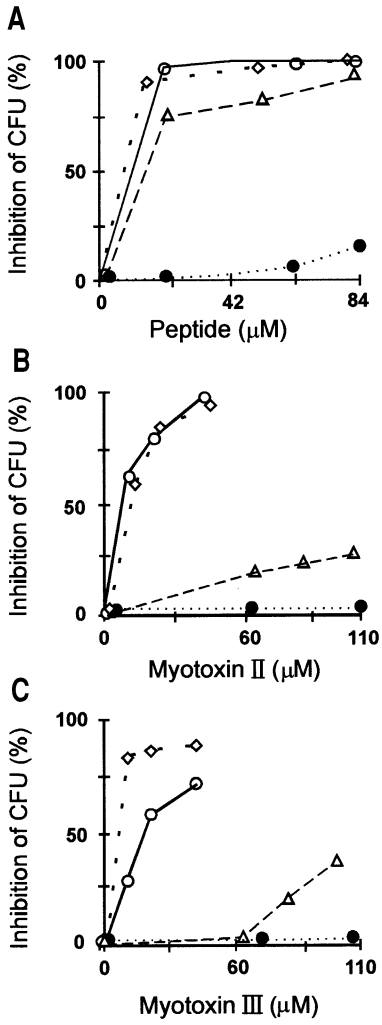

Fig. 1. Bactericidal activity of synthetic myotoxin II-(115–129)-peptide (A) myotoxin II (B), and myotoxin III (C). Dose-response curves against *S. aureus* Cowan-1 (△), *B. abortus* 2308 (●), *E. coli* 29648 (○) and *V. cholerae* Ogawa (◇). Each point represents the mean of quadruplicate assays. The standard deviations of all values were less than 10% of the value.

Construction of bacterial LPS chimeras. The coating of live rough *B. abortus* 45/20 or live rough *E. coli* K-12 with heterologous LPS was carried out as described [42], with some modifications. 20 mg/ml of *E. coli* 29648 or *B. abortus* 2308 LPS in buffer A were sonicated for 20 s at maximum frequency (Branson sonifier 450, Branson Ultrasonics Corp.) and sterilized by filtration (0.2 μm; Millipore Corp.). 200 μl fresh *B. abortus* 45/20 or fresh *E. coli* K-12 cells, adjusted to 4×10^7 CFU/ml in buffer A, 1% peptone, were mixed with 200 μl of different concentrations of heterologous LPS. The mixtures were sonicated briefly (three pulses of 1 s) and incubated for 18 h at 37 °C. Unbound LPS was removed by washing the cells repeatedly with buffer A, at 14000×*g* for 15 min at 25 °C. Bacterial pellets were suspended in 200 μl buffer A, and used immediately for

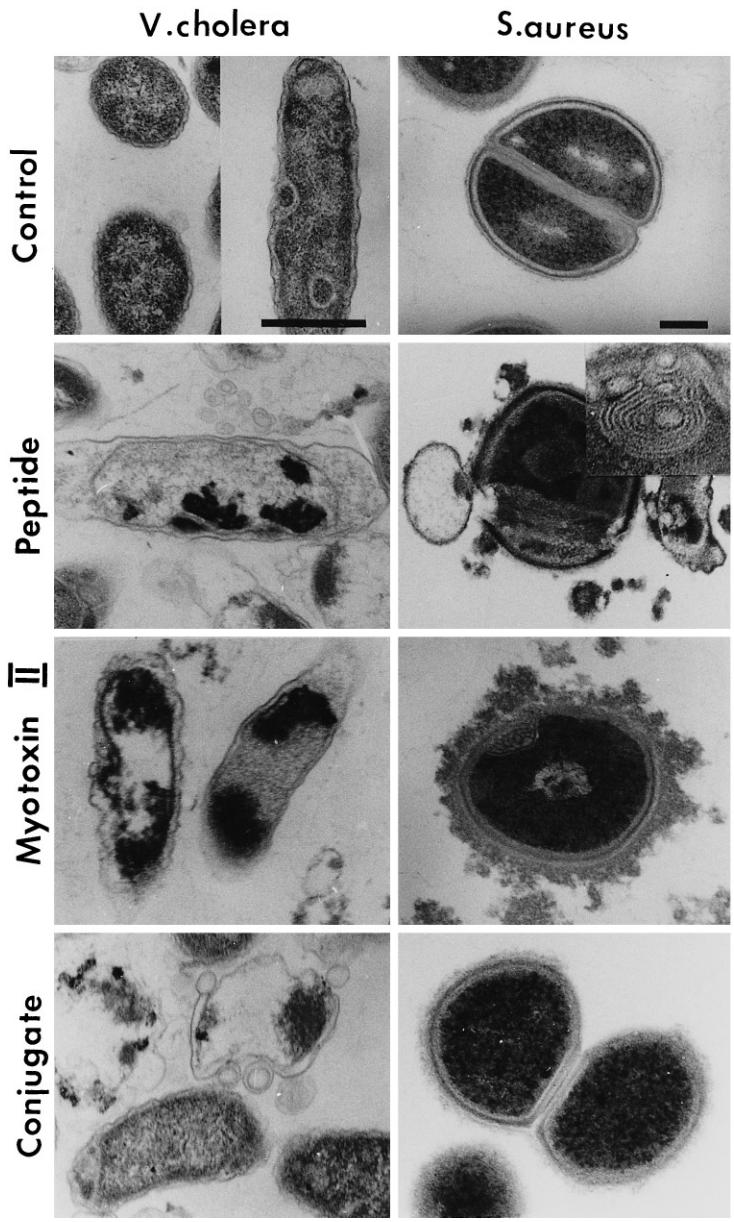

Fig. 2. Transmission electron micrograph of *V. cholerae* Inaba and *S. aureus* Cowan-1 treated with myotoxin II, myotoxin II-(115–129)-peptide (Peptide), or myotoxin II-(115–129)-peptide conjugated to diphtheria toxoid (Conjugate). The insert for *S. aureus* processed with peptide shows a higher-magnification image of the mesosome-like structures, which are evident in treated bacteria. Note the electron-dense precipitated material on the surface of myotoxin II-treated and conjugate-treated *S. aureus*. The cellular damage observed in conjugate-treated and myotoxin II-treated *E. coli* 29648 was similar to that of *V. cholera* (data not shown). The bars correspond to 0.25 μm.

bactericidal assays or electron microscopy. The efficiency of heterologous LPS incorporation into the outer membrane was evaluated by gold immunostaining, agglutination and immunofluorescence [42].

