Mapping of the structural determinants of artificial and biological membrane damaging activities of a Lys49 phospholipase A2 by scanning alanine mutagenesis

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Abstract

Scanning alanine mutagenesis has been used to study the structural determinants of several activities of bothropstoxin-I (BthTx-I), a lysine 49 phospholipase A2 from the venom of Bothrops jararacussu. A total of 31 mutants were generated in the interfacial recognition site and C-terminal loop regions of the protein. The effects of mutagenesis on the in vivo myotoxic activity, the cytolytic activity against cultured C2C12 myoblasts, the bactericidal activity, and the Ca2+-independent membrane damaging activity against liposome membranes were compared. Residues 116–119 and 122–125 in the C-terminal loop region are structural determinants for these activities, indicating that membrane permeabilization by the BthTx-I is an important general property in all the measured effects. The structural determinants of myotoxicity and myoblast membrane permeabilization are highly correlated, demonstrating that cultured C2C12 myoblasts are a good model for the myotoxic effect. However, comparison of the structural determinants for all activities revealed several differences in the structural determinants between the effects against myoblast and bacterial membranes, and further differences when compared to the liposome membrane damaging effect. These membrane dependent effects are interpreted to be the consequence of differences in the activation of the membrane bound form of the protein on biological and artificial membranes.

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1. Introduction

Phospholipases A2 (PLA2-EC 3.1.1.4) catalyze the hydrolysis of the sn-2 acyl bonds of sn-3 phospholipids [1], and are currently classified in 15 groups on the basis of disulphide bonding patterns and amino acid sequence similarity [2]. The hydrolysis of phospholipids by Group I/II PLA2s involves a His48/Asp99 pair in the catalytic site that activate a conserved water molecule, thereby initiating the nucleophilic attack on the sn-2 position of the substrate [3,4]. Many snake venom PLA2s (vPLA2) present pharmacological properties [5], and it is well documented that at least some of these activities are mediated by interactions between the vPLA2 and a specific receptor or acceptor on the target cell membrane [6–10]. Although the details of the mechanisms through which the venom PLA2s exert these effects is not fully understood, the evidence suggests that the catalytic function is not necessary in the case of some of these pharmacological effects [11–16].

Catalytically inactive vPLA2s have been described that present a high degree of amino acid sequence similarity to other group II PLA2s. In these PLA2 variants, the aspartic acid residue at position 49 (Asp49) that is invariable in catalytically active PLA2s, is substituted by a lysine residue (Lys49). Since their...
initial description, the amino acid sequences of 30 Lys49–PLA2s from 23 crotalid and vipersnake venoms have been deposited in the public sequence databases. Comparison of the crystal structures of Lys49–PLA2s demonstrates that the ε-amino group of the Lys49 is located in the position normally occupied by the Ca$^{2+}$ cofactor in the Asp49-PLA2 [17–19], and it has been suggested that the Lys49–PLA2s are catalytically inactive as a consequence of the loss Ca$^{2+}$ cofactor binding. Although the Lys49–PLA2s do not show catalytic activity, interaction with liposomes results in the rapid liberation of the liposome contents via a Ca$^{2+}$-independent mechanism that does not involve the hydrolysis of membrane phospholipids [20–22]. Nevertheless, the in vivo activities of the Lys49–PLA2s include the formation of edema and local myonecrosis [23,24], cytolyis of a wide range of cell types [14,25], bactericidal activity [15,26] and local inflammation and pain [11,12,27,28].

Based on a visual analyses of PLA2 myotoxin sequence alignments, it has been suggested that either an extended surface including polar and cationic residues primarily on helix 3 [29] or that residues 78 to 87 located in the β-wing region of the PLA2s [30] are determinants of the myotoxic effect. More refined methods for analysis of multiple amino acid sequence alignments have been developed which identify the specific amino acid residues that define sub-groups of proteins [31–33]. When applied to Lys49–PLA2s sequences this analysis yielded a list of 12 specific residues that were mapped to 3 amino acid clusters located in the active site region, the hydrophobic fatty acyl chain binding pocket and the tip of the β-wing [34]. Subsequent site-directed mutagenesis results using bothropstoxin-I, a Lys49–PLA2 from the venom of Bothrops jararacussu, have confirmed that all these three regions play important and distinct roles in the Ca$^{2+}$-independent membrane damaging mechanism [35–37].

