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գիտական աստիճանի հայցման ատենախոսության

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Երևան- 2013

MINISTRY OF SCIENCE AND EDUCATION OF RA
YEREVAN STATE UNIVERISTY

GHAZARYAN NARINE ALBERT

MECHANISMS OF *MACROVIPERA LEBETINA OBTUSA* SNAKE VENOM INFLUENCE ON THE ARTIFICIAL BILAYER LIPID MEMBRANES

S Y N O P S I S

of dissertation for conferring of scientific degree of
Candidate of Biological Sciences
in the specialty of 03.00.02 – Biophysics
Yerevan -2013

Ատենախոսության թեման հաստատվել է Երևանի պետական համալսարանում:

Գիտական ղեկավար՝ կենս. գիտ. թեկնածու
Ն.Մ. Այվազյան

Պաշտոնական ընդդիմախոսներ՝ ֆիզմաթ. գիտ. դոկտոր,
պրոֆեսոր Վ.Բ. Առաքելյան
կենս. գիտ. թեկնածու Մ.Հ. Մալաքյան

Առաջատար կազմակերպություն՝ ՀՀ ԳԱԱ Հ. Բունիաթյանի անվ.
կենսաքիմիայի ինստիտուտ

Ատենախոսության պաշտպանությունը տեղի կունենա 2013թ. սեպտեմբերի 10-ին, ժամը 14:00-ին, Երևանի պետական համալսարանում գործող ՀՀ ԲՈՀ-ի Կենսաֆիզիկայի 051 մասնագիտական խորհրդի նիստում (0025, Երևան, Ալեք Մանուկյան փ. 1, ԵՊՀ, կենսաբանության ֆակուլտետ):

Ատենախոսությանը կարելի է ծանոթանալ Երևանի պետական համալսարանի գրադարանում:
Ատենախոսության սեղմագիրն առաքված է 2013թ. հունիսի 28–ին:

051 մասնագիտական խորհրդի գիտ. քարտուղար,
կենս. գիտ. դոկտոր, պրոֆեսոր՝ Լ. Հ. Նավասարդյան

The theme of the dissertation has been approved at Yerevan State University.

Academic advisor: Candidate of Biological Sciences,
N.M. Ayvazyan

Official opponents: Dr. of Physico-mathematical Sciences,
Professor V.B. Arakelyan
Candidate of Biological Sciences,
M.H. Malaqyan

Leading organization: H. Buniatian Institute of Biochemistry NAS RA

The defense of the dissertation will be held on September 10th, 2013, at 14:00, at the session of 051 Biophysics Specialized Council of RA SCC at Yerevan State University (0025, Yerevan, Alex Manoogian str. 1, YSU, Faculty of Biology).

The dissertation is available at the library of Yerevan State University.
The synopsis has been sent on June 28th, 2013.

Scientific Secretary of the 051 Specialized Council,
Dr. of Biological Sciences, Professor L. Navasardyan

INTRODUCTION

Topic significance. The venom as medicine has interested the scientists since ancient times, but it is only in recent years that important achievements have been described (Marcinkiewicz et al., 2003; Calvete, 2013). Snakes have toxins in their venom, which have unique features due to which they are widely used for scientific and medical purposes (LEBETOX which can stop bleeding). As a rule, being complex and biologically active, the greater part of zootoxins is subjected to biotransformation and interacts with biological membranes after it. In this case the interaction of different venom components with the membranes is not always the same.

It is common knowledge that at least one component of venom - phospholipase A_2 (PLA_2), can change membrane permeability, but membrane curvature is essential for this enzyme (Bell et al., 1996; Susana et al. 2002; Gutiérrez and Lomonte, 2013). Besides nowadays research has shown that synergistic action of venom proteins may enhance their activities or contribute to the spread of toxins and the action of one component of venom with membranes can be different with overall venom effect.

In recent years fluorescent probes have been widely used for the study of biological membranes (Kirk al., 2007). It makes possible to study substance transport and structural restoration depending on membrane function (Winnik et al., 1980; Yu et al., 1996; Bagatolli et al., 1997; Cheng et al., 1997; Garda et al., 1997).

Research goals and tasks. The aim of the present study is to obtain detailed information about the mechanism and topology of the *Macrovipera lebetina obtusa* (*MLO*) venom in the membrane-binding process and to find out how the presence of such lipid/protein interaction changes the plastic properties and the ionic permeabilization of membrane.

