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Identification of the myotoxic site of the Lys49 phospholipase A_2 from *Agkistrodon piscivorus piscivorus* snake venom: synthetic C-terminal peptides from Lys49, but not from Asp49 myotoxins, exert membrane-damaging activities

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Abstract

Group II phospholipase A_2 (PLA_2) myotoxins found in the venoms of Crotalidae snakes can be divided into 'Asp49' and 'Lys49' isoforms, the latter being considered catalytically-inactive variants. Previous studies on one Lys49 isoform, myotoxin II from *Bothrops asper*, indicated that its myotoxic activity is due to the presence of a short cationic/hydrophobic sequence (115–129) near its C-terminus, which displays membrane-damaging properties. Since the C-terminal region of different group II PLA_2 myotoxins presents considerable sequence variability, synthetic peptides homologous to region 115–129 of myotoxin II, but corresponding to *B. asper* myotoxin III (Asp49), *Agkistrodon piscivorus piscivorus* Asp49 PLA_2 and Lys49 PLA_2, were studied to determine the possible functional relevance of such region for the toxic activities of these proteins. Results showed that both Lys49-derived peptides (p-BaK49 and p-AppK49) were able to lyse skeletal muscle C2C12 cells in culture, and to induce edema in the mouse footpad assay. Moreover, p-AppK49, which showed a markedly stronger cytotoxic potency than p-BaK49, additionally induced skeletal muscle necrosis when injected into mice. These observations unequivocally identify the sequence 115–129 (KKYKAYFKLKCKK) of the Lys49 PLA_2 of *A. p. piscivorus* as containing the key structural determinants needed for myotoxicity, and represent the first report of an unmodified, PLA_2-derived short synthetic peptide with the ability to reproduce this effect of a parent toxin in vivo. On the other hand, the two Asp49-derived peptides did not show any toxic effects in vitro or in vivo, even at high concentrations. These findings suggests that Lys49 and Asp49 group II PLA_2s might exert their myotoxic actions through different molecular mechanisms, by implying that the latter may not utilize their C-terminal regions as main membrane-destabilizing elements.

Keywords: Phospholipase A_2; Myotoxin; Snake venom; Synthetic peptides; Heparin

1. Introduction

Despite having evolved within the constraints of a conserved architecture with their non-toxic counterparts, many group I and group II phospholipases A_2 (PLA_2s) from venomous animal secretions display a variety of pharmacological/toxic activities (Rosenberg, 1990; Ogawa et al., 1995; Scott, 1997; Kini and Chan, 1999; Jeyaseelan et al., 2000). Snakes of the family Crotalidae secrete a number of basic PLA_2s with myotoxic activity (Gutiérrez and Lomonte, 1995, 1997; Fletcher et al., 1997), which may cause severe muscle damage in human envenomations. These myotoxic PLA_2s are structurally classified as group II (Dennis, 1997), and belong to two main types, commonly referred to as 'Asp49' and 'Lys49' isoforms. The latter, first described in the venom of *Agkistrodon piscivorus piscivorus* (Maraganore et al., 1984), contain critical amino acid substitutions, including the Asp49 → Lys change, that preclude the catalytic mechanism exerted by the former, and are therefore considered enzymatically-inactive PLA_2 homologues (Scott et al., 1992; Arni and Ward, 1996). Since both

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Table 1
C-terminal amino acid sequences of selected class II myotoxic phospholipases A_2. C-terminal amino acid sequences are indicated by single-letter code, where positions identical to the *A. p. piscivorus* Lys49 PLA_2 sequence are represented by dots. Dashes represent gaps introduced to maximize alignments. Sequences of the four synthetic peptides utilized in the present study are indicated by bold type)

Myotoxic PLA_2	Type	Sequence	Reference
Agkistrodon p. piscivorus PLA_2	K49	N**KKYKAYFKLK-CKK**-PDTC	Maraganore and Heinrikson (1986)
Agkistrodon c. laticinctus myotoxin	K49	. . . . . F . - . . . . - . E . .	Selistre de Araujo et al. (1996)
Bothrops asper myotoxin II	K49	. . . . **RY . L . PL- . . .** -A . A.	Francis et al. (1991)
B. jararacussu bothropstoxin I	K49	. . . . RYHL . P F - . . . -A . P.	Cintra et al. (1993)
B. moojeni myotoxin I	K49	. . . RDV . L . P F - . D . -GRD.	Soares et al. (2000a)
B. moojeni myotoxin II	K49	. . . . RYNY LKPF . . . -A . P.	Soares et al. (1998)
B. neuwiedi pauloensis BnSP-7 myotoxin	K49	. . . . RYHL . PF - . . . -A . P.	Soares et al. (2000b)
B. pirajai piratoxin I	K49	. . L . RYHL . PF - . . . -A . D.	Toyama et al. (1998)
Trimeresurus flavoviridis basic protein I	K49	. . . . T I . P . PF - . . . -A . . .	Yoshizumi et al. (1990)
Vipera ammodytes ammodytin L	S49	. . . . . V . L RF . - . . GVSEK.	Krizaj et al. (1991)
Agkistrodon p. piscivorus PLA_2	D49	S **. T .WK. P . KN- . .** EESE P.	Maraganore and Heinrikson (1986)
B. asper myotoxin III	D49	**K . R .M . . PD . L- . . .** PAEK.	Kaiser et al. (1990)
B. jararacussu bothropstoxin II	D49	. . . AYYHL . P F- . . EPAE . .	Pereira et al. (1998)

the Lys49 and Asp49 PLA_2 isoforms display similar myotoxic effects (Gutiérrez and Lomonte, 1995, 1997), it has been of interest to study the structural basis of their mechanisms of action in recent years.

