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Phospholipases a_2 from *Viperidae* snakes: Differences in membranotropic activity between enzymatically active toxin and its inactive isoforms

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ABSTRACT

We describe the interaction of various phospholipases A_2 (PLA_2) from snake venoms of the family Viperidae (*Macrovipera lebetina obtusa*, *Vipera ursinii renardi*, *Bothrops asper*) with giant unilamellar vesicles (GUVs) composed of natural brain phospholipids mixture, visualized through fluorescence microscopy. The membrane fluorescent probes 8-anilino-1-naphthalenesulfonicacid (ANS), LAUDRAN and PRODAN were used to assess the state of the membrane and specifically mark the lipid packing and membrane fluidity. Our results have shown that the three PLA_2s which contain either of aspartic acid, serine, or lysine residues at position 49 in the catalytic center, have different effects on the vesicles. The PLA_2 with aspartic acid at this position causes the oval deformation of the vesicles, while serine and lysine-containing enzymes lead to an appreciable increase of fluorescence intensity in the vesicles membrane, wherein the shape and dimensions of GUVs have not changed, but in this case GUV aggregation occurs. LAURDAN and PRODAN detect the extent of water penetration into the bilayer surface. We calculated generalized polarization function (GP), showing that for all cases (D49 PLA_2, S49 PLA_2 and K49 PLA_2) both LAUDRAN and PRODAN GP values decrease. A higher LAURDAN GP is indicative of low water penetration in the lipid bilayer in case of K49 PLA_2 compared with D49 PLA_2, whereas the PRODAN mainly gives information when lipid is in liquid crystalline phase.

1. Introduction

Modification of giant unilamellar vesicles (GUVs) with different venom proteins which have unique properties and biological activities represents an informative approach to understanding the topology of membrane–protein complexes, and also to gain insights as to how these complexes may alter the state of the lipid membrane in terms of fluidity, thickness, permeability, ion selectivity, and other biophysical properties. There is a specific lipid asymmetry, which plays an important role in the activation of membrane proteins and membranes implementing numerous functions. The structure of the membrane-binding sites in proteins determines the mechanism of their interaction with an asymmetric membrane, leading to either their adsorption on the membrane surface or their insertion into the bilayer. These modifications are immediately reflected in the properties of the lipid bilayer. Application of modern techniques of fluorescence microscopy, which is currently one of the most powerful tools for the visualization of supramolecular interactions, allows detailed investigation of lipid–protein interactions and complex processes such as the formation of lipid rafts and incorporation of proteins into the bilayer [1].

Phospholipases A_2 (PLA_2) are enzymes which catalyze the hydrolysis of the *sn*-2 fatty acid ester bond of *sn*-3 phosphoglycerides, liberating free fatty acids and lysophospholipids [2,3]. PLA_2s are widely distributed in nature. In mammals, PLA_2s lack toxicity and play important roles in various physiological processes and in some pathologies such as rheumatism, osteoarthritis, asthma, and psoriasis [4]. In contrast, many PLA_2s from snake venoms are among the most aggressive toxic proteins, often playing a major role in prey immobilization and killing [5]. In the venom of a single snake, PLA_2 may be represented by several isoenzymes whose pharmacological effect can vary considerably. Most presynaptic neurotoxins and myotoxins from snake venoms are either inherently PLA_2s or contain PLA_2s or PLA_2-like subunits in their molecules [6,7].

Snake venom PLA_2s consist of 120–125 amino acid residues and contain seven disulfide bridges. An aspartic acid residue at position 49 of their amino acid sequence plays a critical role in catalysis and is conserved in the structure of enzymatically active snake venom PLA_2s which are referred to as D49 PLA_2s. However, in some PLA_2s from venom of viper snakes this amino acid is substituted with lysine, serine,

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asparagine or arginine, and the enzymes are classified as K49, S49, N49 and R49 PLA_2 homologues, respectively. Due to the substitution of the critical D49 residue and to changes in the calcium-binding loop, these proteins are no longer able to bind Ca^{2+} and, as a result, they lose the lipolytic activity [8,4]. Nonetheless, these catalytically-inactive isoforms display myotoxic and cytotoxic effects by a mechanism that does not require hydrolysis of phospholipids [9–11].