A method for comparing residue variability in highly similar amino acid sequences has identified positions in the C-terminal loop region of the Lys49–PLA2 myotoxins, which were not identified using previously available sequence analysis tools [5], and were identified as candidates for being the structural determinants of the biological effects of Lys49–PLA2 myotoxins. This prediction is supported by the observation that a synthetic peptide of residues 115–129 in the C-terminal region of the myotoxin-II from Bothrops asper demonstrates biological activity, although at reduced levels when compared to the intact protein [25]. Furthermore, this prediction shows a good correlation with previous results of site directed mutagenesis experiments in BthTx-I, which demonstrated that positions 117 to 122 influence the myotoxic activity in the C-terminal loop region [38]. Here we have extended these studies to further elucidate the structural determinants of the pharmacological activities of the Lys49–PLA2s, and to understand the role of the Ca$^{2+}$-independent membrane damaging activity in these effects.

In group I/II PLA2s, the interfacial recognition site (IRS, [39]) or the i-face [40] is a region of the protein defined by a highly conserved hydrophobic surface cleft that binds the fatty acyl chain of the phospholipid substrate, together with a surrounding ring of more variable polar and charged residues. This report presents the result of the effects of a scanning alanine mutagenesis study of all cationic residues in the IRS and all residues around the C-terminal loop region of the BthTx-I. The effects of a total of 31 mutants on the myotoxicity, membrane permeabilization of cultured C2C12 myoblasts, bactericidal activity and liposome membrane disruption have been evaluated and compared with previous site-directed mutagenesis studies of the BthTx-I.

2. Materials and methods

2.1. Protein purification from crude venom

Bothropstoxin-I (BthTx-I) was purified from crude lyophilized B. jararacussu venom using single step cation-exchange chromatography as previously described [37]. The BthTx-I eluted as a single peak and was dialyzed against 5 mM Tris–HCl, pH 7.5 for 36 h with buffer changes every 12 h and concentrated 10-fold by lyophilization. Protein purity was routinely evaluated by silver staining of SDS-PAGE gels [41], and aliquots of purified protein were stored at 4 °C until further use.

2.2. Site directed mutagenesis

A full-length cDNA encoding BthTx-I has been previously isolated from B. jararacussu venom gland cDNA by RT-PCR (GenBank Acc. No. X78599) [42], and sub-cloned into the expression vector pET3-d [43]. Nucleotide sequencing has confirmed the construct in which Ser1 of the BthTx-I is preceded by a Met, and a stop codon immediately follows Cys133 (homology numbering as reported previously [44] is used throughout this report). After linearization of this construct with SacI, site directed mutagenesis of the BthTx-I was performed by PCR mutagenesis [45] to introduce a total of 31 single mutations (shown in Table 1). The final PCR reactions were performed using oligonucleotides complementary to the vector sequences flanking the BthTx-I insert which contained restriction sites for XhoI (5’-extremity) and BamHI (3’-extremity). After digestion with these enzymes, the amplified fragments were subcloned into the equivalent sites in the expression vector pET3d and fully sequenced.

2.3. Recombinant protein expression and purification

Four hundred millilitres of growth medium (per litre, 25 g yeast extract; 15 g tryptone; 10 mM MgSO$_4$; 35 mg chloramphenicol; 100 mg ampicillin; pH 7.5) was inoculated with Escherichia coli strain BL21(DE3)pLysS transformed with the native or mutant constructs in pET3d, and grown at 37 °C to an A$_{600}$ of 0.6. Recombinant protein expression was induced by addition of 0.6 mM IPTG, and the culture grown for an additional 7 h. Inclusion bodies were isolated from bacterial pellets by repeated rounds of sonication in 20 mL of lysis buffer (50 mM Tris–HCl, pH 8.0; 1 mM EDTA; 0.4 M urea; 1% Triton X-100) followed by centrifugation at 12,000 g. The protocol for the solubilization and refolding of recombinant BthTx-I in the presence of a gel filtration medium was performed as previously described [43]. The refolded protein was applied directly to the cation exchange column and eluted as described earlier for the purification of the native BthTx-I from crude venom.