RESULTS

Bactericidal activity of myotoxins and myotoxin II-(115–129)-peptide. Fig. 1 shows the bactericidal activity of myotoxins II and III, and synthetic myotoxin II-(115−129)-peptide, derived from the myotoxin II sequence. Myotoxin II-(69−80)-peptide did not show bactericidal activity, and therefore no further experiments were carried out with it. By mass, myotoxin II-(115−129)-peptide was more active than myotoxins. On a molar basis, the three molecules displayed bactericidal activity against susceptible gram-negative bacteria in the micromolar range. For *S. aureus*, the activity of myotoxin II-(115−129)-peptide was higher than that of myotoxins. *B. abortus* was the most resistant organism tested to the action of myotoxins and myotoxin II-(115−129)-peptide (Fig. 1).

Bacterial damage induced by myotoxin II and myotoxin II-(115−129)-peptide was evaluated additionally by direct observation of ultrathin sections under the transmission electron microscope. Treatment of *Vibrio cholerae* with myotoxin II or myotoxin II-(115−129)-peptide produced severe cell decay (Fig. 2). In general, the cellular damage was characterized by detachment of the inner membrane, blisters of the outer membrane, formation of internal electron-dense granules, and vacuolization. Morphological alterations in *S. aureus* seemed to be less severe than in *Vibrio*; nevertheless, disruption of the membrane,

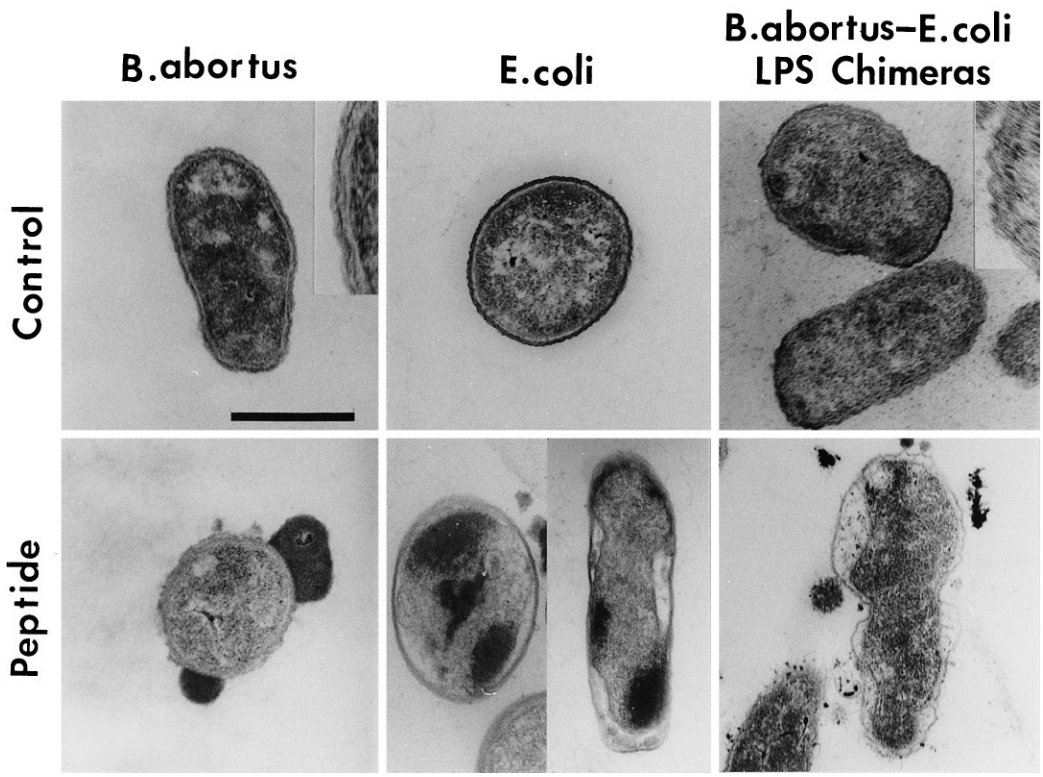

Fig. 3. Transmission electron micrograph of rough *B. abortus* 45/20, *E. coli* 29648, and *B. abortus* 45/20-*E. coli* 29648 LPS chimeras treated with myotoxin II-(115–129)-peptide. Higher-magnification images of the cell envelope of *B. abortus* 45/20 and *B. abortus* 45/20-*E. coli* 29648 LPS chimera are shown. Note the wave-like appearance of the chimeric bacteria envelope in comparison to that of the control.

formation of electron-dense granules, and rolling of the membrane to the interior of the cell was often observed (Fig. 2). The formation of mesosome-like structures as a consequence of membrane rolling was the most conspicuous and frequent alteration observed in *S. aureus*, even at low doses of myotoxin II-(115−129)-peptide. Treatment of *V. cholerae* or *S. aureus* with myotoxin II produced similar damage to that caused by myotoxin II-(115−129)-peptide. However, a precipitate in the boundary of the bacterial surface was detected with myotoxin II (Fig. 2). A similar cellular decay was observed with *E. coli* (Fig. 3) and other bacteria (data not shown), but not with *B. abortus*, which was resistant to the bactericidal action of these agents (Fig. 3). However, some electron-dense precipitate appeared on the surface of *B. abortus* treated with myotoxin II.