The molecular mechanisms of catalytically-inactive isoform action on membranes are yet not completely clear. Recently we have reported in a short communication about the membranotropic effects of PLA_2s from different viper venoms [12]. In this study we investigated in detail the effects of D49, K49, and S49 PLA_2s on GUVs using fluorescence microscopy techniques. In addition, we have shown that the synergic action of D49 and S49 PLA_2s in the presence of Ca^{2+} ions significantly alters the giant unilamellar vesicles (GUVs) morphology, and that PLA_2s induce changes in bilayer fluidity and lipid packaging, as evidenced by the fluorescent probes PRODAN and LAURDAN.

2. Experimental procedure

2.1. Animals

Adult outbread male rats (weighing 180–220 g) were used in all experiments; the animals were kept in a 12-h light:dark cycle at 22 ± 2 °C and fed with laboratory chow and tap water *ad libitum*. Experiments were carried out between 08.00 and 09.00 AM. All procedures were done according to our institution's animal care rules and the IACUC's ethical guidelines for Decapitation of Unanaesthetized Mice and Rats (http://www.utsouthwestern.edu/utsw/cda/dept238828/files/469088.html).

2.2. Phospholipid processing

Lipid fractions were isolated from brain of rats, according to the original Kates method [13]. The chloroform–methanol mixture was removed under reduced pressure, and then lipid residue was dissolved in nonane (3% solution).

2.3. Giant unilamellar vesicles (GUVs)

GUVs were prepared by the electroformation method, developed by Angelova and Dimitrov [14]. GUVs were formed in a temperature-controlled chamber that allows a working temperature range from 20 °C to 50 °C. GUVs were prepared using the following steps: ~2 μl of the phospholipid stock solution was spread on each of the two sample chamber platinum wires. The chamber was then dried for ~1 h to remove any remaining trace of organic solvent (nonane). The chamber and the buffer (Tris–HCl 0.5 mM, pH 7.4) were separately equilibrated to temperatures above the lipid mixture phase transition(s) (~10 °C over the corresponding transition temperature) and then 2 mL of buffer was added to cover the wires. Immediately after buffer addition, the platinum wires were connected to a function generator and a low-frequency AC (*alternating current*) field (sinusoidal wave function with a frequency of 10 Hz and amplitude of 2 V) was applied for 90 min. The mean diameter of these GUVs should be ~300 μm, as previously reported [15]. PLA_2s were added to the electroformation chamber before the vesicles were formed, for spectrofluorometric studies.

2.4. Phospholipases A_2

D49 PLA_2 (VL-PLA_2) was isolated from the venom of *Macrovipera lebetina obtusa* using gel filtration and reverse-phase chromatography. Crude venom was separated on Superdex 75 column (10 × 300 mm, GE Healthcare) and fraction containing PLA_2s was further purified by reversed phase chromatography on a Discovery BIO Wide Pore C18 column (10 × 250 mm, Supelco) using a gradient described earlier [16]. Mass spectrometry identified it as an analogue of acidic PLA_2 (UniprotKB accession number C3W4R6). S49 PLA_2 (Vur-S49) was isolated from the venom of *Vipera ursinii renardi* gel-filtration as described [17] with further separation of enzyme-containing fraction by reversed phase and ion exchange HPLC on the same column. K49 PLA_2 was purified from the venom of *Bothrops asper* by cation-exchange chromatography, followed by RP-HPLC as described [18,19] on a C8 semipreparative column (10 × 250 mm; Vydac). For experiments in present of Ca-ions, two concentrations of Ca-ions were added to the sample (1.1 and 2.2 μl of 0.1 M $CaCl_2$).

2.5. Fluorescence labeling and measurements

The membrane fluorescence probe ANS (8-anilino-1-naphthalenesulfonicacid) was used for visualization of GUVs. 1 μM ANS added to the sample chamber after the vesicle formation and then incubated for 5 min at 25 °C [20]. Images were collected on a fluorescence microscope FM320-5M (AmScope, USA). The excitation and emission wavelengths for ANS were 360 nm and 490 nm, respectively [21].