2.4. Release of entrapped fluorescent markers from liposomes

Membrane damaging activity was evaluated by the release of the liposome entrapped self-quenching fluorescent dye calcein. The loss of liposome membrane integrity results in dilution of the fluorophore with a consequent increase in the fluorescence signal [21,22]. Unilamellar liposomes composed of either mixtures of egg yolk phosphatidylcholine (EYPC):dimyristoyl phosphatidic acid (DMPA) or EYPC: egg yolk phosphatidylglycerol (EYPG) at a 9:1 molar ratio containing 25 mM calcein (Sigma) were prepared by reverse phase evaporation as described previously [22], and mixed with BthTx-I to a final protein:lipid molar ratio of 1:400. Membrane damage kinetics were monitored by the increase in fluorescence emission at 520 nm with excitation at 490 nm,
Table 1
Oligonucleotide sequences of primers used for the site-directed mutagenesis experiments

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>K7A</td>
<td>GCAAGATGATGCCCTGATGTC</td>
</tr>
<tr>
<td>K15A</td>
<td>GACGCGGCTGCGGCTGCTCT</td>
</tr>
<tr>
<td>K36A</td>
<td>CTTTGGCGGCCCTGGGCC</td>
</tr>
<tr>
<td>P37A</td>
<td>GGCTGCCCTTGGATGCTTGG</td>
</tr>
<tr>
<td>K38A</td>
<td>GGCTCCCCTGCTGCTGGCC</td>
</tr>
<tr>
<td>D39A</td>
<td>CAGTGCGGCCCTTGCTGGC</td>
</tr>
<tr>
<td>R43A</td>
<td>AGCAACAGGGCTGACCTGTC</td>
</tr>
<tr>
<td>Y46A</td>
<td>TTTGTCACGGCCAGCAAGGG</td>
</tr>
<tr>
<td>I48Q</td>
<td>GAAACCGATTTCCGACTGCTG</td>
</tr>
<tr>
<td>K53A</td>
<td>CATTTTGGGTATACGAGCCT</td>
</tr>
<tr>
<td>K54A</td>
<td>CGAAGGCTGAGTCTTATTTAAC</td>
</tr>
<tr>
<td>I-K57/R58</td>
<td>GAGGCTGAGGCTGCTTGGCT</td>
</tr>
<tr>
<td>K80A</td>
<td>GATAGTGGGCTGCTGCTCC</td>
</tr>
<tr>
<td>E86G</td>
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</tr>
<tr>
<td>K92A</td>
<td>ACAACACTGGAGCCGGC</td>
</tr>
<tr>
<td>K100A</td>
<td>TGCCAGGCCTGCGGGACTCT</td>
</tr>
<tr>
<td>T121A</td>
<td>TTGTCATGCGGCGCAATGT</td>
</tr>
<tr>
<td>N114A</td>
<td>ATTTTGGCGTCTAGGCTGCG</td>
</tr>
<tr>
<td>K115A</td>
<td>CCTATATTGCCTGTTGATCC</td>
</tr>
<tr>
<td>K116A</td>
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<tr>
<td>Y117A</td>
<td>GATACCTAGCTCGTTTGT</td>
</tr>
<tr>
<td>Y117W</td>
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</tr>
<tr>
<td>R118A</td>
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<tr>
<td>Y119A</td>
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<tr>
<td>Y119W</td>
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<td>H120A</td>
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<tr>
<td>L121A</td>
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<tr>
<td>K122A</td>
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</tr>
<tr>
<td>F125A</td>
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</tr>
<tr>
<td>F125W</td>
<td>CTTTTGGGCAAGGAGTGTGTCAG</td>
</tr>
<tr>
<td>K129A</td>
<td>GGGCGTGGGCGCTGCTGGCAAA</td>
</tr>
</tbody>
</table>

All sequences are shown in the 5′ to 3′ direction, and the underlined bases indicate the codon that was introduced during the mutagenesis.

a Generation of mutant previously described (Ward et al., 2002).

b Generation of mutant previously described (Chioato et al., 2002).

and the signal was expressed as the percentage of total calcein liberation on addition of 5 mM Triton X-100. The effect of each mutant was measured in 6 independent experiments, and the data were analyzed using ANOVA with a subsequent Tukey test.