Our objective was to determine changes in optical spectroscopic and electrical properties accompanying venom-bilayer complexation and study the influence of venom binding on the energetics of gel-to-liquid transitions of lipids as a function of base sequence and amino acid composition of peptides.

Constituted tasks of the research were to:

1. reveal *in vivo* influence of venom on electrical properties of bilayer lipid membranes in univalent ion media (Na^+, K^+, Li^+, J^-, Cl^-, Br^-) for the study of membrane electropermeabilization, electroporation and ionic permeability;
2. study membrane potential and hydration degree change (with ANS and LAURDAN fluorescent probes) to estimate energy changes accompanying the peptide-bilayer binding events;
3. study changes of plastic properties of membrane during lipid/peptide interaction with the help of membrane fluorescence probes (ANS, pyrene, LAURDAN, PRODAN);
4. study morphological changes of giant unilamellar vesicles (GUVs) after modification with venom components.

Scientific novelty and applied value of the study. We have chosen a number of different types of membrane models and fluorescent probes which being sensitive criteria of the physiological condition of lipids and lipid-contained structures make it possible to create simple and real models of lipid rafting or lipid/peptide interactions.

In the present study we have shown that *MLO* venom has a significant influence on different electrical parameters of artificial lipid membrane and changes the plastic properties of the membrane. Addition of different snake venoms (*Montivipera raddei, Naja kaoutia*) apparently changes the brain obtained BLM conductivity, but in case of *MLO* this change occurs during high concentration and has a cumulative nature. The venom influence is more evident on GUVs, which is the evidence of the curvature role during such an interaction.

For the first time artificial membrane modeling formed from different tissue lipids was applied after *in vivo* injection of snake venom, which enables us to study the influence of secondary processes following the intoxication of the membrane structures. The obtained results are also new in terms of the study of the GUVs in the venom media, since their fluorescence microscopy makes it possible to visualize the morphological properties of liposomes with damaged membranes.

The minimal amino acid content change in the catalytic center of proteins leads to the activity loss of PLA_2 which is one of the major components of *MLO* venom and it has a vital impact on its interaction with the lipid bilayer.

Main points to present at defense.

1. *MLO* venom influences lipid bilayer electropermeabilization, electroporation and ionic permeability changing the morphology of their supramolecular structures as well.
2. *in vivo* intoxication of *MLO* venom results in the change of electrical parameters of different artificial lipid membranes, which is evidence of ongoing cascade and rapid processes in the organism.
3. The interaction of the components of *MLO* venom, particularly PLA_2 with membranes requires a curvature and it depends on the amino acid sequence in the catalytic center.

Work approbation. The main results of the dissertation were discussed at seminars and at international scientific conferences.

Publications. According to experimental data observed in dissertation 16 works, including 6 papers in peer-reviewed journals were published.

Volume and structure of dissertation. The dissertation contains the following chapters: introduction, literature review (Chapter 1), experimental part (Chapter 2), results and discussion (Chapter 3), concluding remarks, conclusions and cited literature (total 119 papers and books). The document consists of 101 pages, 8 tables and 34 figures.

MATERIALS AND METHODS

Animal preparations. Adult (weighing 180–220g) non-purebred male rats were used in all experiments; they were kept in standard conditions of light (on between 07.00 and 19.00 h) and temperature (22 ± 2°C) and fed with laboratory chow and tap water *ad libitum.* The animals to be used for *in vivo* studies were handled gently daily, for a week, to minimize stress conditions. Experiments were carried out between 08.00 and 09.00 h. All procedures were done according to our institution's animal care rules and the IACUC's ethical guidelines for Decapitation of Unanesthetized Mice and Rats (http://www.utsouthwestern.edu/utsw/cda/dept238828/files/469088.html).

Tissue Processing. The venom of the *Macrovipera lebetina obtusa, Montivipera raddei* and *Naja kaouthia* was tested for its ability to induce supramolecular changes in rats after short-term (10 min) intramuscular injection of the venom (0.35 mg/kg approx. 0.5 LD 50), by modeling of artificial membranes from native lipid content from some organs (liver, heart, brain and muscle). For this purpose rats were killed by decapitation at either 10 min after venom injection and tissues were quickly removed (-4°C) for lipid purification. We tried to compare the data of *in vitro* and *in vivo* experiments. For *in vitro* experiments dried lyophilized toxin of snakes was dissolved in Tris-HCl buffer (pH 7.4) with a concentration of $3*10^{-5}$M.