First clues towards identifying the molecular region responsible for the toxic actions of a Lys49 myotoxic PLA_2 emerged from neutralization studies utilizing heparins, and subsequent mapping of the heparin-binding site on *Bothrops asper* myotoxin II (Lomonte et al., 1994a,b). A 13-mer synthetic peptide corresponding to its region 115–129 (numbering of Renetseder et al., 1985) was shown to mimic the cytolytic effect of this toxin on cultured endothelial cells (Lomonte et al., 1994b), and its in vitro bactericidal activity (Páramo et al., 1998). Although injection of peptide 115–129 did not reproduce myonecrosis (B. Lomonte, unpublished data; and present study), antibodies against it were found to inhibit the cytolytic and myotoxic effects of myotoxin II (Calderón and Lomonte, 1998, 1999). Moreover, a variant of peptide 115–129 with a triple Tyr → Trp substitution (Y117W/Y119W/Y120W) expressed enhanced membrane-damaging activities, inducing myonecrosis and edema in vivo, as well as cytotoxicity and bactericidal activity in vitro (Lomonte et al., 1999a). Altogether, these lines of evidence have supported the conclusion that, in the case of *B. asper* myotoxin II, the amphiphylic region 115–129 is the key structural determinant of its myotoxic action. Nevertheless, the C-terminal region of group II myotoxic PLA_2s shows considerable sequence diversity, not only between Asp49 and Lys49 isoforms, but also within individual members of these two groups of proteins (Selistre de Araujo et al., 1996; Ward et al., 1998). Therefore, the aim of the present study was to investigate whether the region 115–129 would also be of functional relevance to the activities of other PLA_2 myotoxins. For this purpose, four myotoxin sequences were selected, corresponding to two Lys49 and two Asp49 isoforms from *B. asper* and *A. p. piscivorus*, respectively. Synthetic peptides homologous to region 115–129 of myotoxin II, but corresponding to *B. asper* myotoxin III (Asp49; Kaiser et al., 1990), *A. p. piscivorus* Asp49 PLA_2, and Lys49 PLA_2 (Maraganore and Heinrikson, 1986) were comparatively analyzed regarding their properties and possible toxic activities, in vitro and in vivo.

2. Materials and methods

2.1. Synthetic peptides

Four synthetic peptides were selected, corresponding to the sequence 115–129 of *B. asper* myotoxin II (KKYRYYLKPLCKK; Francis et al., 1991), or homologous regions of *B. asper* myotoxin III (KKRYMAYPDLLCKK; Kaiser et al., 1990), *A. p. piscivorus* Lys49 PLA_2 (KKYKAYFKLKCKK), and *A. p. piscivorus* Asp49 PLA_2 (KTYWKYPKKNCK; Maraganore and Heinrikson, 1986), respectively (Table 1). These peptides will be referred to as p-BaK49, p-BaD49, p-AppK49, and p-AppD49, respectively. Peptides (5 mg) were synthesized by Fmoc chemistry, with native endings, by a commercial provider (Chiron Mimotopes, Victoria, Australia). Their estimated molecular masses were in agreement with corresponding calculated values, and final purity levels were at least 95% by RP-HPLC analysis. Peptides were kept dry at —20°C, and dissolved in 0.12 M NaCl, 40 mM sodium phosphate (PBS), pH 7.2, immediately before being tested for their activities.

2.2. Cytotoxic activity

The effect of peptides on the viability of the C2C12 skeletal muscle cell line was tested in a 96-well format assay, as previously described (Lomonte et al., 1999b). The activity of lactic dehydrogenase (LDH, kit 500-C; Sigma Chemical

Company, USA) released from damaged cells to the supernatant was quantified 3 h after their exposure to variable concentrations of peptides. Results were expressed as percentage values of LDH release, considering culture medium alone or 0.1% Triton X-100 as 0 and 100% reference values, respectively. All samples were assayed in duplicate cultures, on at least two independent experiments.

2.3. Myotoxic activity

Peptides (250 μg dissolved in 50 μl PBS) were injected into the right gastrocnemius of groups of three mice, in order to evaluate necrosis of skeletal muscle in vivo (Lomonte and Gutiérrez, 1989). This dose was selected on the basis of previous studies characterizing the myotoxic synthetic peptide variant p115–W3 (Lomonte et al., 1999a). As a control, another group of mice received an i.m. injection of 50 μl of PBS alone. After 3 h, tail blood samples were collected into heparinized capillary tubes, and the plasma creatine kinase (CK, kit 520-C; Sigma) activity was determined as an estimator of necrosis. Values of CK activity were expressed in U/ml, one unit resulting in the phosphorylation of one nanomole of creatine per min at 25°C.

The myotoxic effect of p-AppK49, which screened positive by the plasma CK assay, was additionally evaluated by intravital microscopy on a murine cremaster preparation (Lomonte et al., 1994c). In brief, the cremaster muscle of anesthetized male mice ($n = 3$) was surgically exposed and covered with a thin (6 μm) Mylar® sheet for microscopical observation and video recording. After stabilizing the preparation for 5 min, 100 μg of p-AppK49, dissolved in 20 μl of PBS, were directly applied onto the tissue with a micropipet, and the morphological alterations were microscopically observed during the next 30 min under 100 × and 200 × magnification.