Relative bactericidal activity of myotoxin II-(115–129)-peptide. The bactericidal activity of myotoxin II-(115−129)-peptide compared to polymyxin B, for a variety of gram-negative and gram-positive bacteria, is presented in Table 1. For each strain, the minimal dose of polymyxin B causing 90−100% reduction in the number of CFU after 20 min exposure was determined, and the myotoxin II-(115−129)-peptide assayed at the same concentration, under identical conditions. In most cases, the peptide showed a bactericidal activity similar to that of polymyxin B, although in others it had a weaker effect (Table 1). *V. cholerae* strains and *S. aureus* were the most-susceptible gram-negative and gram-positive bacteria, respectively. Among the *Brucella* strains, which were partially resistant, the rough strain was the most susceptible (Table 1).

The potencies of myotoxin II-(115−129)-peptide, myotoxin II and myotoxin III against *E. coli* ATCC 29648, compared with those of other known bactericidal cationic peptides and proteins, are presented in Table 2. Under our experimental conditions, the relative bactericidal potencies exhibited by myotoxin II-(115−129)-peptide and myotoxins II and III were similar or stronger than the activities displayed by other cationic peptides or proteins. By mass, the lethal activities of the peptides were higher than those of the proteins.

Fluorimetry. To study the actions of myotoxin II-(115−129)-peptide and myotoxins II and III on the outer membrane of gram-negative bacteria, the transfer of the fluorescent probe Ph-NH-Nap was evaluated in intact and in treated bacteria. In the absence of bactericidal molecules, only a limited amount of Ph-NH-Nap partitioned into the outer membrane of *E. coli*, as shown by the small increase in fluorescence after addition of the probe (Fig. 4). Upon exposure of *E. coli* cells to myotoxin II-(115−129)-peptide, myotoxin II or myotoxin III, a rapid increase in the entry of Ph-NH-Nap was detected. Similar Ph-NH-Nap−uptake kinetics and levels of transfer were observed for the three molecules (Fig. 4). In contrast to *E. coli*, Ph-NH-Nap partitioned spontaneously into *B. abortus* outer membranes, and the kinetics of Ph-NH-Nap uptake were not altered by myotoxin II-(115−129)-peptide or the myotoxins (Fig. 4). The abrupt and steep Ph-NH-Nap uptake by native *S. aureus* precluded using this bacterium in fluorimetric assays.

Bactericidal activity of myotoxin II-(115–129)-peptide conjugated to diphtheria toxoid. That myotoxins, with a molecular mass close to 30 kDa (as dimers), displayed bactericidal activity suggested that myotoxin II-(115−129)-peptide was acting on the membrane, without crossing to the interior of the cell. To test this hypothesis, a conjugate of myotoxin II-(115−129)-peptide and diphtheria toxoid (molecular mass 60 kDa) was constructed, and its bactericidal activity tested at equivalent amounts of free peptide and carrier-bound peptide. The peptide-carrier conjugate killed the different bacteria (Fig. 5), although at lower levels than the free peptide. For *S. aureus*, the conjugate was more

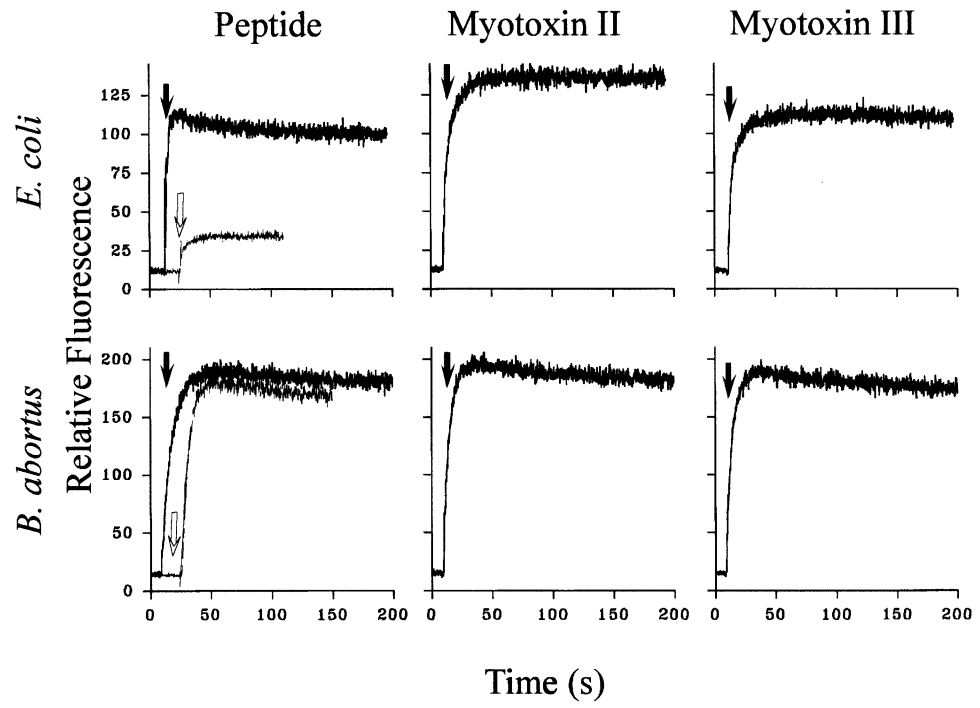

Fig. 4. Fluorimetry assays of sensitive *E. coli* 29648 and resistant *B. abortus* 2308 treated with synthetic myotoxin II-(115–129)-peptide, myotoxin II, or myotoxin III. Arrows indicate the time of addition of Ph-NH-Nap (⇨), or Ph-NH-Nap plus the peptide or myotoxins (➡). The hydrophobic probe Ph-NH-Nap partitioned spontaneously into *B. abortus* strains because their outer membranes are not barriers to hydrophobic permeants [49].

Table 2. Bactericidal activity of cationic peptides and proteins on *E. coli* ATCC 29648. The assay was carried out with 10 μg peptide for 20 min, or 200 μg protein for 180 min. Assays were performed in triplicate. n.d., not determined. Minimal bactericidal concentrations refer to peptide concentrations inhibiting growth in agar plates.