Phospholipid processing. Lipids fractions were isolated from marked tissues of rats, according to the original Kates method (Kates, 1972). Then a vacuum pump was used to remove the chloroform-methanol mixture. For *in vitro* experiments they were incubated with venom solution and held at a constant temperature of 37°C for 10 min. Then lipid sediments were dissolved in nonane (3% solution).

Preparation of pure proteolipids. Proteolipids fraction was separated from the bovine brain according to the Folch method (Zobolenski et al., 1984). White matter was dissected from fresh adult bovine brain, and scraped free from gray matter. The white matter is homogenized with 1:1 chloroform-methanol mixture (1 g of tissue/ 20 ml). The suspension was washed with distilled water to remove non-lipid substances.

The water which remained between the two phases went into the solution when 1-2ml methanol was added. Then we added 1/2 chloroform volume (compared to the extract) and dried in vacuum pump at room temperature (20-22°C). After that distilled water was added in proportion 33 times as much as the weight of the sediment and the latter was centrifuged for an hour at 4600g. This procedure was repeated four times. The last one lasted 10 min 200g (2°C). The sediment was crude proteolipids which was dried in a vacuum pump after which a mixture (1:1) of sulfur ether and ethanol (150 times as much as the sediment weight) was added and centrifuged for 10 min 4600g (-10°C). The sediment was pure proteolipids.

The crude proteolipids contain 40-50 % of protein and 60-50% of lipids. The content of phospholipids is about 40%. Purified proteolipids contain 70% of protein and 30% of lipids. The crude and purified proteolipids (as compared with the original lipid extracts from which they were isolated) are enriched in acidic phospholipids: phosphatidylserine, phosphatidylinositol and diphosphatidylglycerol. Acidic phospholipids in total lipid extract make up 15-20% of total phospholipids, in crude proteolipids 25-30% and in purified proteolipids 70-75% where the greater part is phosphatidylserine (35-40 %).

Planar bilayer lipid membranes (BLMs). The lipid bilayer membranes were formed from the total lipid fractions of the bovine brain on a Teflon aperture by means of the Muller method (Mueller et al., 1962). A Teflon cylindrical cup having a 0.8mm hole was coupled to a glass chamber; so the cup separated two compartments filled with 5 ml electrolyte each. Electrical access to the baths was through a pair of Ag/AgCl electrodes.

Optical reflectance, electrical resistance and capacitance indicated the formation of planar lipid bilayers. The electrical parameters of the BLMs were determined on a device equipped with a Keithley 301 differential feedback amplifier (United States) in a voltage-fixation mode, which

let keep up a membrane potential on any level independently of ionic streams. The potential setting on exit of generator completely falls on membrane, the resistance of which is much higher than that of resistance of electrodes, electrolyte and effective resistance of current's gauge. Electrometric device can measure a current through membrane under fixed value of transmembrane difference of potentials. The own resistance of membrane amounting by following formula:

$$R_m = R_f U_m / U_f \text{ (Ohm)},$$

Where

U_f - potential from source of direct current;

U_m - difference of potentials on membrane;

R_m- membrane resistance;

R_f- chain resistance

Membrane conductivity:

$$g_m = 1 / R_m \text{ (Ohm}^{-1}\text{)}$$

Specific electrical conductance (g) is expressed in Ohm^{-1}/mm^2, and 0.1 M KCl, NaCl, LiCl and $CaCl_2$ serve as ionic media.

The breaking-potential of membrane recorded in the experiments in shielded camera was taken as the threshold value of the voltage applied. Potential of membrane rapture was a criterion for valuation of natural defects of model membranes. Under electrical potential, radius of these holes increased. There was a critical value of radius (r_o) and when $r_\varphi > r_o$ membrane was destroyed.

Giant unilamellar vesicles (GUV). GUVs were prepared according to the electroformation method, developed by Angelova and Dimitrov (Angelova et al., 1992). 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 and proteolipid 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. 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 field (sinusoidal wave function with a frequency of 10 Hz and amplitude of 2V) was applied for 90 min (Bagatolli et al., 1999). The mean diameter of the GUVs was up to 300 µm.

Fluorescence labeling and measurements. The fluorescence emission was observed from 350 nm to 600 nm. The membrane fluorescence probes, ANS and pyrene, were used to assess the state of the membrane and specifically mark the phospholipid domains. ANS and pyrene allow us to quantify the fluidity changes in the membrane by measuring of the fluorescence intensity. These probes were added to the sample chamber after the vesicles formation. 1 µM ANS was added to the sample and then incubated for 5 min in 25°C (Verstraeten et al., 2005). The fluorescence intensity of ANS is inversely proportional to the value of the

membrane potential. 3 μM pyrene was added to the samples and then incubated by stirring constantly in 25°C for 2min (Galla et al., 1974).