2.4. Edema-forming activity

Groups of four mice received a subcutaneous injection of the peptides (100 μg dissolved in 50 μl of PBS) in their right footpad. Fifty μl of PBS alone were injected in their left footpad, utilized as a control. After 1 h, mice were sacrificed, and the increase in wet weight of the right footpad, relative to the contralateral control, was determined and expressed as percent edema (Yamakawa et al., 1976).

2.5. Heparin-binding activity

Heparin with low affinity for antithrombin (LA-heparin; Pharmacia, Sweden) was added in gradual increments to a cuvette containing 200 μg of the peptides (approximately 55 μM), in 2 ml of 0.05 M Tris-HCl, 0.01 KCl, pH 7.0 buffer. Absorbance changes due to turbidity were monitored at 340 nm, allowing 1 min of incubation after each heparin addition, as described (Lomonte et al., 1994b).

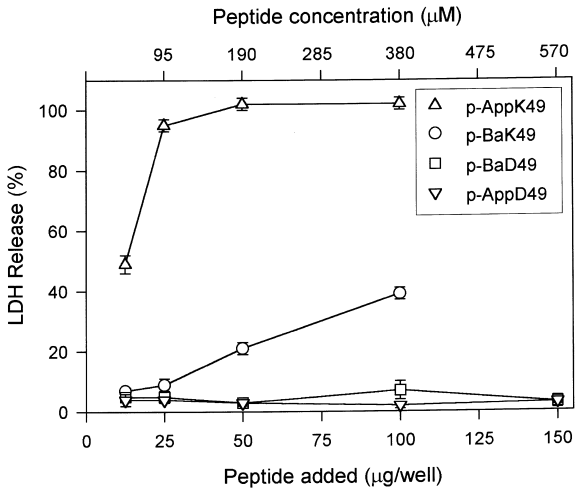

Fig. 1. Evaluation of the cytotoxic activity of synthetic peptides derived from myotoxic phospholipases A_2 upon C2C12 skeletal muscle cells in vitro. Myoblasts were grown in 96-well plates until near confluence, and allowed to partially differentiate and fuse into myotubes for 4–5 days. Variable amounts of peptides were added to duplicate cultures in a final volume of 150 μl/well, and after 3 h the lactate dehydrogenase (LDH) activity of cell supernatants was determined as described in Section 2. Values are expressed as percentages, considering medium alone or 0.1% Triton X-100 as reference values for 0 and 100% release, respectively. Sequences of peptides from *Bothrops asper* (p-BaK49 and p-BaD49) or *Agkistrodon piscivorus piscivorus* (p-AppK49 and p-AppD49) myotoxins are described in Table 1. Each point represents mean ± SD.

3. Results

When synthetic peptides were tested for cytotoxic activity upon cultured C2C12 cells, a clear difference was observed between those derived from Lys49 PLA_2s and Asp49 PLA_2s, respectively (Fig. 1). In particular, p-AppK49 showed a strong cytotoxic effect, causing half-maximal LDH release at approximately 12 μg/well (~45 μM), whereas p-BaK49 induced a weaker, although clearly significant cell-damaging effect (Fig. 1). In contrast, neither p-BaD49 or p-AppD49 caused any cell damage, as judged by the release of LDH, even at concentrations as high as 150 μg/well (~580 μM). Microscopical observation of the cell cultures confirmed the lack of cytotoxic activity of these Asp49 PLA_2-derived peptides, since no morphological alterations were evident in treated cells, in comparison to control cultures incubated with medium alone. On the other hand, p-AppK49 and p-BaK49 induced drastic morphological alterations of the C2C12 cultures, which were identical to those previously described for several myotoxic PLA_2s in this assay system (Lomonte et al., 1999b).

When synthetic peptides were injected by the i.m. route in vivo, p-AppK49 induced a significant increase of plasma CK activity, in contrast to the other peptides (Fig. 2). The

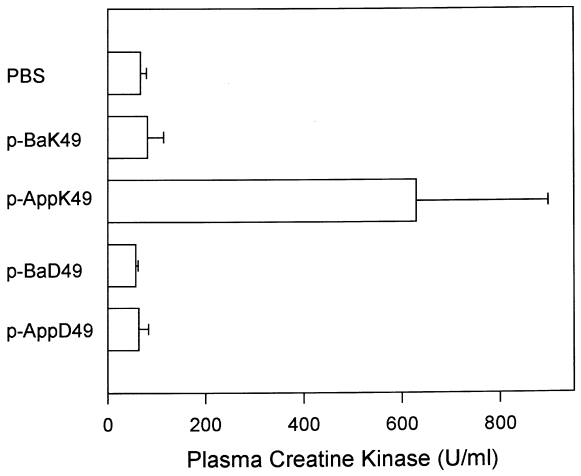

Fig. 2. Evaluation of the myotoxic activity of synthetic peptides derived from myotoxic phospholipases A_2 in vivo. Groups of three mice received an intramuscular injection of 250 μg of peptides, in their right gastrocnemius, in 50 μl of phosphate-buffered saline (PBS). Controls received 50 μl of PBS alone. After 3 h, plasma creatine kinase was determined as described in Section 2. Peptide legends are as in Fig. 1. Each bar represents mean ± SD.