To detect polarity changes induced by PLA_2 within membrane, LAURDAN (6-lauroyl-2-(dimethylamino)naphthalene) and PRODAN (6-propionyl-2-(dimethylamino)naphthalene) probes were used. LAURDAN and PRODAN were dissolved in dimethyl sulfoxide (DMSO) and added to the liposomes at a probe:lipid molar ratio of 1:10,000,

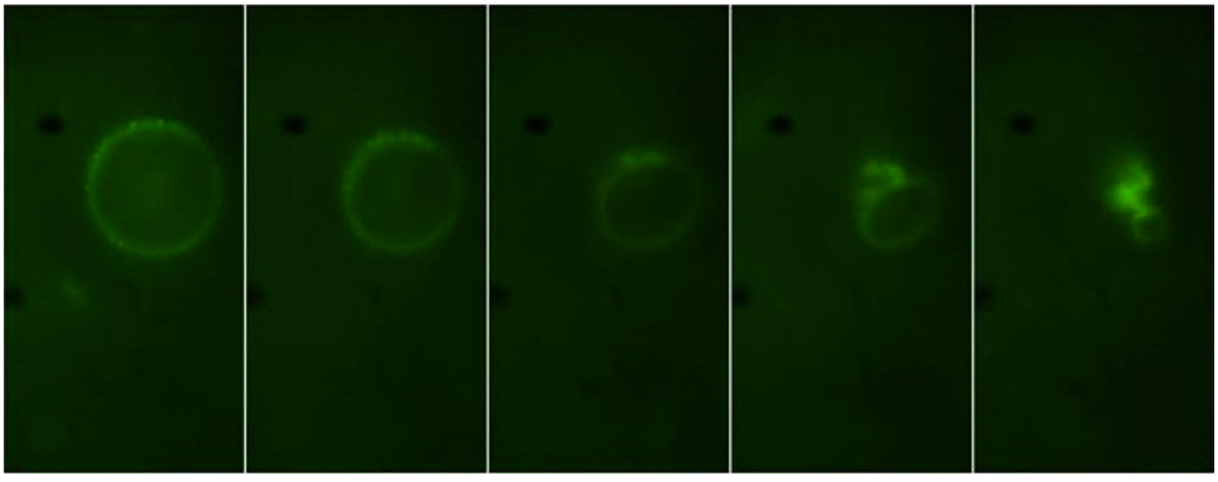


Fig. 1. *Macrovipera lebetina obtusa venom* effect on the ANS-containing GUVs. The *Macrovipera lebetina obtusa* venom was dissolved in Tris–HCl buffer (pH 7.4) to a concentration of 1 mg/mL and added to the sample under a microscope to a final concentration of 0.10 μg/mL (duration—3 min from first to last image).