2.5. Bactericidal activity

The bactericidal effect of BthTx-I and mutants was evaluated against the Gram-negative bacteria E. coli (K12). Five mL of LB bacterial growth medium was inoculated with a freshly grown colony and incubated for 12 h with agitation at 37 °C. One mL of this culture was used to inoculate 50 mL of fresh LB, and the culture was grown at 37 °C to an OD600 of 0.7. The bacteria were diluted to a cell density of 4.5 × 10^6 cells mL^-1 with sodium phosphate buffer, pH 7.6 containing 1% tryptone. One hundred μL of this cell suspension were incubated with BthTx-I and mutants at a final concentration of 3 μg mL^-1 for 2 h at 37 °C, and plated on solid LB medium. After incubation at 37 °C for 12 h, the number of colony forming units was counted. The percentage inhibition of colony forming units was calculated relative to the number of colonies present in the negative control, which consisted of bacterial incubation with buffer alone. The effect of each mutant was measured in 4 independent experiments, and the data were analyzed using ANOVA with a subsequent Tukey test.

2.6. Myotoxic activity

The plasma creatine kinase (CK) activity was determined using the CK-UV kinetic kit (Sigma Chemical Co.) after injection of 30 μg of BthTx-I in a total volume of 50 μL of PBS into the gastrocnemius muscle of male Swiss mice (wt 18–22 g) as previously described [35,38]. CK activity was expressed in U L^-1, where one unit is defined as the amount of enzyme that produces 1 mMol of NADH min^-1 under the standard conditions used in the assay. Animals used as negative controls received 50 μL of PBS, and the CK activity data were analyzed using ANOVA with a subsequent Tukey test from a minimum of 4 independent experiments.

2.7. Cytotoxic activity against C2C12 myoblasts

Mouse myogenic cells C2C12 (American Type Culture Collection; Manassas, VA) were cultured as exponentially growing myoblasts at 37 °C in a 5% CO2–95% air humidified atmosphere in a supplemented Dulbecco’s modified Eagle’s medium (DMEM) growth medium containing 10% fetal bovine serum (FBS), 4 mM l-glutamine and 100 UI mL^-1 penicillin and 100 mg mL^-1 streptomycin. Cells were harvested from subconfluent monolayers after detachment by exposure to a trypsin solution (1500 U ml^-1) containing 5.3 mM EDTA, for 5 min at 37 °C. The resuspended cells were seeded in 96-well microplates, at an approximate initial density of between 1 to 4 × 10^5 cells per well, in the same growth medium. After reaching approximately 75% confluence, undifferentiated myoblasts were used directly in the cytotoxicity assay. In this assay, the cells were washed once with RPMI without phenol red and then incubated with RPMI without phenol red + 10 μM Sytox Green (Molecular Probes, Oregon, USA) at 37 °C for 15 min. The cytotoxicity assay was initiated by the addition of BthTx-I or mutant protein to each well at a final concentration of 50 μg mL^-1. Permeabilization of the cell membrane was monitored by the increase in the fluorescence signal using a Gemini EM microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) using 485 nm excitation and 538 nm emission filters respectively. The fluorescence signal recorded after 30 min incubation was normalized to that measured after complete permeabilization of the cells after the addition of 0.5% Triton X-100. The effect of each mutant was measured in the minimum in 6 independent experiments, and the data were analyzed using ANOVA with a subsequent Tukey test.

3. Results

Dideoxynucleotide sequencing of all coding sequences of the BthTx-I revealed that the differences observed between the native DNA nucleotide sequences and their respective mutants (see Table 1) were only those introduced during the mutagenesis procedure. After expression and purification of the heterologous BthTx-I and all mutants, SDS-PAGE of the recombinant protein showed the presence of a single band of the correct molecular weight on silver staining (results not shown). Furthermore, the farUVCD spectra of all mutant proteins presented a spectral profile that was similar to the native BthTx-I purified from the venom of B. jararacussu (results not shown). These results demonstrate that the refolding and purification protocols yielded protein with native-like secondary structure, and are similar to those previously reported for the expression and purification of the recombinant BthTx-I [35,36,38,43,46].