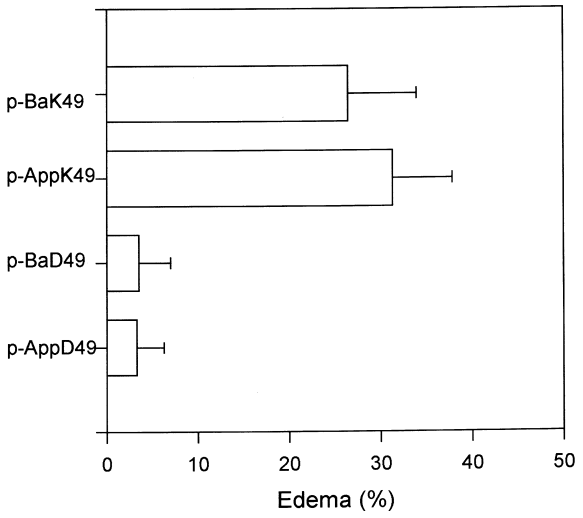

Fig. 3. Evaluation of the edema-forming activity of synthetic peptides derived from myotoxic phospholipases A_2 in vivo. Groups of four mice received a subcutaneous injection of 100 μg of peptides in their right footpad, in 50 μl of phosphate-buffered saline (PBS), and 50 μl of PBS alone in their left footpad as a control. After 1 h, mice were sacrificed, and the increase in wet weight of the right footpad, relative to the contralateral control, was expressed as percent edema. Peptide legends are as in Fig. 1. Each bar represents mean ± SD. The edema values for p-BaD49 and p-AppD49 are not statistically significant ($P > 0.05$).

plasma CK values recorded after injection of 250 μg of p-AppK49 represent approximately a 10-fold increment over levels measured in the control group after injection of PBS alone. Macroscopically, it was observed that mice flexed and momentarily immobilized the limb a few minutes after injection of p-AppK49, but not after injection of the other three peptides.

Intravital microscopical observation of the effects of p-AppK49 clearly confirmed its myotoxic activity. Starting between 7 and 10 min after exposure to the peptide, a number of muscle fibers underwent a slow longitudinal retraction process and eventually ruptured, leaving visible areas of hypercontraction alternating with translucent spaces apparently devoid of myofibrillar material. No bleedings or alterations in the microcirculation and its blood flow were observed after exposure to the peptide, in any of the experiments.

Subcutaneous injection of the peptides in the footpad of mice showed differences in their ability to induce edema. After injection of both Lys49-derived peptides, p-BaK49 and p-AppK49, a significant ($P < 0.05$) increase in footpad weight was recorded. In contrast, p-BaD49 and p-AppD49 did not induce any significant edema in this assay (Fig. 3).

Turbidimetric analyses of the heparin-binding properties of synthetic peptides in solution are summarized in Fig. 4. The four peptides were able to form macromolecular complexes with heparin, leading to variable degrees of turbidity, which was maximal at heparin/peptide molar ratios in the range 0.03–0.04. By adding a heparin excess, a partial resolubilization of the complexes was observed with all four peptides (Fig. 4). In order to determine if the interaction between heparin and the highly cytotoxic p-AppK49 resulted in neutralization of its effect, both were mixed (at a 1:10 heparin:peptide molar ratio), incubated at room temperature for 15 min, and subsequently added to C2C12 cultures (at a dose of 50 μg p-AppK49/well). Heparin completely abolished the cytotoxic activity of p-AppK49 (data not shown).

4. Discussion

The present results provide further insights towards understanding the structure-function relationship of myotoxic PLA_2s. The 13-mer peptide KKYKAYFKLKCKK, from the C-terminal region of *A. p. piscivorus* Lys49 PLA_2, clearly reproduced its cytotoxic action upon skeletal muscle cells in vitro, and its myotoxic effect after intramuscular injection in mice. Although the membrane-damaging activity of this 13-mer peptide is weaker than that of the whole toxin (unpublished data), the present findings unequivocally identify the sequence 115–129 of this Lys49 PLA_2 as containing the key structural determinants needed for myotoxicity, and constitute the first report of an unmodified, PLA_2-derived short synthetic peptide with the ability to reproduce this effect of a parent toxin in vivo.