The I_3/I_1 ratio is quantity that is calculated from fluorescence spectrum from pyrene. The I_3/I_1 ratio has been shown to provide information about the polarity of the medium surrounding pyrene (Scales et al., 2006, Rao et al., 2007). This ratio is calculated as the ratio of the intensities of the third (I_3, λ=386 nm) to first (I_1, λ=375 nm) monomer peaks of the fine vibronic structure of the steady-state emission spectrum.

For determination of lipid phases we used PRODAN and LAURDAN probes. The PRODAN mainly gives information when lipid is in liquid crystalline phase. LAURDAN is used as a membrane probe because of its ability to report the extent of water penetration into the bilayer surface. Water penetration has been strongly correlated with lipid packing and membrane fluidity. The emission spectrum of LAURDAN in a single phospholipid bilayer is centered at 440 nm when the membrane is in the gel phase and at 490 nm when in the liquid crystalline phase. The GP gives a mathematically convenient and quantitative way to measure the emission shift. The function is given by:

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

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

The fluorescent spectra were acquired on a Varian Eclipse spectrofluorometer instrument; the excitation wavelength for the pyrene monomers and dimeres are 286nm and 334nm ($L_{emission}$ are 395nm and 470 nm, respectively); the excitation and emission wavelengths for ANS are 360nm and 490nm respectively (Bordushkov et al., 1993). Images were collected on an epi-fluorescent microscope FM320-5M (AmScope, USA).

Statistical Analysis. For quantitative analysis of electrical parameters of BLMs, a Student's test was used to compare differences at each time point, considering $P< 0.05$ as significant. All data will be presented as mean ±SEM (n=number of experiments).

RESULTS AND DISCUSSION

The changes of electrical properties of planar bilayer lipid membranes (BLMs) in the course of snake venom intoxication

Electrical measurements of planar bilayers provide a means of measuring the changes in conductance, due to the formation of channels, pores or defects on the membranes, caused by the binding, insertion and translocation of peptides, at lowest concentrations, at which transient events can be detected. It also enables the determination of other features as: open probability, ionic selectivity of the pores and an estimative of pore's size (Ayvazyan et al., 2012). **Table 1** shows the results obtained with the *in vitro* action of three types of snake venom on the electrical conductance of BLMs from brain lipids of rats in media of K^+ ions. For the first series of experiment aliquots of 0.05ml venom stock solution (concentration 10mg/ml in Tris/HCl buffer, pH 7.4) was added to the both sides of membrane. After a few minutes of equilibration, -100mV potential was applied in order to monitor changes in electrical properties of BLMs. Starting at 0.2ml, venoms of MLO and NK showed dramatic changes of the membrane conductance

(macroscopic integral conductance) and venom of MR showed significant activity (Table 1). An excess venom added in both sides of BLM sharply raised the membrane conductance and witnessed about cumulative mode of venom-membrane interaction. For the next series of experiments, after removing the chloroform-methanol mixture the lipid fraction was incubated with venom stock solution and held at a constant temperature of 37^0C for 10 min. Then venom solution was washed out and BLMs were formed from lipid mixture in nonane. This procedure didn't lead to any significant changes in the means of conductance of bilayers induced by MLO and MR venoms, but BLMs from brain lipids of rats incubated with cobra venom showed a dependence of electrical resistance of bilayers from processing time (**Fig.1, right**) and venom concentration (**Fig.1, left**). Collectively these results indicate that components of MLO and MR venom demonstrate surface activity during membrane-peptide interaction, while at least one or more components of cobra venom definitely penetrate the bilayer. Indeed, single-channel current events induced by NK venom have been recorded in BLMs at lowest concentrations ($<8*10^{-4}$mg/ml).

Table 1.

Snake venoms *in vitro* action on the electrical properties of BLMs (resistance R_m, conductivity g_m and breaking-potential U_r) formed from brain lipids in media with K^+.