Fig. 1A shows the effects of the mutagenesis on the myotoxic activity of the BthTx-I. The results demonstrate that the C-terminal loop region of the protein is a structural determinant of the activity, and that with the exception of residues 120 and 121, residues at positions 117 to 125 are key determinants of this effect together with position 37. The effect on myotoxic activity is also dependent on the type of amino acid substitution. Whereas the introduction of alanine at positions 117, 119 and 125 reduces the myotoxic activity, the conservative substitution of threonin by tryptophan has no effect demonstrating that aromatic
residues at these positions are important for conservation of activity. Fig. 1B shows the localization of the residues that significantly alter the myotoxic activity as shaded regions on a solid surface representation of the BthTx-I molecule. The three orientations shown in Fig. 1C present the view from (i) the C-terminal loop, (ii) the interfacial recognition site and (iii) the beta wing. These figures demonstrate that the residues that are the structural determinants of the myotoxic activity are all located in a single surface cluster centered on the C-terminal loop region.
The effects of the BthTx-I and site-directed mutants on the permeabilization of cultured C2C12 myoblasts are presented in Fig. 2. Fig. 2A shows that (with the exception of residues 120 and 121) the C-terminal region between residues 115 and 125 is a structural determinant of the membrane permeabilization, along with residues 37 and 38. As is the case with the in vivo myotoxic activity, conservative mutation of the aromatic residues has no significant effect on the membrane permeabilizing activity against myoblasts. Fig. 2B shows the solid surface representation of these results, which when compared to Fig. 1B, shows that there is a strong correlation between the structural determinants of the activity against myoblasts and the structural determinants of the myotoxic effect. This result suggests that the underlying mechanisms of membrane damage in myoblasts and the myotoxic activities are correlated.

Fig. 3 shows the effect of mutagenesis on the bactericidal activity of the BthTx-I, which reveals that mutation of all residues in the C-terminal loop between positions 115 and 125 together with mutation of positions 7, 86 and 100 reduce the effect of the toxin. It is noteworthy that this activity is not affected by mutations around position 37, and furthermore that conservative mutations of the aromatic residues in the C-terminal region have similar effects on the reduction of the bactericidal activity as the non-conservative mutations at these positions. These differences are readily visualized in the solid surface representations shown in Fig. 3B, which demonstrates that the regions of the protein surface which acts as the

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**Fig. 2.** (A) Membrane permeabilization of C2C12 myoblasts comparing the native and mutants BthTx-I. Increase in the Sytox Green fluorescence signal 30 min after the addition of native BthTx-I and mutants to C2C12 cell cultures at a final concentration of 50 μg mL⁻¹. The results show the mean ± s.d. of 6–9 independent assays, in which those mutants indicated by an asterisk (*) present an altered activity at a significance level of *p* < 0.05. (B) Solid surface representations of the BthTx-I monomer in three orientations showing the location of the mutations that significantly alter (*p* < 0.05) the membrane permeabilizing activity. The three orientations shown are the same as described in the legend to Fig. 1.
structural determinants of the bactericidal activity partially overlap with the structural determinants of the myotoxic and myoblast membrane permeabilizing activities.

Fig. 4 presents the effects of mutagenesis on the marker release from liposomes composed of the phospholipid mixture EYPC/DPPG at molar ratio of 9:1. These data show that mutation of residues in the C-terminal loop region between positions 115–127, together with residues in the region 36–43 and positions 46 and 53, all reduce the membrane damaging activity of the BthTx-I. The representation of these results shown in Fig. 4B reveals that the determinants of the liposome membrane damaging activity present an extended surface that includes most of the residues at the C-terminal extremity of the molecule. Fig. 5 shows that most of the positions that define the structural determinants of the membrane damaging activity against EYPC/DPPG liposomes are also determinants of the membrane of damaging activity against liposomes composed of a EYPC/DMPA phospholipid mixture at a molar ratio of 9:1.

A comparison of Figs. 4B and 5B indicates that the residues around the C-terminal region appear to contribute to a conserved surface that determines the activity against both types of artificial membrane. Despite the similarities in the pattern of the structural determinants of the membrane damaging activity against the EYPC/DMPA and EYPC/DPPG liposomes, several differences may be observed. In addition to the extended surface at the C-terminal region that includes residues 115–125 and 36–43 the mutations that decrease the membrane damaging activity against the EYPC/DMPA liposome membrane also include residues 7, 15, 53, 54, 80 and 100. These additional positions show that the surface that acts as the structural determinant of the membrane damaging activity
against the EYPC/DMPA liposomes is more extensive, and is distributed across the IRS region of the protein. In addition to this more extensive surface, conservative mutation of the aromatic residues at positions 117 and 125 cause a respective increase and decrease in the membrane damaging effect against the EYPC/DMPA liposomes.