The direct identification of the myotoxic site of a Lys49

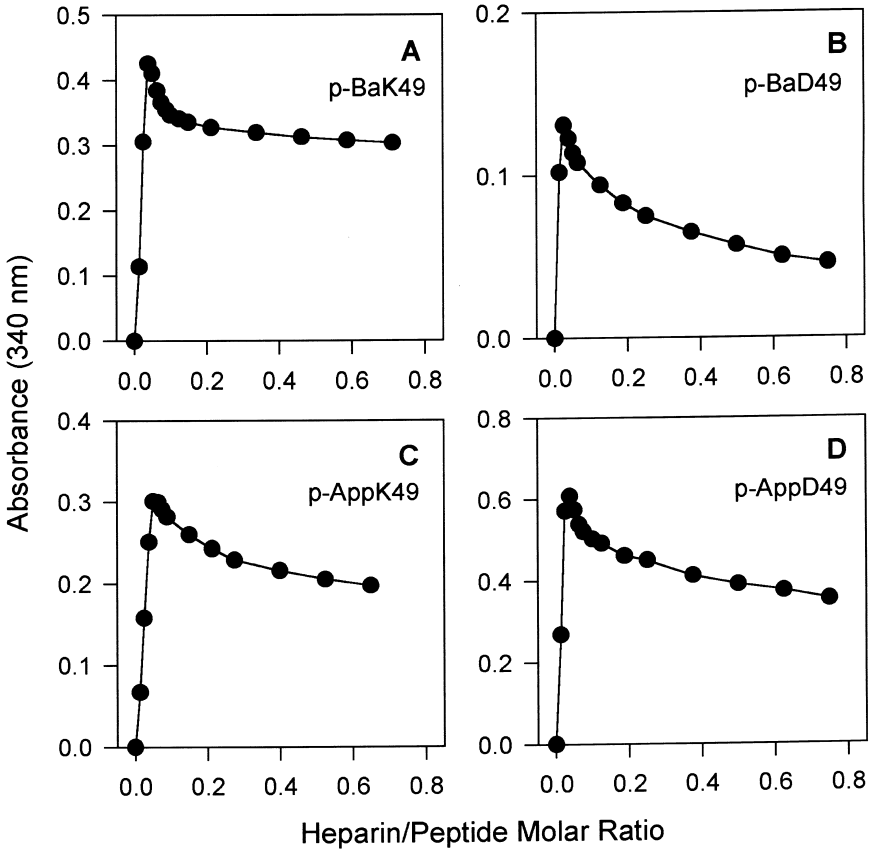

Fig. 4. Evaluation of the heparin-binding activity of synthetic peptides derived from myotoxic phospholipases A_2 in solution. Heparin was added in gradual increments to a cuvette containing 200 μg of peptide (approximately 55 μM), in 2 ml of 0.05 M Tris-HCl, 0.01 KCl, pH 7.0 buffer. Absorbance changes at 340 nm were monitored, allowing 1 min of incubation after each heparin addition. Peptide legends are as in Fig. 1.

PLA_2 from *A. p. piscivorus* by its synthetic peptide 115–129 is in agreement with the evidence that indirectly identified such site in *B. asper* myotoxin II (Lomonte et al., 1994b, 1999a; Calderón and Lomonte 1998, 1999). Although p-BaK49 did not reproduce myonecrosis in vivo, a weak, but clear cytotoxic activity upon skeletal muscle C2C12 cells was demonstrated. The weak toxic potency of p-BaK49 upon C2C12 cells is comparable to that originally reported using endothelial cells as targets (Lomonte et al., 1994b), being one order of magnitude lower than that of p-AppK49. This low membrane-damaging potency would explain the lack of a detectable myotoxic action of p-BaK49 in vivo, in contrast to the clear effect exerted by p-AppK49. Interestingly, the higher cytotoxic potency of p-AppK49 is comparable to that of a triple Tyr → Trp-substituted variant of p-BaK49 (referred to as p115–W3), a modified synthetic peptide which, in similarity with p-AppK49, also displays myotoxicity in mice (Lomonte et al., 1999a).

The structural basis for the different membrane-damaging potencies of p-AppK49 and p-BaK49 is not easily evident, since these peptides differ in five out of their 13 residues (Table 1). A relatively high number of synthetic peptide variants should be characterized to approach this question. Interestingly, an inspection of the three-dimensional structure of region 115–129 in the corresponding myotoxin crystals reveals a difference in the type of aromatic side chain oriented towards the interfacial recognition face (Fig. 5). Myotoxin II exposes Tyr119, in contrast to the more hydrophobic Phe121 of the *A. p. piscivorus* Lys49 PLA_2 lying next to the highly exposed Lys122 (Fig. 5). Two other Tyr residues of region 115–129, Tyr117 and Tyr120, are almost superimposed in both proteins. It was previously shown that substitution of the three Tyr residues of p-BaK49 by Trp markedly increases its membrane-damaging activity (Lomonte et al., 1999a). Therefore, it is tempting to speculate that Phe121 of p-AppK49, by increasing the hydrophobic character of the peptide, might be related to its higher membrane-damaging potency. Hydrophobic interactions between PLA_2s and membrane interfaces are key elements for their binding affinity (Gelb et al., 1999). It would be of interest to characterize the activities of the alternative form of p-BaK49 with Leu124 replaced by