	Control	***Vipera lebetina***	***Montivipera raddei***	***Naja kaoutia***
R_m (Ohm)	$(1\pm0,7)\cdot10^{11}$	$(6\pm0,8)\cdot10^{8}$	$(1.9\pm0,3)\cdot10^{9}$	$(3,3\pm0,2).10^{8}$
g_m (Ohm^{-1})	$(2\pm0,2)\cdot10^{-11}$	$3.3\cdot10^{-9}$	$4\cdot10^{-10}$	$3\cdot10^{-9}$
U_r (mV)	448±12	270±8	580±10	280±9

Average value (±SEM) of minimum 7different experiments

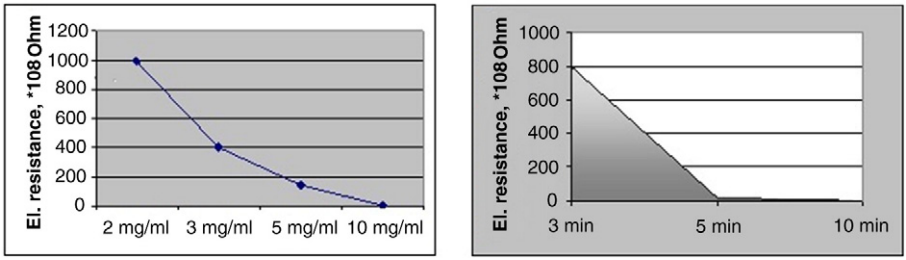


Figure 1. A dependence of electrical resistance of rat's brain lipids BLMs (incubated with cobra venom) on the processing time (right) and venom concentration (left).

Planar lipid bilayer modeling for *in vivo* experiments and ionic permeability of BLMs

Planar lipid bilayers for these series of experiments were formed from solutions of native lipid mixtures from different tissues of rats (liver, heart, brain and muscle) after short-term (10

min) intramuscular injection of the venom (0.35 mg/kg approx. 0.5 LD 50). The presence of viper venom in the organism leads to the increase of the electrical resistance of BLMs from liver and muscle lipids approximately on a sequence, while the BLMs from brain lipids have not shown noticeable differences of plastic properties compare with the control (**Table 2**). The same concentration of cobra venom leads to the decrease of electrical resistance of BLMs from 10^{11}Ohm till 10^{8}Ohm. The low concentration of venom leads to the appearance of channel activity. It is especially noticeable in liver lipids in media of bivalent ions.

Table 2. Snake venom *in vivo* effect on the electrical properties of BLMs (resistance R_m, conductivity g_m and breaking-potential U_r) formed from different tissue lipids in K^+ medium.

	Brain			Heart			Liver			Muscle		
	R_m *Ohm*	g_m *Ohm -1*	U_r mV	R_m *Ohm*	g_m *Ohm -1*	U_r mV	R_m *Ohm*	g_m *Ohm^{-1}*	U_r mV	R_m *Ohm*	g_m *Ohm -1*	U_r mV
Control	**1.3* 10^{10}**	**0.7* 10^{-11}**	**355**	**1.3* 10^{9}**	**16* 10^{-10}**	**200**	**2* 10^{9}**	**5* 10^{-10}**	**219**	**2* 10^{9}**	**5* 10^{-10}**	**180**
Vipera lebetina	**5.6* 10^{10}**	**1.8* 10^{-11}**	**309**	**6* 10^{10}**	**1.6* 10^{-11}**	**228**	**5.6* 10^{10}**	**1.7* 10^{-11}**	**291**	**4.7* 10^{10}**	**2* 10^{-11}**	**225**
Vipera raddei	**7.3* 10^{10}**	**1.3* 10^{-11}**	**313**	-	-	-	**5.3* 10^{10}**	**1.9* 10^{-11}**	**239**	**4.6* 10^{10}**	**2.1* 10^{-11}**	**304**
Naja kaoutia	**7.4* 10^{10}**	**1.3* 10^{-11}**	**407**	**2.3* 10^{10}**	**4.3* 10^{-11}**	**252**	**4.2* 10^{10}**	**2.4* 10^{-11}**	**249**	**2.9* 10^{10}**	**4.2* 10^{-11}**	**232**

Each group contained 20 BLMs from four tissues. P>0.01 by Student's t-test relative to the corresponding control.

Fluidity changes of GUVs membrane in the course of snake venom intoxication

In the first stage of our experiments we studied the ANS fluorescence intensity with the liposomes which were formed by the heterogeneous group of lipids and modified by venom. As you can see in **Fig. 2, left** ANS fluorescence in the buffer solution (518 nm) undergoes minor changes under the influence of venom in the same buffer (524 nm). But the decrease of the fluorescence intensity and the change of the peak to large specter range show that ANS and venom might be connected. Hence an apparent increase in the surface potential could be caused by a deeper penetration of ANS probe between the lipid headgroups.