4. Discussion

The scanning alanine mutagenesis strategy has enabled us to map of the structural determinants of various activities of the BthTx-I. Previous results of site-directed mutagenesis studies of 9 residues in the C-terminal loop region of the protein demonstrated that the residues at positions 118, 119 and 122 are determinants of the myotoxic activity [38], and here we have expanded this study to include 23 additional mutations at positions distributed over the IRS of the protein. In addition to the previously known positions, this study has demonstrated that the additional structural determinants of myotoxicity are all limited to a single cluster of residues centered on the C-terminal loop, and includes residues around position 37. Furthermore, the importance of the aromatic nature of the residue at position 125 has been established, which extends the region of the C-terminal loop that contributes to the myotoxic site. Comparison of the residues that were identified in the present study with a list of positions previously predicted to be determinants of the myotoxic activity by the residue variability analysis of the Lys49–PLA₂s [5], confirms that 4 of the 8 predicted residues are located in the surface cluster that determines the myotoxic activity.

The results of the present study show a striking coherence with previous reports of the myotoxic and bactericidal effects
produced by a synthetic peptide of residues 115–129 from the C-terminal regions of myotoxin II from *B. asper* (BaspMT-II, [15,25]) and other Lys49–PLA₂s [47–50]. The C-terminal peptide from BaspMT-II shares 85% identity with the homologous region in the C-terminus of BthTx-I, and although this peptide reproduces the effects observed in the C-terminal mutants of the BthTx-I, the concentration of the peptide needed to elicit a given effect is approximately 100-fold higher than that for the intact protein. The higher activity of the full protein may be due to the contribution by the other regions in addition to the C-terminal region that have been identified in this study. Furthermore, the C-terminal loop region is tethered to the body of the protein by two disulphide bridges, and the absence of these restraints in the synthetic peptide is likely to result in higher entropy of the peptide and consequently a reduced stability of the active conformation.

This report includes the first systematic study by site-directed mutagenesis of the bactericidal activity of a Lys49–PLA₂, which demonstrates that the structural determinants of the bactericidal effect are more extensive and only partially superposed with those of the C2C12 cell membrane damaging and myotoxic activities. Comparison of the influence of mutagenesis on the bactericidal effect and liposome membrane damage reveals that although the C-terminal region of the protein is a structural determinant of both activities, other regions of the protein are also involved. The mutation K7A resulted in a significant reduction in both the bactericidal activity and the Ca²⁺-independent activity against membranes.

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**Fig. 5.** (A) Calcium independent damaging activity of the native and mutant BthTx-I against EYPC:DMPA liposomes. Release of trapped calcein from liposomes (EYPC:DMPA at a 9:1 molar ratio) 150 s after mixing with 3 μg/mL of protein (at a final protein:lipid ratio of 1:400). The results show mean percentage ± s.d. of 6 independent measurements, in which those mutants indicated by an asterisk (*) present an altered activity at a significance level of *p* < 0.05. The data are normalized to the release observed after addition of 5 mM Triton X-100. (B) Solid surface representations of the BthTx-I monomer in three orientations showing the location of the mutations that significantly alter (*p* < 0.05) the Ca²⁺-independent membrane damaging activity. The three orientations shown are the same as described in the legend to Fig. 1.
containing the PA. These results may be compared with previous reports, in which acetylation or cleavage of the N-terminal octapeptide of the Lys49-PLA2 BNSP-7 inhibited the bactericidal activity against E. coli [26]. It is noteworthy that the same mutations did not influence either the myotoxic activity or the membrane permeabilization of C2C12 myoblasts, and is in agreement with previous results which have demonstrated that incubation with monoclonal antibodies against the N-terminal region of the Lys49-PLA2 did not influence biological activity [51]. These results emphasize that the composition of the target membrane exerts a significant influence on the observed activities of the BthTx-I. Although the results indicate substantial differences in the structural determinants of the liposome membrane damaging effects in comparison to the biological membranes, the experiments with model membranes yield insights with respect to the membrane damaging mechanism that provide guidelines for the interpretation of the results in the more complex biological systems.