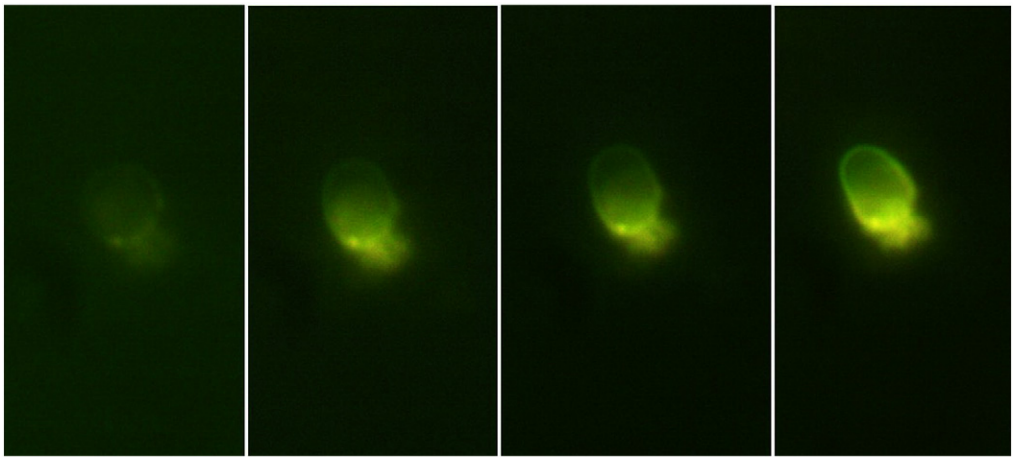


Fig. 2. ANS-containing GUVs "oval" deformation induced by the action of D49 of PLA_2 isolated from *Macrovipera lebetina obtusa* venom. Lyophilized D49 PLA_2 was dissolved in Tris–HCl buffer (pH 7.4), final concentration 30 μM, and 1.1 μl of this solution was added to the fluorescent microscope sample (duration—3 min from first to last image).

keeping the concentration of the organic solvent low enough to avoid altering the biological samples.

The fluorescence spectra were acquired on a Varian Eclipse spectrofluorometer; the LAURDAN and PRODAN excitation wavelength was set to 340–360 nm. To determine the membrane polarity more accurately, a generalized polarization function (GP) was used [22]. The GP was calculated as

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490}),$$

where I_{440} and I_{490} are the blue and green emission intensities, respectively.

2.6. Statistical analysis

For quantitative analysis of LAURDAN/PRODAN GP values, results are reported as means ± SEM. The significance of differences between the means was assessed by ANOVA followed by Bonferroni's test when various experimental groups were compared with the control group. A value of $P < 0.05$ indicated significance.

3. Results

3.1. Phospholipases A_2

PLA_2s containing three different amino acid residues at position 49 of amino acid sequence were used in this study. Enzymatically active D49 PLA_2 was isolated from *M. lebetina obtusa* venom. For this purpose crude venom was first separated by gel-filtration on Superdex 75 column. Then fraction containing PLA_2 was further separated by reversed phase HPLC using gradient of acetonitrile in water described earlier [16]. Fractions corresponding to #19 and 20 on Fig. 1 in Sanz et al. (2008) were analyzed by mass spectrometry. Their molecular masses determined by electrospray ionization mass spectrometry were 13,645 and 13,675 Da for #19 and 20, respectively. These values are close to those determined by Sanz et al. [16]. Peptide mass fingerprinting identified these proteins as very close analogues of acidic PLA_2 from *M. lebetina obtusa* venom (UniprotKB accession number C3W4R6). The difference is in C-terminus as peptides corresponding to the fragment 123-138 of C3W4R6 amino acid sequence were not detected. PLA_2 with molecular mass of 13,645 was used in this work.

PLA_2s S49 was isolated from *V. ursinii* venom. For this purpose, crude venom was separated by gel-filtration as described [23] and fraction 3 + 4 (Fig. 3A in Tsai et al., 2011) was further purified by reversed phase HPLC. The protein that possessed the molecular mass of 13,935 Da (that is similar to the calculated mass of Vur-S49) was subjected to peptide mass fingerprinting. The mass spectrometry data showed that the masses of the peptides obtained after trypsinolysis corresponded to those derived from Vur-S49; therefore the isolated protein is Vur-S49. It is referred to as PLA_2 S49 in this work.

3.2. Visualization of GUV morphological changes by fluorescence measurements

The rapid changes of GUV's morphology under the influence of whole venom of *M. lebetina obtusa* have been demonstrated previously [24,25], and Fig. 1 illustrates the time course of liposome shrinking after venom addition (2.2 μl of 1 mg/ml solution in Tris-HCl buffer; pH 7.4).