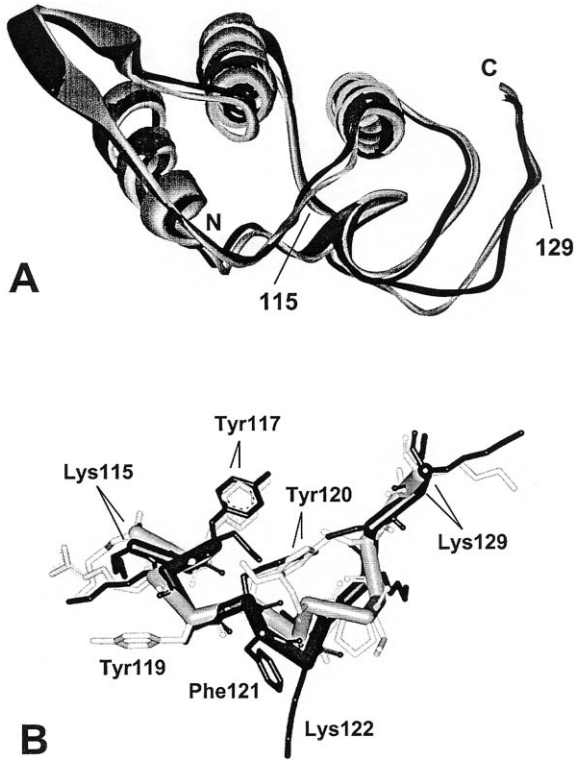

Fig. 5. Comparison of the three-dimensional structures of *Agkistrodon piscivorus piscivorus* Lys49 phospholipase A_2 and *Bothrops asper* myotoxin II monomer. (a) Ribbon representations of superimposed *A. p. piscivorus* Lys49 PLA_2 (black), and *B. asper* myotoxin II monomer (gray), showing the location of C-terminal region 115–129. The crystal structures correspond to PDB entries 1 ppa (Holland et al., 1990) and 1 clp (Arni et al., 1995), respectively, superimposed with the Swiss PDB Viewer 3.51 software (http://www.expasy.ch/spdbv/mainpage.html). The molecules are oriented with their interfacial recognition face approaching the membrane in a horizontal plane at the bottom (Zhou and Schulten, 1996; Snitko et al., 1997). (b) Backbone representation showing the amino acid side chains of the superimposed regions 115–129, as in (a).

Phe, reported as a natural microheterogeneity in myotoxin II preparations (Francis et al., 1991), since several myotoxic PLA_2s contain at least one Phe residue in their C-terminal region (Table 1), or other aromatic residues such as Tyr or Trp (Janssen et al., 1999). In this regard, Pungercar et al. (1999) demonstrated that Phe124, located at the C-terminal region of the group II PLA_2 ammodytoxin A from *Vipera ammodytes*, is essential for its presynaptic neurotoxicity, and Ivanovski et al. (2000) obtained evidence for a key role of region 115–129 in this toxic activity.

The strong (p-AppK49) and moderate (p-BaK49) membrane-damaging actions of the two Lys49-derived C-terminal peptides studied, were in sharp contrast to the complete lack of activity of their Asp49-derived counterparts, p-AppD49 and p-BaD49, even when tested at very high concentrations in a sensitive cell culture assay. In addition, edema was induced by the Lys49-derived, but not by the Asp49-derived peptides. These results suggests that Lys49 and Asp49 group II PLA_2s might exert their cytotoxic/myotoxic actions through different mechanisms, by implying that the latter may not utilize their C-terminal regions as main membrane-destabilizing elements. This hypothesis would seem plausible in the light of (a) the fundamental difference in catalytic activity between Asp49 and Lys49 isoforms; and (b) the significant structural divergence between Asp49 and Lys49 proteins, as it has been shown that the similarity of Lys49 PLA_2s from different snake species is higher than the similarity between Asp49 and Lys49 isoforms within the same species (Francis et al., 1991; Ogawa et al., 1995). In studies on the mechanisms of liposome disruption, Rufini et al. (1992) demonstrated essential differences between the actions of *B. asper* myotoxins II and III, observing that the action of the latter (Asp49) was attributable to catalytic phospholipid hydrolysis rather than to the calcium-independent membrane destabilization caused by the former. A relevant role of the enzymatic activity of Asp49 PLA_2s in the mechanism of membrane damage was also evidenced by Díaz et al. (1991). These findings, together with the striking differences observed between the membrane-damaging activities of two Lys49- and Asp49-derived peptides, support the possibility that the molecular mechanisms of myotoxicity utilized by Lys49 and Asp49 group II PLA_2s might be substantially different, a hypothesis that deserves further investigation.

The four C-terminal peptides bound heparin in solution, forming macromolecular complexes which increased turbidity by variable extents. Heparin is known to inhibit the myotoxic activity of myotoxins II and III from *B. asper*, as well as of the crude venom of *A. p. piscivorus* (Lomonte et al., 1994a), which contains both Asp49 and Lys49 PLA_2 isoforms. The observed interaction of heparin with C-terminal peptides of the two Asp49-type myotoxins suggests that this region, despite not showing a direct membrane-damaging effect when represented by the corresponding synthetic peptides of these isoforms, might still somehow be involved in steps of their toxic mechanism, perhaps by participating in interfacial binding. On the other hand, abolition of the cytotoxic effect of p-AppK49 caused by heparin extends similar observations originally made with peptide 115–129 (p-BaK49) of *B. asper* (Lomonte et al., 1994b), and rules out the possibilily that the toxic activities demonstrated for p-AppK49 in this study would be due to non-specific chemical contaminants of the particular peptide preparation.

On the basis of previously reported evidence for *B. asper* myotoxin II, and present results for *A. p. piscivorus* Lys49 PLA_2, it is concluded that the myotoxic site of these catalytically-inactive Lys49 PLA_2s, and possibly of other members of this protein family, lies within their 115–129 C-terminal region, which combines cationic and hydrophobic amino acids that are responsible for the damaging mechanism to cell membranes. The cationic residues would interact

electrostatically with anionic groups of a (still unidentified) acceptor site, while the hydrophobic residues, and especially the bulkier aromatic ones, would interact with, and possibly penetrate, the phospholipid bilayer, altogether resulting in its destabilization. On the other hand, the lack of direct membrane-damaging activity observed for two myotoxic Asp49 PLA_2-derived C-terminal peptides suggests that the toxic mechanism exerted by this type of proteins, which probably involves their catalytic activity as a relevant step, might differ from that utilized by the Lys49 PLA_2 myotoxins.

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References

Arni, R.K., Ward, R.J., 1996. Phospholipase A_2—a structural review. Toxicon 34, 827–841.

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.