The activity of all mutants was evaluated against liposome membranes composed of both EYPC/DMPA and EYPC/DPPG. Although the activity is higher against EYPC/DMPA membranes and modulation of the effect of specific mutations is also observed, the structural determinants of the damaging effect against both membrane types are broadly similar (compare Figs. 4 and 5). These differences demonstrate that the roles of individual amino acids may be modulated by the composition of the target membrane, which in turn suggests that the activity of the BthTx-I against a phospholipid membrane is mediated by the overall physico-chemical properties of the IRS rather than specific phospholipid binding sites. Mapping of the structural determinants of the bactericidal effect reveals a distribution more similar to that observed for the liposome membranes, suggesting a non-specific lipid-mediated interaction with the target membrane. In comparison, the close clustering of the structural determinants of the myotoxic and myoblast membrane permeabilization activities are suggestive of a more specific interaction of the BthTx-I with these membranes, perhaps mediated by a membrane associated acceptor.

Our results give a deeper understanding as to the structural basis of the mechanism of the Lys49-PLA2s. Comparison of 18 crystal structures of Lys49-PLA2s from 8 Agkistrodon and Bothrops species has shown that residues in the C-terminal region have an enhanced flexibility [17], and that the binding of amphiphilic molecules to the protein is generally correlated with the exposure of conserved hydrophobic residues in the region 120–125 (the so-called hydrophobic knuckle) [17]. These observations have led to the proposal that the binding of lipids to the active site results in a conformation change of the C-terminal loop exposing residues L121 and F125, and that this altered surface hydrophobicity contributes to activation of the membrane damaging activity of the protein [17]. This is consistent with the previous suggestion that the hydrophobic residues in the C-terminal region play a role in target membrane permeabilization [47–50]. Furthermore, mutants of BthTx-I with an altered topology of the substrate-binding site show a reduced membrane damaging activity, providing supportive evidence for a functional link between the substrate-binding region and residues in the C-terminal loop involved with the Ca\(^{2+}\)-independent liposome membrane damaging activity [36]. Here we have not only demonstrated that the region 122–125 is a structural determinant of all functions that were studied, but also that the hydrophobicity of position 125 may influence the activity of the protein. These results add support to the proposal that conformation change in the C-terminal loop on substrate binding contributes to the activation of the membrane damaging activity of the Lys49-PLA2s.

On the basis of experiments using erythrocytes enriched with phosphatidylserine and lipopolysaccharide from Gram-negative bacteria, it has been suggested that the permeabilization of the cell membranes by the Lys49-PLA2s is a key event in the pathological mechanism [52]. The close correlation observed between the structural determinants of the in vivo myotoxicity and the membrane permeabilization effect against cultured C2C12 myoblasts adds support to this idea. However, previous reports have demonstrated that the myotoxic and artificial membrane damaging activities may be separated [37,38]. The absence of any effect of active site mutants on the myotoxic activity suggests either that the conformation of this region is conserved on activation of the membrane damaging activity on membrane binding, or that any change that is induced has no effect on the myotoxic activity of the protein. This implies that the previously observed separation of the myotoxic and membrane permeabilizing [37,38] is a consequence of differences in the mechanism of binding and activation of the membrane damaging effects against muscle and liposome membranes. We propose that on interaction with liposome membranes containing anionic lipids, the direct lipid binding to the active site activates the BthTx-I and under these circumstances the binding and activation are concomitant events. This compares to the binding of the toxin to muscle cell membranes where the membrane binding and protein activation are separated. This explains why the knuckle region appears as a determinant in all assays, since the interaction of hydrophobic surface patch is a conserved feature of the activity against all membranes. However, in the case of the myotoxic and myoblast activities the activation event is separated from the lipid binding, and is triggered by additional events occurring after association with the membrane. Previous results have clearly separated the binding of Lys49-PLA2 to the target membrane from the cytotoxic effects of the protein [49], suggesting that the events after membrane binding are responsible for the manifestation of cytotoxic effects of the protein. The results of this study lead us to conclude that membrane-binding motifs are important for myotoxic and membrane damaging activities, however the underlying mechanism of activation on muscle cell membranes is different from that on bacterial membranes and liposomes.

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