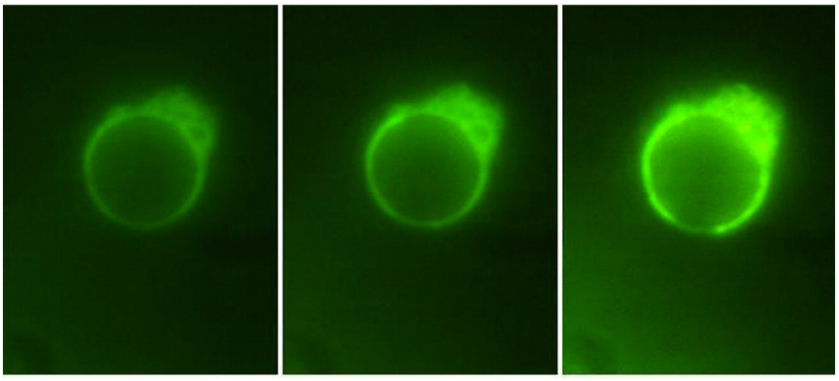


Fig. 3. ANS-containing GUV interaction with K49 PLA_2 isolated from *Bothrops asper* snake venom. Lyophilized K49 PLA_2 was dissolved in Tris–HCl buffer (pH 7.4), final concentration 30 μM, and 1.1 μl of this solution was added to the fluorescent microscope sample (duration—3 min from first to last image).

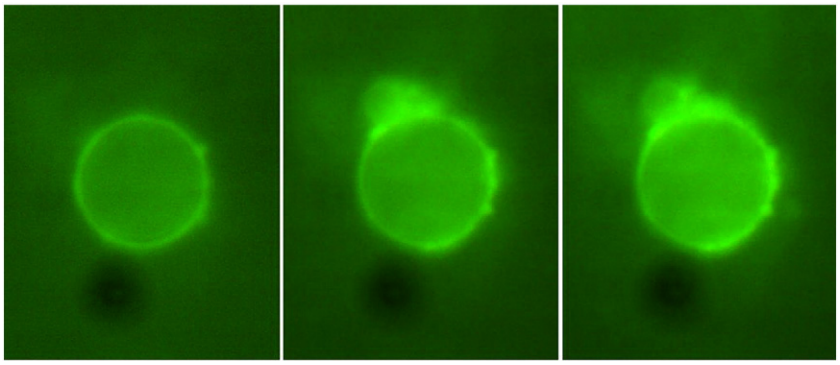


Fig. 4. ANS-containing GUVs interaction with S49 PLA_2 isolated from *Vipera ursinii renardi* venom. Lyophilized S49 PLA_2 was dissolved in Tris–HCl buffer (pH 7.4), final concentration 30 μM, and 1.1 μl of this solution was added to the fluorescent microscope sample (duration—3 min from first to last image).

The membrane-disrupting effect of this protein cocktail was demonstrated with ANS fluorescent probe, which is a classical assay for protein–lipid binding visualization.

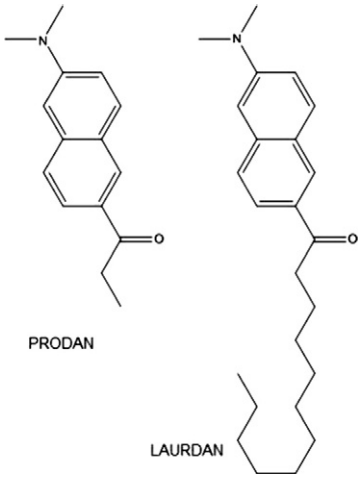
The membranotropic activities of the D49, K49 and S49 PLA_2s were studied using ANS probe. Fig. 2 illustrates the oval deformation of circular GUVs caused by D49 PLA_2. No significant differences in the condition of GUVs were observed after addition of K49 and S49 toxins (Figs. 3 and 4), and these data provide further evidence of the non-enzymatic nature of these two PLA_2 isoforms. At the same time, we could see a noticeable accumulation of the fluorescent probe on the membrane surface and these data suggest that the interaction of proteins with lipid bilayer still occurs, and the presence of Ca^{2+} did not induce (activate) any changes in GUV morphology.

Intriguingly, the overabundance of Ca^{2+} ions (2.2 μl of 0.1 M $CaCl_2$) leads to the slight tendency of ovalization in case of S49 PLA_2 toxin (Fig. 5). This result is in contrast with our previous observations, but some other authors also highlight earlier the often-contradictory nature of reports concerning the lipolytic activities of PLA_2s purified from crude venom [8].