It is interesting to follow the above interactions when we get GUVs from pure proteolipids instead of usual vesicles: according to the division method, after chemical purification the proteolipids mainly consist of acid phospholipids (mainly of phosphatidylserine) and membrane proteins (Zabolenski et al. 1984). Negatively charged phospholipid GUVs interaction with ANS which is also negatively charged is very superficial. (**Fig.2, right**).

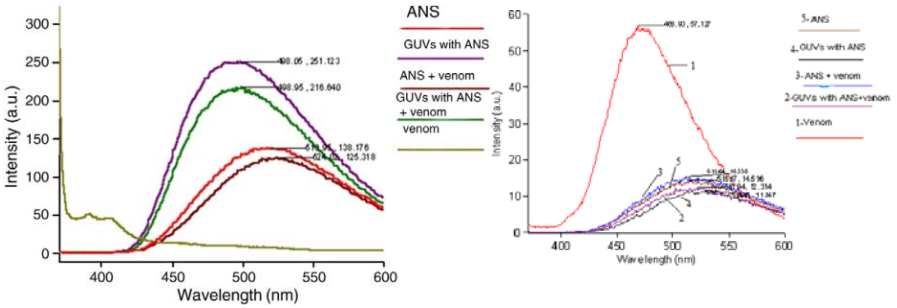


Figure 2. ANS fluorescence intensity in phospholipid GUVs (left) and proteolipid GUVs (right) membrane modified with viper venom.

The study of venom influence on liposome fluidity with the help of pyrene made it possible to find out certain features of the interaction between fluorescence probe and liposomes modified by venom. Being a hydrophobic probe pyrene isn't solved in the buffer solution and its fluorescence intensity is very weak. But after incubation in liposome solution pyrene fluorescence intensity increases sharply. As you can see in **Fig. 3, left** there are two peaks which prove the formation of pyrene excimer in the bilayer lipid membrane. As was noted earlier by Kalayanasundaram and Thomas, the relative intensity of peak III (I_3, λ=386nm) to peak I (I_1, λ=375nm) is a characteristic parameter, which is used to discuss the environmental effects on pyrene monomer fluorescence. In our case the peak ratios are higher for GUVs without venom (I_3/I_1=1.09), indicating a smaller water penetration in these liposomes compared to GUVs modified with venom (I_3/I_1=0.95). The fluorescence of venom itself at 340nm doesn't undergo visible changes after incubation with pyrene as pyrene doesn't interact with protein. We have absolutely different results in case of pyrene incubation with liposomes modified by venom. The emission spectrum enables to divide three main interaction parts, one of which is almost entirely like the pyrene fluorescence in control liposomes (370-400 nm). Besides we can see fluorescence peak in 346 nm range, which is evidently the pyrene monomer emission spectrum in the area of lipid- protein contact. A new higher intensity fluorescence peak comes into being in 457nm range, which corresponds to the pyrene dimmer emission range in the lipid bilayer. The ratio of the excimer (I´,λ=457nm) to monomer intensity (I,λ=394nm) of the embedded pyrene is an index of fluidity parameter of its environment and also of its magnitude of incorporation (Nandy et al., 1983). At a constant temperature the I´/I ratio is directly proportional to the effective concentration of the probe incorporated within the membrane (I´/I, λ=2.61). These data enable to conclude that the bilayer fluidity changes as a result of interaction with venom. As you can see the bilayer fluidity decreases abruptly in those areas where the bilayer interacts with the proteins. But at the same time the bilayer fluidity fairly increases in the free lipid bilayer areas**.**

Unlike ANS, pyrene interacts with proteolipid GUVs and this hydrophobic interaction is an interesting information source for lipid/protein interaction. Pyrene fluorescence was not so intense, but as a result of proteolipid GUVs, we got two peaks with high intensity which corresponded to monomer and dimer fluorescence peaks in the lipid/lipid and lipid/protein contact points. The study of venom influence on GUVs fluidity with the help of pyrene made it possible to find out certain features of the interaction between fluorescence probe and GUVs

modified by venom. Being a hydrophobic probe, pyrene isn't solved in the buffer solution and its fluorescence intensity is very weak. But after incubation in GUVs solution pyrene fluorescence intensity sharply increases (**Fig. 3, right**). Pyrene monomer fluorescence is more intense, which is due to the great number of lipid/protein contacts. It is natural, as according to the chemical structure of the proteolipids, there are fewer free lipid bilayer areas in these vesicles, as these phospholipids are hydrophobic. If we compare pyrene interaction in the GUVs modified by venom and in proteolipid GUVs, we can see that having protein properties, the components of the venom could play the role of membrane protein themselves. But in case of negatively charged proteolipids the venom binding mechanisms are superficial.