Calderón, L., Lomonte, B., 1998. Immunochemical characterization and role in toxic activities of region 115–129 of myotoxin II, a Lys49 phospholipase A_2 from *Bothrops asper* snake venom. Archs. Biochem. Biophys. 358, 343–350.

Calderón, L., Lomonte, B., 1999. Inhibition of the myotoxic action of *Bothrops asper* myotoxin II in mice by immunization with its synthetic peptide 115–129. Toxicon 37, 683–687.

Cintra, A.C.O., Marangoni, S., Oliveira, B., Giglio, J.R., 1993. Bothropstoxin-I: amino acid sequence and function. J. Prot. Chem. 12, 57–64.

Dennis, E.A., 1997. The growing phospholipase A_2 superfamily of signal transduction enzymes. Trends Biochem. Sci. 22, 1–2.

Díaz, C., Gutiérrez, J.M., Lomonte, B., Gené, J.A., 1991. The effect of myotoxins isolated from *Bothrops* snake venoms on multilamellar liposomes: relationship to phospholipase A_2, anticoagulant and myotoxic activities. Biochim. Biophys. Acta 1070, 455–460.

Fletcher, J.E., Selistre de Araujo, H.S., Ownby, C.L., 1997. Molecular events in the myotoxic action of phospholipases. In: Kini, R.M. (Ed.). Venom phospholipase A_2 Enzymes: Structure, Function, and Mechanism. John Wiley & Sons, England, pp. 455–497.

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. Archs. Biochem. Biophys. 284, 352–359.

Gelb, M.H., Cho, W., Wilton, D.C., 1999. Interfacial binding of secreted phospholipases A_2: more than electrostatics and a major role for tryptophan. Curr. Opin. Struct. Biol. 9, 428–432.

Gutiérrez, J.M., Lomonte, B., 1995. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. Toxicon 33, 1405–1424.

Gutiérrez, J.M., Lomonte, B., 1997. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. In: Kini, R.M. (Ed.). Venom Phospholipase A_2 Enzymes: Structure, Function, and Mechanism. John Wiley & Sons, England, pp. 321–352.

Holland, D.R., Clancy, L.L., Muchmore, S.W., Ryde, T.J., Einspahr, H.M., Finzel, B.C., Heinrikson, R.L., Watenpaugh, K.D., 1990. The crystal structure of a lysine 49 phospholipase A_2 from the venom of the cottonmouth snake at 2.0-Å resolution. J. Biol. Chem. 265, 17649–17656.

Ivanovski, G., Copic, A., Krizaj, I., Gubensek, F., Pungercar, J., 2000. The amino acid region 115–129 of ammodytoxins plays an important role in neurotoxicity. Biochem. Biophys. Res. Comm. 276, 1229–1234.

Janssen, M.J.W., Burghout, P.J., Verheij, H.M., Slotboom, A.J., Egmond, M.R., 1999. Introduction of a C-terminal aromatic sequence from snake venom phospholipases A_2 into the porcine pancreatic isozyme dramatically changes the interfacial kinetics. Eur. J. Biochem. 263, 782–788.

Jeyaseelan, K., Armugam, A., Donghui, M., Tan, N.H., 2000. Structure and phylogeny of the venom group I phospholipase A_2 gene. Mol. Biol. Evol. 17, 1010–1021.

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*. Archs. Biochem. Biophys. 278, 319–325.

Kini, R.M., Chan, Y.M., 1999. Accelerated evolution and molecular surface of venom phospholipase A_2 enzymes. J. Mol. Evol. 48, 125–132.

Krizaj, I., Bieber, A.L., Ritonja, A., Gubenšek, F., 1991. The primary structure of ammodytin L, a myotoxic phospholipase A_2 homologue from *Vipera ammodytes* venom. Eur. J. Biochem. 202, 1165–1168.

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.

Lomonte, B., Tarkowski, A., Bagge, U., Hanson, L.Å., 1994. Neutralization of the cytolytic and myotoxic activities of phospholipases A_2 from *Bothrops asper* snake venom by glycosaminoglycans of the heparin/heparan sulfate family. Biochem. Pharmacol. 47, 1509–1518.

Lomonte, B., Moreno, E., Tarkowski, A., Hanson, L.Å., Maccarana, M., 1994. Neutralizing interaction between heparins and myotoxin II, a Lys-49 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, 29867–29873.

Lomonte, B., Lundgren, J., Johansson, B., Bagge, U., 1994. The dynamics of local tissue damage induced by *Bothrops asper* snake venom and myotoxin II on the mouse cremaster muscle: an intravital and electron microscopic study. Toxicon 32, 41–55.

Lomonte, B., Pizarro-Cerdá, J., Angulo, Y., Gorvel, J.P., Moreno, E., 1999. Tyr → Trp-substituted peptide 115–129 of a Lys49 phospholipase A_2 expresses enhanced membrane-damaging

activities and reproduces its in vivo myotoxic effect. Biochim. Biophys. Acta 1461, 19–26.

Lomonte, B., Angulo, Y., Rufini, S., Cho, W., Giglio, J.R., Ohno, M., Daniele, J.J., Geoghegan, P., Gutiérrez, J.M., 1999. Comparative study of the cytolytic activity of myotoxic phospholipases A_2 on mouse endothelial (tEnd) and skeletal muscle (C2C12) cells in vitro. Toxicon 37, 145–158.

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.