Molecule	Reduction of CFU	Minimal bactericidal concentration
	%	μg/ml
Myotoxin II-(115−129)-peptide	99 ±2.3	2.7± 0.3
Myotoxin II-(69−80)-peptide	0	>100
Bactenecin 5-(1−31)-peptide	76.8±5.2	24.4± 5.8
Bactenecin 7-(1−35)-peptide	97.3±0.7	12.1± 1.1
Cationic protein 18 A	91.7±3.0	3.7± 0.1
Cecropin A	100 ±0.0	14.7
Cecropin Pl	97.8	17.4
Defensin NP-2 C	94.0±0.7	n.d.
Lactoferricin B	94.0±1.2	2.8± 0.3
Lactoferrin (bovine)	83.0±2.0	n.d
Lysozyme (bovine)	30.1±2.54	n.d
Magainin 2 amide	84.9	39.7
Magainin 2b	93.7	n.d.
Mellitin	100 ±0.0	3.7± 0.3
Myotoxin II (*B. asper*)	95.4±1.5	n.d
Myotoxin III *(B. asper)*	75.0±1.5	n.d
Poly-L-lysine	99.6±0.3	34.8±14.6
Poly-L-ornithine	99.6±0.4	22.3± 3.8
Polymyxin B sulfate	100 ±0.0	1.0± 0.1

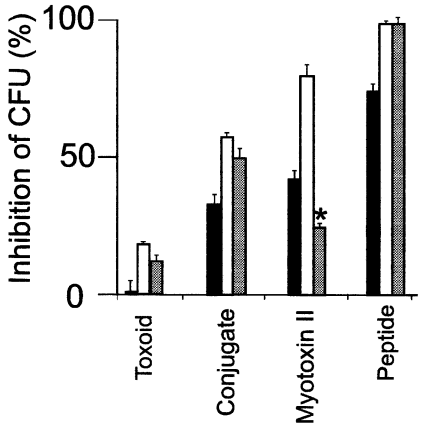

Fig. 5. Comparison of the bactericidal activity of myotoxin II-(115–129)-peptide (peptide), peptide conjugated to diphtheria toxoid conjugate (Conjugate), and myotoxin II. All samples were assayed at equivalent amounts of peptide represented in the conjugate or in the native myotoxin II sequence. Unconjugated diphtheria toxoid (Toxoid) was used as a control for the conjugate preparation. Bars represent means ±SD of quadruplicate assays using *S. aureus* Cowan-1 (▨), *V. cholera* Ogawa (□), or *E. coli* 29648 (■). ★, statistically significant ($P < 0.001$) difference between myotoxin-II-treated and conjugate-treated *S. aureus* Cowan-1.

active than myotoxin II. A low bactericidal effect of the diphtheria toxoid carrier alone was observed, which was attributed to traces of thimerosal (added by the manufacturer), which remained after dialysis (Fig. 5). Electron microscopy of *V. cholerae* and *S. aureus* treated with conjugated peptide showed cell damage similar to that caused by free peptide and myotoxin II (Fig. 2). No conspicuous cell damage was observed in *B. abortus* strains treated with conjugated peptide (data not shown).

Binding of myotoxin II-(115–129)-peptide to bacterial cells and molecules. Intact *S. aureus* and *E. coli* were the most efficient bacteria in adsorbing myotoxin II-(115−129)-peptide (Fig. 6). This property correlated with the higher ability of their corresponding lipoteichoic acids, LPS and lipid A to adsorb this

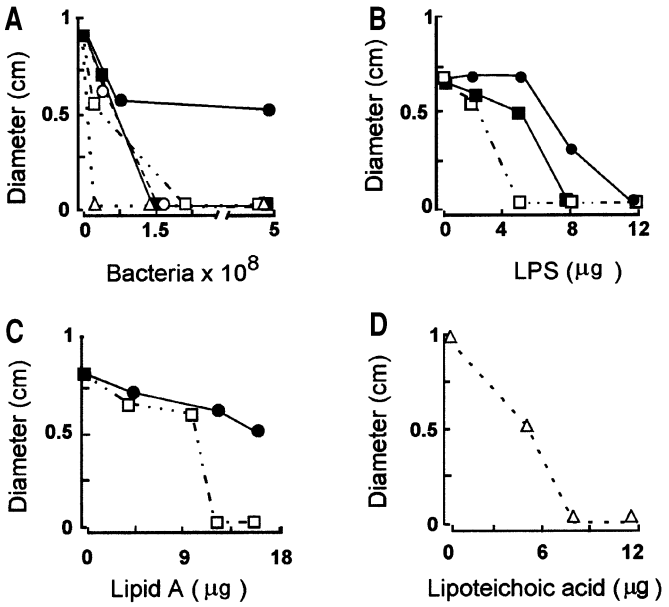

Fig. 6. Binding of myotoxin II-(115–129)-peptide to whole bacterial cells, purified LPS, lipid A or lipoteichoic acid. Binding was estimated indirectly, by comparing the diameters of inhibition halos caused by the peptide, either alone or after incubation with each test substances, as described in Materials and Methods. ●, *B. abortus* 2308; □, *E. coli* 29648; ○, *V. cholerae* Ogawa; ■, *S. montevideo* SH94; △, and *S. aureus* Cowan-1. The standard deviation at all points was less than 8% of the values.