The data repeat the results obtained by prof. Bagatolli's in the recent studies on PLA_2 mechanisms in liposomes through probes LAURDAN and PRODAN, but it is worth mentioning that unlike prof. Bagatolli's lab results we study the complex structure of venom and the interaction of its separate particles on bilayers may be different.

At the same time the studies with the fluorescence probes (PRODAN and LAURDAN) show that PLA_2 changes bilayer fluidity and lipid packaging.

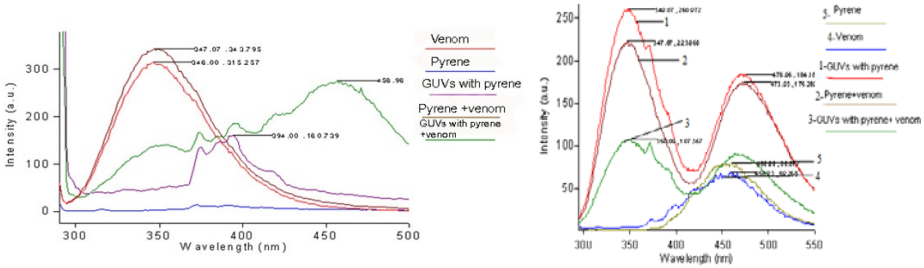


Figure 3. Pyrene fluorescence intensity in phospholipid GUVs (left) and proteolipid GUVs (right) membrane modified with viper venom.

To better understand the interaction between lipid membranes and venom components we have made use of probes LAURDAN and PRODAN, which makes it possible to understand the water penetration into the bilayer and gel-liquid condition of hydrophobic segments. LAURDAN emission intensily increases after incubation with GUVs. In this case GP value is 0,156 which decreases in case of venom modified liposomes GP= 0.096. This is proof of the water penetration into the bilayer. We observe the similar effect in case of PRODAN.

We also conducted experiments with PLA_2 enzyme isolated from Bothrops jararacussu snake venom in order to see GUVs interaction and in this case GP value also underwent significant changes in control GUVs and after PLA_2 modification.

The changes of electrical properties of planar bilayer lipid membranes (BLMs) reconstructed with liposomes modified by *Macrovipera lebetina obtusa* venom

Our studies also emphasize the importance of a membrane surface curvature for its interaction with enzymatic components of venom. And we worked out the following scheme relying on the fact that some principal components of the venom such as PLA_2 can't go into the flat bilayer. That is, under the influence of electric current these vesicles approach the bilayer flat membrane of Teflon aperture and become part of BLMs.

The introduction of the modified GUVs into the BLMs is easier, as a lipid/lipid interaction takes place and as a result BLMs is modified by venom components. The addition of modified GUVs leads to the channel-like activity, which is periodic in case of high voltage (200 mV). In fact the penetration of GUVs bilayer is larger and results in the described effect. It is worth mentioning that the insertion of clean proteolipids with BLMs leads to a different kind of interaction. The BLMs modified with such proteolipids have low resistance (10^7-10^8Ohm, U_m=50mV) and channel-like activity, which is not periodic and show the work of different membrane proteins.

Morphological changes of GUVs during snake venom interaction and their visualization with ANS probe and fluorescence microscope

GUVs were visualized with the membrane fluorophore ANS during experiments on MLO venom hydrolysis. ANS integrates into the membrane and emits with sufficient intensity to follow the changes produced on the GUVs after the addition of the venom. **Fig.4** shows the noticeable increase of liposomes in the course of modification with a low concentration of venom at 21^{o}C in the liquid phase. The higher concentration of venom leads to the very fast increase of the fluorescent intensity, after which the vesicle shrinks until there is virtually nothing of the vesicle remaining (**Fig.5**).