Further experiments were conducted to examine the possible synergistic action of enzymatic D49 and non-enzymatic S49 PLA_2s on the liposome membrane. A synergism in cytolytic activity between D49 and K49 PLA_2s has been previously observed in the myogenic cell line C2C12 in [26]. Taking into consideration the usual presence of different isoforms of PLA_2 (both enzymatic and non-enzymatic) in the same venom, it was reasonable to explore the mutual dependence of their biological activities. The results showed that the addition of D49 PLA_2 together with S49 PLA_2, in the presence of Ca^{2+} ions in the GUVs media, leads to rapid changes of vesicle size, morphologically very similar to the membranotropic effect of the crude venom of *M. lebetina obtusa* (Fig. 6) [24,25 and Fig. 1]. In the concentration range of toxins tested, the liposome shrinking occurred within the first 20 s, and the fluorescence intensity remained almost constant, indicating both that the effect was instantaneous and not cumulative.

3.3. Influence of PLA_2s on membrane polarity

For determination of lipid phases we used PRODAN and LAURDAN probes (see picture). The PRODAN mainly gives information when lipid is in liquid crystalline phase. The advantage of using LAURDAN as a membrane probe is proved by its ability to report the extent of water penetration into the lipid bilayer. The penetration of water molecules into the membrane surface is strongly correlated with lipid packing and membrane fluidity. LAURDAN and PRODAN emission spectrum in a single bilayer is centered at 440 nm for the membrane in the gel phase and at 490 nm for the membrane in liquid crystalline phase [27,28]. Thus the GP could give a mathematically convenient and quantitative way to measure the shift of emission.



In order to study the perturbation effects of PLA_2s on the bilayer membrane, GP values of PRODAN and LAURDAN fluorescent probes

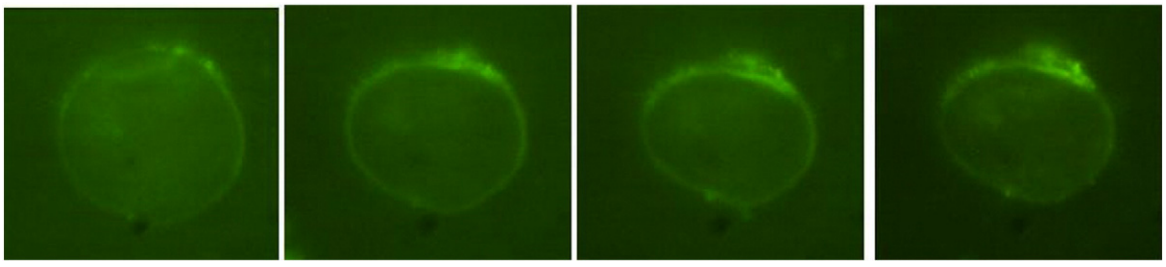


Fig. 5. ANS-containing GUV interaction with S49 PLA_2 isolated from *Vipera ursinii renardi* venom. Lyophilized S49 PLA_2 was dissolved in Tris–HCl buffer (pH 7.4), final concentration 300 μM, and 2.2 μl of this solution was added to the fluorescent microscope sample (5 min).

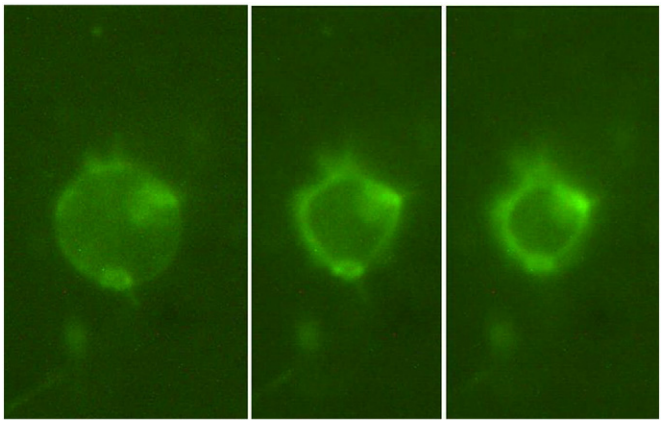


Fig. 6. ANS-containing GUV interaction with a mixture of D49 and S49 PLA_2s in the presence of Ca^{2+} ions. The mixture was dissolved in Tris–HCl buffer (pH 7.4), final concentration of overall PLA_2 mix is 30 μM, and 1.1 μl of this solution was added to the fluorescent microscope sample (5 min).