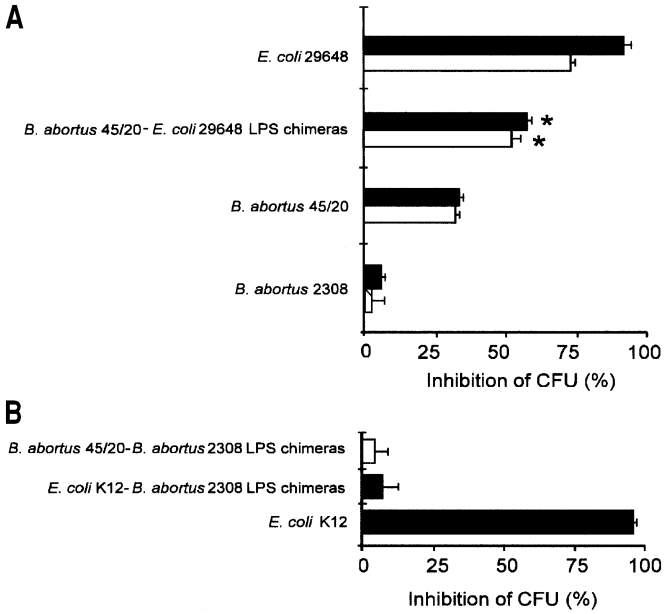

Fig. 7. Sensitivity of native bacteria and bacterial-LPS chimeras to 40 μg (□) and 60 μg (■) myotoxin II-(115–129)-peptide. The reduction of CFU was calculated with respect to control bacterial suspensions without peptide. (A) *B. abortus* 45/20-*E. coli* 29648 LPS chimeras. (B) *B. abortus* 45/20-*B. abortus* 2308 LPS, and *E. coli* K12-*B. abortus* 2308 LPS chimeras. ★, statistically significant difference ($P < 0.001$) between *B. abortus-E. coli* LPS chimeras and native *B. abortus* 45/20 treated with the peptide.

peptide. *Brucella* cells or their LPS and lipid A bound less peptide than other bacteria, in agreement with its resistance to the bactericidal action of peptide. None of the O-polysaccharide chains tested adsorbed peptide (data not shown), and the lipid A preparations were less efficient than LPS in the binding of this molecule.

Sensitivity of the LPS chimeras to the myotoxin II-(115–129)-peptide. To evaluate the role of LPS in the susceptibility or resistance of gram negative bacteria to the action of myotoxin II-(115−129)-peptide, different bacterial LPS chimeras were constructed and assayed for their resulting sensitivity (Fig. 7). Compared with the native controls, the coating procedure with heterologous LPS had no detectable effect on the growth rate, nor did it cause any decrease in the number of CFU during 8−48 h culture [42]. Electron microscopy showed no major differences between chimeras and native bacterial cells (Fig. 2). Rough *B. abortus* 45/20 was more sensitive to myotoxin II-(115−129)-peptide than smooth *B. abortus* 2308, but considerably more resistant than the *E. coli* strains (Fig. 7). Compared with the native rough *B. abortus* 45/20 cells and *E. coli* K-12, the chimeras were more resistant or more susceptible to the peptide, depending on the LPS used in the coating procedure. When the heterologous LPS corresponded to the less-sensitive smooth *B. abortus* 2308, the chimeras became significantly more resistant. On the other hand, when the LPS was from *E. coli* ATCC 29648 the chimeras were more susceptible than the uncoated controls (Fig. 7).

The LPS chimeras treated with myotoxin II-(115−129)-peptide were observed by electron microscopy (Fig. 3). Treatment of *B. abortus* with a dose of peptide lethal for *E. coli* did not affect the structure of the cells. The use of large quantities of peptide resulted in the deposition of an electron-dense layer on the surface of *B. abortus*, with no detectable cell alterations. No differences were observed between smooth and rough *B. abortus* strains (data not shown). In contrast, exposure of *B. abortus-E. coli* LPS chimeras to myotoxin II-(115−129)-peptide produced severe cell damage, which was comparable to that observed for *E. coli* and *V. cholerae* cells. The detrimental effect produced by the peptide was characterized by the detachment of the inner membrane, formation of internal electron-dense granules, and vacuolization (Fig. 3). These alterations were conspicuous at higher peptide doses and correlated with CFU reductions measured by the bactericidal assay (Fig. 7).

DISCUSSION

This work demonstrates that myotoxin II, a catalytically inactive Lys49 PLA_2 found in the venom of *B. asper*, is directly bactericidal to a variety of gram-negative and gram-positive bacteria. Previous studies have shown that PLA_2 enhance the degradation of bacteria treated with bactericidal/permeability-increasing factor [18, 19], or induce direct bactericidal action dependent on their enzymatic activity [17, 22]. Therefore, the bactericidal activity of myotoxin II represents a mechanism for group II PLA_2 that does not depend on catalytic activity.

Since Lys49 myotoxin II and Asp49 myotoxin III showed similar bactericidal potencies, the observed effect of myotoxin II cannot be ascribed to contamination with catalytically active isoforms during isolation. Furthermore, results identified the region of myotoxin II responsible for its bactericidal action, which comprises a relatively short stretch of amino acids (115−129) near the C-terminal loop, and which reproduces the effect of the whole protein. On the other hand, for catalytically active myotoxin III, a contribution of its PLA_2 activity to the bactericidal effect cannot be ruled out with the present data. However this seems to be of little, if any, relevance, since the overall bactericidal activity exerted by this protein is not greater than that mediated by myotoxin II.