Maraganore, J.M., Merutka, G., Cho, W., Welches, W., Kezdy, F.J., Heinrikson, R.L., 1984. A new class of phospholipases A_2 with lysine in place of aspartate 49. J. Biol. Chem. 259, 13839–13843.

Ogawa, T., Kitajima, M., Nakashima, K., Sakaki, Y., Ohno, M., 1995. Molecular evolution of group II phospholipases A_2. J. Mol. Evol. 41, 867–877.

Páramo, L., Lomonte, B., Pizarro-Cerdá, J., Bengoechea, J.A., Gorvel, J.P., Moreno, E., 1998. 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. Eur. J. Biochem. 253, 452–461.

Pereira, M.F., Novello, J.C., Cintra, A.C.O., Giglio, J.R., Landucci, E.T., Oliveira, B., Marangoni, S., 1998. The amino acid sequence of bothropstoxin-II, an Asp-49 myotoxin from *Bothrops jararacussu* (jararacucu) venom with low phospholipase A_2 activity. J. Prot. Chem. 17, 381–386.

Pungercar, J., Krizaj, I., Liang, N.S., Gubenšek, F., 1999. An aromatic, but not a basic, residue is involved in the toxicity of group-II phospholipase A_2 neurotoxins. Biochem. J. 341, 139–145.

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, 11627–11634.

Rosenberg, P., 1990. Phospholipases. In: Shier, W.T., Mebs, D. (Eds.). Handbook of Toxinology. Marcel Dekker, New York, pp. 67–277.

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, 12424–12430.

Scott, D.L., 1997. Phospholipase A_2: structure and catalytic properties. In: Kini, R.M. (Ed.). Venom Phospholipase A_2 Enzymes: Structure, Function, and Mechanism. John Wiley & Sons, England, pp. 97–128.

Scott, S.L., Achari, A., Vidal, J.C., Sigler, P.B., 1992. Crystallographic and biochemical studies of the (inactive) Lys-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. J. Biol. Chem. 267, 22645–22657.

Selistre de Araujo, H.S., White, S.P., Ownby, C.L., 1996. cDNA cloning and sequence analysis of a lysine-49 phospholipase A_2 myotoxin from *Agkistrodon contortrix laticinctus* snake venom. Archs. Biochem. Biophys. 326, 21–30.

Snitko, Y., Koduri, R.S., Han, S.K., Othman, R., Baker, S.F., Molini, B.J., Wilton, D.C., Gelb, M.H., Cho, W., 1997. Mapping the interfacial binding surface of human secretory group IIA phospholipase A_2. Biochemistry 36, 14325–14333.

Soares, A.M., Rodrigues, V.M., Homsi-Brandeburgo, M.I., Toyama, M.H., Lombardi, F., Arni, R.K., Giglio, J.R., 1998. A rapid procedure for the isolation of the Lys-49 myotoxin II from *Bothrops moojeni* (caissaca) venom: biochemical characterization, crystallization, myotoxic and edematogenic activity. Toxicon 36, 503–514.

Soares, A.M., Andrião-Escarso, S.H., Angulo, Y., Lomonte, B., Gutiérrez, J.M., Toyama, M.H., Marangoni, S., Arni, R.K., Giglio, J.R., 2000. Structural and functional characterization of myotoxin I, a Lys49 phospholipase A_2 homologue from *Bothrops moojeni* (caissaca) snake venom. Archs. Biochem. Biophys. 373, 7–15.

Soares, A.M., Guerra-Sá, R., Caroline, R., Borja-Oliveira, C.R., Rodrigues, V.M., Rodrigues-Simioni, L., Rodrigues, V., Fontes, M.R.M., Lomonte, B., Gutiérrez, J.M., Giglio, J.R., 2000. Structural and functional characterization of BnSP-7, a Lys49 myotoxic phospholipase A_2 homologue from *Bothrops neuwiedi* pauloensis venom. Archs. Biochem. Biophys. 378, 201–209.

Toyama, M.H., Soares, A.M., Novello, J.C., Oliveira, B., Giglio, J.R., Marangoni, S., 1998. Amino acid sequence of piratoxin-I, a myotoxin from *Bothrops pirajai* snake venom and its biological activity after alkylation with p-bromophenacyl bromide. J. Prot. Chem. 17, 713–718.

Ward, R.J., Rodrigues Alves, A., Ruggiero Neto, J., Arni, R.K., Casari, G., 1998. A SequenceSpace analysis of Lys49 phospholipases A_2: clues towards identification of residues involved in a novel mechanism of membrane damage and in myotoxicity. Prot. Engin. 11, 285–294.

Yamakawa, M., Nozaki, M., Hokama, Z., 1976. Fractionation of Sakishima-habu (*Trimeresurus elegans*) venom and lethal, hemorrhagic and edema-forming activities of the fractions. In: Ohsaka, A., Hayashi, K., Sawai, Y. (Eds.). Animal, Plant and Microbial Toxins, vol. 1. Plenum Press, New York, p. 97.

Yoshizumi, K., Liu, S.Y., Miyata, T., Saita, S., Ohno, M., Iwanaga, S., Kihara, H., 1990. Purification and amino acid sequence of basic protein I, a lysine-49 phospholipase A_2 with low activity, from the venom of *Trimeresurus flavoviridis* (Habu snake). Toxicon 28, 43–54.

Zhou, F., Schulten, K., 1996. Molecular dynamics study of phospholipase A_2 on a membrane surface. Proteins 25, 12–27.