were measured to monitor overall changes in membrane anisotropy. Because of the different lengths of their acyl residues, the partitioning of the two probes between water and the membrane bilayer differs profoundly. PRODAN is more sensitive to polarity variations occurring closer to the bilayer surface than those detected by LAURDAN, and thus there is a substantial preferential partitioning in the liquid-crystalline phase. Hence, PRODAN is also sensitive to the pretransition in the polar head region due to its different location in the membrane closer to the aqueous surface of the bilayer.

The advantages of the GP method reside in its sensitivity to the properties of the membrane [28] and in the possibility of determining and quantifying the phase state of the membrane [27]. The data for GP measurements are shown in Fig. 7. A high LAURDAN GP is indicative of low water penetration; therefore both crude venom and isolated PLA_2 components acted on the membrane and changed the condition of the lipid bilayer crucially, but the interaction of D49 and S49 PLA_2 with membrane is more significant ($P < 0.05$). The lowest changes occurred after K49 PLA_2 addition (Fig. 7). Interestingly, all these dramatic changes occurred for a deep stratum of membrane, but not in the polar head level, where with respect to the PRODAN GP values, changes of lipid packaging were not so significant.

4. Discussion

The binding of venom components to lipid interfaces is dependent on many properties of the membrane surface. It has been a generally

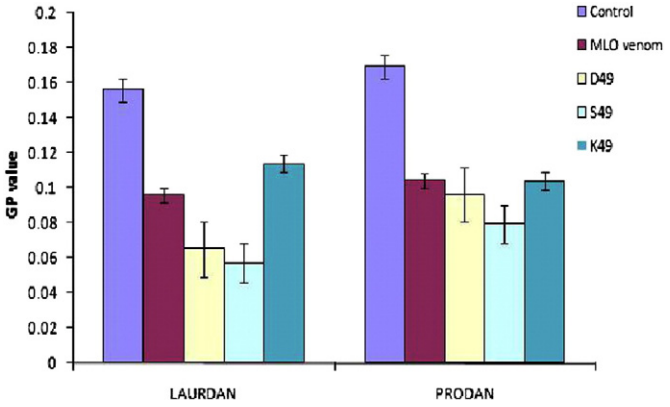


Fig. 7. LAURDAN and PRODAN GP values in phospholipid GUV membrane modified with *Macrovipera lebetina obtusa* crude venom, D49 PLA_2 isolated from *Macrovipera lebetina obtusa* venom, S49 PLA_2 isolated from *Vipera ursinii renardi* and K49 PLA_2 isolated from *Bothrops asper* snake venoms.

accepted hypothesis that the secreted PLA_2s are particularly active in the presence of transient "membrane defects," and borders between coexisting lipid phases have been postulated to be a source of these defects [29–31]. One of the recurrent questions in this respect is that concerning the preferential interactions of this enzyme and other proteins from snake venom with different lipid domains or with the lipid domain borders. Our experimental approach can help answer this question by direct visualization of the proteins on unsupported lipid bilayers.

The results presented in this work demonstrate that two catalytically-inactive isoforms of PLA_2, K49 and S49, interact with phospholipid membranes in a way to induce leakage of liposomes without changing the shape of GUVs through a Ca^{2+}-independent mechanism. It is known that the hydrolysis of phospholipids by venom PLA_2s involves a His48–Asp49 pair in the catalytic site, which activates a structurally conserved water molecule, thereby initiating the nucleophilic attack on the *sn*-2 position of the substrate. During catalysis, the tetrahedral intermediate is stabilized by a Ca^{2+} ion cofactor, which is bound by the carboxyl oxygen atoms of Asp49 and carbonyl main-chain oxygens of the neighboring calcium-binding loop [31–33]. In the currently accepted model of phospholipid hydrolysis, it is suggested that charge neutralization of the oxyanion intermediate alone is not sufficient to promote catalysis, and it has been proposed that an additional Ca^{2+} ion acts as an auxiliary electrophile, polarizing the amide of the Cys29-Gly30 peptide bond, which enables completion of the catalytic cycle [8]. At the replacement of Asp49 by lysine or serine abrogation of catalytic activity results from either the re-orientation of the Cys29–Gly30 peptide bond [34,35], or the reduced binding affinity of the Ca^{2+} cofactor [35].