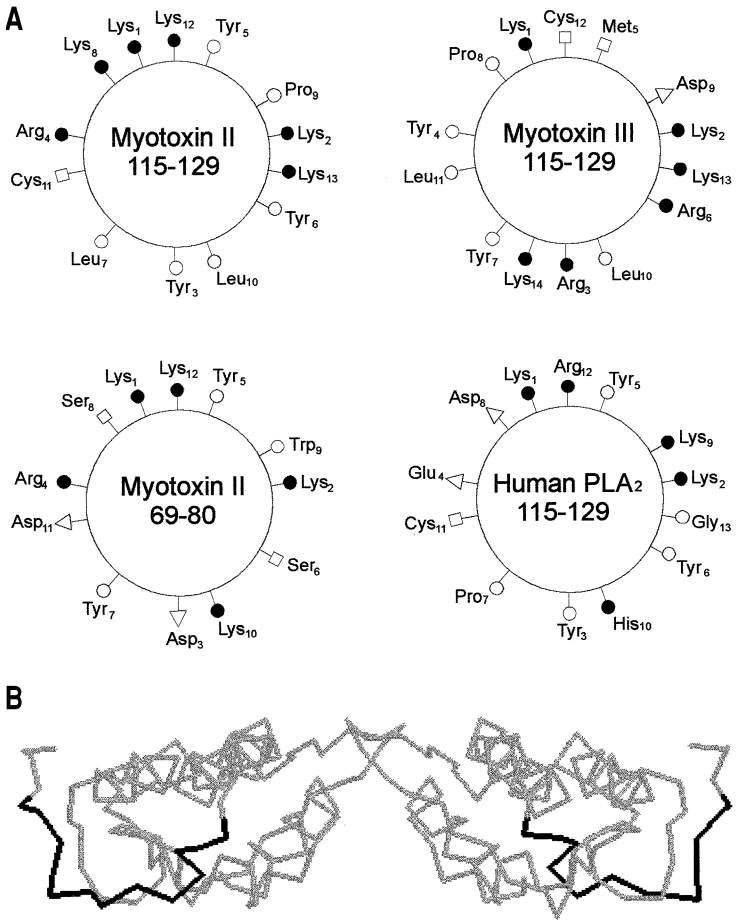

Fig. 8. Schematic representations of axial projections of different PLA_2 peptides (A) and of myotoxin II, showing the location of residues 115–129 (B). The positively charged amino acids of peptides 115−129 of myotoxins II and III are clustered in one of the axial faces, whereas the positively charged amino acids in the corresponding peptide of human PLA_2, or a non-bactericidal cationic peptide derived from a different region of myotoxin II (residues 69−80), are more dispersed around the axial projections. The design of peptides based on residues 115−129 of myotoxins II and III is potentially compatible with a model of amphipathic molecules having antimicrobial activities, although their three-dimensional structures have not been determined (A). The exposed location of residues 115−129 in the myotoxin II dimer (B), near to the C-terminus, presumably facilitates its interaction with bacterial membranes, although the disulfide bond between Cys133 and Cys50 precludes the formation of an helix in the intact protein.

The bactericidal effect of myotoxin II and myotoxin II-(115−129)-peptide was observed on a variety of gram-negative and gram-positive organisms, and differences in individual susceptibilities were evident. The bactericidal potency of the peptide is, in general, comparable to those of other well characterized cationic peptides such as defensins, cecropins and polymyxins (Table 2; [17]). The susceptibility of the different bacteria tested followed the same pattern as that observed with other cationic peptides. For example, the intracellular *Brucella*, which have evolved to resist the arsenal of antimicrobial mechanisms presented by eukaryotic hosts, including lysosomal peptides and proteins [28], was the most resistant to the action of myotoxins and myotoxin II-(115−129)-peptide.

The bactericidal effect of myotoxins and myotoxin II-(115−129)-peptide was confirmed by direct microscopical observation, revealing major alterations in cell morphology of susceptible but not of resistant bacteria. Cell-damage morphology resembled that described for lactoferricin B, but was different from that induced by polymyxin B [28, 42]. The drastic alterations observed rule out the possibility of a bacteriostatic effect being responsible for the decrease in CFU caused by the myotoxins or myotoxin II-(115−129)-peptide.

The experiments performed with myotoxin II-(115−129)-peptide conjugated to a protein carrier (diphtheria toxoid) strongly suggest that the bactericidal mechanism of myotoxin II and its active peptide is exerted at the membrane surface, rather than involving an internalization process. Results obtained in fluorimetry experiments using the hydrophobic probe Ph-NH-Nap are in agreement with this conclusion, demonstrating a rapid membrane permeabilization of susceptible gram-negative bacteria caused by myotoxin II or myotoxin II-(115−129)-peptide. In comparison with myotoxin II-(115−129)-peptide, myotoxins displayed a lower bactericidal effect against *S. aureus* than against susceptible gram-negative bacteria. This difference may be the consequence of the thicker cell wall of gram-positive bacteria, which may hamper the access of the cationic peptide domain of the protein to the bacterial membrane.

The bactericidal mechanism of myotoxin II-(115−129)-peptide might be exerted in a similar fashion to that proposed for other cationic peptides, such as lactoferricin B and defensins [42], i.e. by displacing metal ions such as Ca^{2+} and Mg^{2+} from negatively charged groups of the cell surface, namely core and lipid A moieties (for gram-negative bacteria) or phosphate groups of lipoteichoic acids (for gram-positive bacteria). Because these cationic molecules are much bulkier than metal ions, the membrane is destabilized, allowing the eventual insertion of the hydrophobic domain of the peptide into the bilayer, followed by membrane permeabilization, and ultimately cell death. The

three-dimensional structure of myotoxin II-(115−129)-peptide in solution has not been investigated. Nevertheless, its axial projection [44] (Fig. 8) suggests that this molecule has the potential to form a helix that displays the hydrophobic and cationic domains in opposite sides of the coil. This design appears to be a common feature of a variety of membrane-disturbing peptides of diverse origins that act upon bacteria [45, 46]. The axial projection of myotoxin III-(115−129)-peptide conserves this hydrophobic/cationic distribution, similarly to myotoxin II-(115−129). In contrast, another cationic peptide of myotoxin II [myotoxin II-(69−80)-peptide], which lacked bactericidal effect, or residues 115−129 of the human group-II PLA_2, in which bactericidal action correlates with enzymatic activity, do not display this distribution in their axial projections (Fig. 8).

Binding experiments using purified bacterial components support the concept of an interaction between myotoxin II-(115−129)-peptide and negatively charged surface moieties. Binding of peptide to the lipid A and LPS of *E. coli*, or to lipoteichoic acids of *S. aureus*, correlated with their observed susceptibility to bactericidal action. Likewise, resistance of *Brucella* species correlated with the low binding between their whole cells or isolated components and myotoxin II-(115−129)-peptide. The reduced divalent-cation stabilization of LPS in the outer membrane of *Brucella* species [47] and the comparatively low number of negatively charged groups in this molecule [48] may account for the low affinity of *Brucella* outer membrane to myotoxin II-(115−129)-peptide, and in consequence, to the resistance of these bacteria.

In general, since lipid A was less efficient than LPS in binding myotoxin II-(115−129)-peptide, and the O-chain did not bind peptide at all, it was feasible that the negatively charged core sugars (e.g. 3-deoxy-D-manno-octulosonic acid) contributed to the interaction of this cationic molecule with the outer membrane. The importance of LPS in the bactericidal mechanism induced by myotoxin II-(115−129)-peptide in gram-negative bacteria was demonstrated by the results obtained with LPS chimeras, in which resistance or susceptibility depended upon the foreign LPS inserted in the outer membrane of the receptor bacteria.

The direct bactericidal activity of myotoxin II and myotoxin II-(115−129)-peptide (on gram-positive and gram-negative bacteria) reinforces the notion that group-II Lys49 PLA_2 display membrane-perturbing effects on a wide variety of biological targets [7] independently of an intrinsic enzymatic action, and that the C-terminal region (residues 115−129) plays a relevant role in these effects [14].

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REFERENCES

1. Dennis, E. A. (1994) Diversity of group types, regulation, and function of phospholipase A_2, *J. Biol. Chem. 269*, 13 057−13 060.
2. Kini, R. M. (1997) Phospholipase A_2 − a complex multifunctional protein puzzle, in *Venom phospholipase A_2 enzymes: structure, function and mechanism* (Kini, R. M., ed.) pp. 1−28, John Wiley & Sons, Chichester.
3. Nakashima, K., Nobuhisa, I., Deshimaru, M., Nakai, M., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Hattori, M., Sakaki, Y., Hattori, S. & Ohno, M. (1995) Accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland phospholipase A_2 isozyme genes, *Proc. Natl Acad. Sci. USA 92*, 5605−5609.
4. Maraganore, J. M., Merutka, G., Cho, W., Welches, W., Kézdy, F. J. & Heinrikson, R. L. (1984) A new class of phospholipases A_2 with lysine in place of aspartate 49, *J. Biol. Chem. 259*, 13 839−13 843.
5. Maraganore, J. M. & Heinrikson, R. L. (1986) The lysine-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. Relation of structure and function to other phospholipases A_2, *J. Biol. Chem. 261*, 4797−4804.
6. Lomonte, B. & Gutiérrez, J. M. (1989) A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo), *Toxicon 27*, 725−733.
7. Lomonte, B., Tarkowski, A. & Hanson, L. Å. (1994) Broad cytolytic specificity of myotoxin II, a lysine-49 phospholipase A_2 of *Bothrops asper* snake venom, *Toxicon 32*, 1359−1369.
8. Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J. & Sigler, P. B. (1985) A comparison of the crystal structures of phospholipase A_2 from bovine pancreas and *Crotalus atrox* venom, *J. Biol. Chem. 260*, 11 627−11 634.
9. Arni, R. K., Ward, R. J., Gutiérrez, J. M. & Tulinsky, A. (1995) Structure of a calcium-independent phospholipase-like myotoxic protein from *Bothrops asper* venom, *Acta Cryst. D 51*, 311−317.
10. Arni, R. K. & Ward, R. J. (1996) Phospholipase A_2 − a structural review, *Toxicon 34*, 827−841.
11. Francis, B., Gutiérrez, J. M., Lomonte, B. & Kaiser, I. I. (1991) Myotoxin II from *Bothrops asper* (terciopelo) venom is a lysine-49 phospholipase A_2, *Arch. Biochem. Biophys. 284*, 352−359.
12. Rufini, S., Cesaroni, P., Desideri, A., Farias, R., Gubensek, F., Gutiérrez, J. M., Luly, P., Massoud, R., Morero, R. & Pedersen, J. Z. (1992) Calcium ion independent membrane leakage induced by phospholipase-like myotoxins, *Biochemistry 31*, 12 424−12 430.
13. Pedersen, J. Z., De Arcuri, B. F., Morero, R. & Rufini, S. (1994) Phospholipase-like myotoxins induce rapid membrane leakage of non-hydrolyzable ether-lipid liposomes, *Biochim. Biophys. Acta 1190*, 177−180.
14. Lomonte, B., Moreno, E., Tarkowski, A., Hanson, L. Å. & Maccarana, M. (1994) Neutralizing interaction between heparins and myotoxin II, a Lys49 phospholipase A_2 from *Bothrops asper* snake venom. Identification of a heparin-binding and cytolytic toxin region by the use of synthetic peptides and molecular modeling, *J. Biol. Chem. 269*, 29 867−29 873.
15. Díaz, C., Alape, A., Lomonte, B., Olamendi, T. & Gutiérrez, J. M. (1994) Cleavage of the NH_2-terminal octapeptide of *Bothrops asper* myotoxic lysine-49 phospholipase A_2 reduces its membrane-destabilizing effect, *Arch. Biochem. Biophys. 312*, 336−339.
16. Gutiérrez, J. M. & Lomonte, B. (1997) Phospholipase A_2 myotoxins from *Bothrops* snake venoms, in *Venom phospholipase A_2 enzymes: structure, function and mechanism* (Kini, R. M., ed.) pp. 321−352, John Wiley & Sons, England.
17. Harwig, S. S. L., Tan, L., Qu, X., Cho, Y., Eisenhauer P. B. & Lehrer R. I. (1995) Bactericidal properties of murine intestinal phospholipase A_2, *J. Clin. Invest. 95*, 603−610.
18. Elsbach, P. & Weiss, J. (1992) Oxygen-independent antimicrobial systems of phagocytes, in *Inflammation, basic principles and clinical correlates* (Gallin, J. I., Goldstein, I. M. & Snyderman, R., eds) pp. 603−636, Raven Press, New York.
19. Forst, S., Weiss, J., Maraganore, J. M., Heinrikson, R. L. & Elsbach, P. (1987) Relation between binding and the action of phospholipases A_2 on *Escherichia coli* exposed to the bactericidal/permeability-increasing protein of neutrophils, *Biochim. Biophys. Acta 920*, 221−225.
20. Weiss, J., Wright, G., Bekkers, A., van den Bergh, C. J. & Verheij, H. M. (1991) Conversion of pig pancreas phospholipase A_2 by protein engineering into enzyme active against *Escherichia coli* treated with the bactericidal/permebility-increasing protein, *J. Biol. Chem. 266*, 4162−4167.
21. Weiss, J., Inada, M., Elsbach, P. & Crowl, R. M. (1994) Structural determinants of the action against *Escherichia coli* of a human inflammatory fluid phospholipase A_2 in concert with polymorphonuclear leukocytes, *J. Biol. Chem. 269*, 26 331−26 337.