The changes of membrane condition, its fluidity and microviscosity, were studied using different fluorescent probes and a comparative mode. Our primary observation of venom-dependent GUV morphology change has been the shrinking response at high concentrations of venom. Gradual loss of vesicle size because of crude venom is surprising, because the twice lower concentrations (addition of 1.1 μl of venom solution (1 mg/ml in Tris-HCl buffer; pH 7.4)) caused the increasing of vesicle's size [25].

Generally it is accepted that such model membranes, as SUVs, LUVs (small and large unilammeliar vesicles, respectively), or erythrocytes, remain intact and unaffected in course of hydrolysis of the outer leaflet phospholipids. [36–38]. Because usually GUVs are stable for many hours in the absence of PLA_2s, it is quite obvious that the observed mass loss clearly must be dependent from the formation of fatty acid and lysophospholipid products of the enzymatic digestion by PLA2s. So the presented results of our investigation are in agreement with theoretical considerations and previous experimental data [1,27–29,36] both for enzymatic and non-enzymatic PLA_2s: both the crude venom and the catalytically-active D49 PLA_2 caused drastic morphological alterations in the vesicles, whereas no significant changes were observed when the catalytically-inactive PLA_2s (S49 or K49) interacted with vesicles.

Concerning K49 we would like to make two general remarks. First, *B. asper* K49 PLA_2 is a dimeric protein unlike the other two PLA_2s here studied and should have its own unique membrane association and interactions, characteristic of this protein subfamily [9,10,37]. K49 PLA_2s have previously been shown to associate with cell membranes in a charge-dependent manner and to require negative surface charges for interaction with liposomes [38]. Present results show that the interaction of D49 PLA_2 is different as compared to K49 PLA_2. One possible explanation for such differences might be the significant change in the topology of the proteins binding to the lipid bilayer, the cause of which might be the replacement of an aspartic acid residue at position 49 to lysine. The dimeric form of K49 is relevant, although not indispensable, for the manifestation of biological activity [39–41] but at the same time, most D49 PLA_2 are monomeric [42]. This probably explains the differences we observed in the actions of D49 and K49 PLA_2s on GUVs.

Thus, the minimal change of amino acid residue in the catalytic center of enzyme leads to the activity loss of PLA_2 which is one of the major

components of *M. lebetina obtusa* venom. This charge has a vital impact on PLA_2 interaction with the lipid bilayer which can be seen from the absence of morphological changes during the interaction catalytically inactive PLA_2s with GUVs. At the same time the studies with the fluorescent probes (PRODAN and LAURDAN) show that the three types of PLA_2s studied change bilayer fluidity and lipid packaging. The parallel use of LAURDAN and PRODAN to monitor structural and dynamical changes of membrane properties, gives the possibility of calculating the probe partition coefficient between the membrane and water. Less values of the dipolar relaxation could be attributed to a less damages in the bilayer at the level of glycerol backbone, where the PRODAN naphthalen moieties reside. For LAURDAN, the longer acyl chain prevents this probe from partitioning in water. The more hydrophobic interaction of D49 and also S49 with the bilayer renders these toxins responsible to the changes of packing properties of the membrane core, but not the surface.

In conclusion, the most important result of this work could be the evidence of membranotropic properties amplification in course of synergistic effect of D49 and S49 proteins in the presence of Ca^{2+} ions. It would be a very attractive hypothesis about heterodimerization of these two proteins from different venoms, which could enhance their powerful action on the lipid bilayer, but this opinion is quite speculative and needs direct structural investigations to prove or reject the idea.

Acknowledgments

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