22. Weinrauch, Y., Elsbach, P., Madsen, L. M., Foreman, A. & Weiss, J. (1996) The potent anti-*Staphylococcus aureus* activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A_2, *J. Clin. Invest. 97*, 250−257.
23. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
24. Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962) Disk electrophoresis of basic proteins and peptides on polyacrylamide gels, *Nature 195*, 281−283.
25. Gutiérrez, J. M., Avila, C., Rojas, E. & Cerdas, L. (1988) An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica, *Toxicon 26*, 411−413.
26. Kaiser, I. I., Gutiérrez, J. M., Plummer, D., Aird, S. D. & Odell, G. V. (1990) The amino acid sequence of a myotoxic phospholipase from the venom of *Bothrops asper*, *Arch. Biochem. Biophys. 278*, 319−325.
27. Valerio, R. M., Bray, A. M., Campbell, R. A., DiPasquale, A., Margellis, C., Rodda, S. J., Geysen, H. M. & Maeji, N. J. (1993) Multipin peptide synthesis at the micromole scale using 2-hydroxyethyl methacrylate grafted polyethylene supports, *Int. J. Pept. Protein Res. 42*, 1−9.
28. Martínez de Tejada, G., Pizarro-Cerdá, J., Moreno, E. & Moriyón, I. (1995) The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides, *Infect. Immun. 63*, 3054−3061.
29. Lennette, E. H., Spaulding, E. H. & Truant, J. P. (1974) *Manual of clinical microbiology*, 2nd edn, American Society for Microbiology, Washington DC.
30. Hultmark, D., Engström, Å., Bennich, H., Kapur, R., Boman, H. G. (1982) Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from *Cecropia* pupae, *Eur. J. Biochem. 127*, 207−217.
31. Cherwonogrodozki, J. W., Dubray, G., Moreno, E. & Mayer, H. (1990) Antigens of *Brucella*, in *Animal brucellosis* (Nielsen, K. & Duncan, J. R., eds) pp. 19−64, CRC Press, Boca Raton.
32. Rojas, N., Freer, E., Weintraub, A., Ramírez, M., Lind, S. & Moreno, E. (1994) Immunochemical identification of *Brucella abortus* lipopolysaccharide epitopes, *Clin. Diagn. Lab. Immunol. 1*, 206−213.
33. Baker, P. J. & Wilson, J. B. (1965) Chemical composition and biological properties of endotoxin of *Brucella abortus*, *J. Bacteriol. 90*, 895−902.
34. Moreno, E., Pitt, W., Jones, L. M., Shurig, G. G. & Berman, D. T. (1979) Purification and characterization of smooth and rough lipopolysaccharides from *Brucella abortus*, *J. Bacteriol. 138*, 361−369.
35. Westphal, O. & Jann, K. (1965) Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure, *Methods Carbohydr. Chem. 5*, 83−96.
36. Khun, H., Samanta, B. & Mayer, H. (1987) Comparison of enterobacterial common antigen from different species by serological techniques, *Eur. J. Biochem. 162*, 69−74.
37. Freer, E., Rojas, N., Weintraub, A., Lindberg, A. & Moreno, E. (1995) Heterogeneity of *Brucella abortus* lipopolysaccharides, *Res. Microbiol. 146*, 569−578.
38. Galanos, C., Lüderitz, O. & Westphal, O. (1971) Preparation and properties of antisera against the lipid A-component of bacterial lipopolysaccharides, *Eur. J. Biochem. 24*, 116−122.
39. Aragón, V. R., Díaz, R., Moreno, E. & Moriyón, I. (1996) Characterization of *Brucella abortus* and *Brucella melitensis* native haptens outer membrane O-type polysaccharides independent from the smooth lipopolysaccharide, *J. Bacteriol. 178*, 1070−1079.
40. Wicken, A. J. & Knox, K. W. (1970) Studies on the group F antigen of lactobacilli: isolation of a teichoic acid-lipid complex from *Lactobacillus fermenti* NCTC 6991, *J. Gen. Microbiol. 60*, 293−301.
41. Tsai, C. & Frash, C. E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels, *Anal. Biochem. 119*, 115−119.
42. Freer, E., Moreno, E., Moriyon, I., Pizarrro-Cerdá, J., Weintraub, A. & Gorvel, J. P. (1996) *Brucella-Salmonella* lipopolysaccharide chimeras are less permeable to hydrophobic probes and more sensitive to bacterial cationic peptidess than their native *Brucella* spp. counterparts, *J. Bacteriol. 178*, 5867−5876.
43. Träuble, H. & Overath, P. (1973) The structure of *Escherichia coli* membranes studied by fluorescence measurements of lipid phase transitions, *Biochim. Biophys. Acta 307*, 491−512.
44. Schiffer, M. & Edmundson, A. B. (1967) Prediction of alpha-helices in glucagon, *Biophys. J. 7*, 121−135.
45. Kini, R. M. & Evans, H. J. (1989) A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides, *Int. J. Peptide Prot. Res. 34*, 277−286.
46. Blondelle, S. E. & Houghten, R. A. (1992) Design of model amphipathic peptides having potent antimicrobial activities, *Biochemistry 31*, 12688−12694.
47. Moriyón, I. & Berman, D. T. (1982) Effects of nonionic, ionic, and dipolar ionic detergents and EDTA on the *Brucella* cell envelope, *J. Bacteriol. 152*, 822−828.
48. Moreno, E., Stackebrandt, E., Dorsch, M., Wolters, J., Busch, M. & Mayer, H. (1990) *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class Proteobacteria, *J. Bacteriol. 172*, 3569−3576.
49. Martínez de Tejada, G. & Moriyón, I. (1993) The outer membranes of *Brucella* spp. are not barriers to hydrophobic permeants, *J. Bacteriol. 175*, 5273−5275.
50. Alton, G. G., Jones, L. M., Angus, R. D. & Verger, J. M. (1988) *Techniques for the brucellosis laboratory*, Institut National de la Recherche Agronomique, Paris.