The other series of experiments was done with a visualization of GUVs modified with MLO venom in the course of liposome formation. This was done to ensure the involvement of venom components in the membrane and investigation of the properties and morphological characteristics of GUVs with damaged lipid bilayer. **Fig.6** shows a time sequence of fluorescence intensity images obtained from GUVs formed in the environment of low concentration of venom (at 21^{o}C in the liquid phase). After the addition of the venom to the ionic media of GUVs in the course of formation, the vesicles demonstrated time-dependent changes in the estimated surface area of the GUVs as hydrolysis proceeds. Liposomes were aggregating and merging within approximately 15-20 min and then disappearing. We tried to form giant vesicles also in the media of high concentration of MLO venom, but there was no visible micelliar structure. The image of this non-structured lipid mixture obtained after the electroformation in the venom environment is plotted in **Fig.7.**

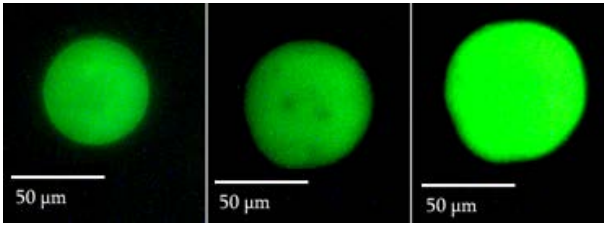


Figure 4. Macrovipera lebetina obtusa venom-dependent increase of the GUV size is shown. Dried lyophilized toxin of *MLO* was dissolved in Tris–HCl buffer (pH 7.4) with a final concentration of $3*10^{-5}$ M, and 1.1μl of this solution were added to the fluorescent microscope sample.

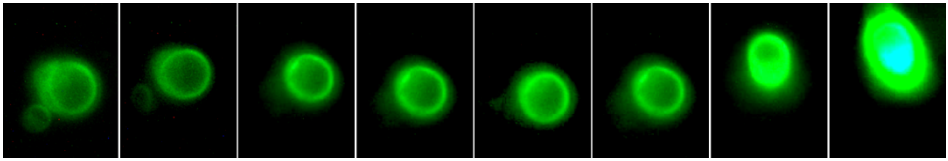


Figure 5. Rapid changes of the fluorescent intensity of the ANS-containing GUVs in the course of MLO venom processing (30 s). Dried lyophilized toxin of *MLO* was dissolved in Tris-HCl buffer (pH 7.4) with a final concentration of $3*10^{-5}$ M, and 2.2μl of this solution were added to the fluorescent microscope sample.

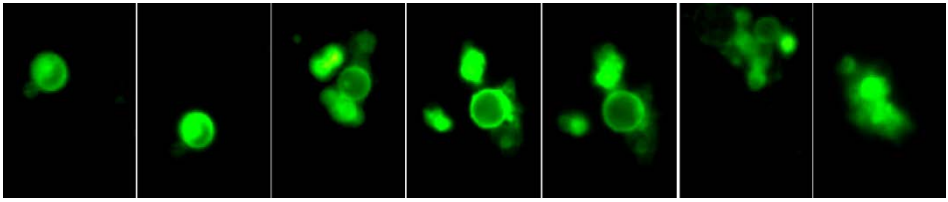


Figure 6. Time-dependent aggregation and merging of the ANS-containing GUVs, modified with *MLO* venom in the course of liposome formation. GUVs were prepared according to the electroformation method in a temperature- chamber containing Tris-HCl buffer (pH 7.4) with a final concentration of *MLO* 10^{-4} M.

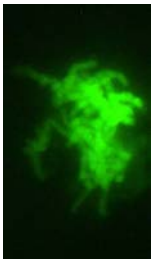


Figure 7. The fluorescent image of non-structured ANS-containing lipids, damaged with *MLO* venom in course of liposome formation. GUVs were tried to prepare according to the electroformation method in a temperature-controlled chamber containing Tris-HCl buffer (pH 7.4) with a final concentration of *MLO* 10^{-3} M.

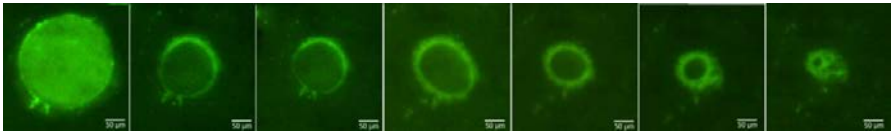


Figure 8. Rapid changes of the size of the ANS-containing p-GUVs in the course of *MLO* venom addition (47s). Dried lyophilized toxin of *MLO* was dissolved in Tris-HCl buffer (pH 7.4), final concentration $3*10^{-5}$M, 1.1 μl of this solution were added to the fluorescent microscope sample. The initial size of GUV was 249 micron.

So, in the next stage of our research we combined the proteolipid GUVs (p-GUVs) formation technique and fluorescence microscopy for the visualization of the above mentioned interaction. **Fig. 8** shows the obvious decrease of p-GUVs size in the course of modification with venom (low concentration) at 21^0C in the liquid phase. During 47 second the p-GUVs shrink until nothing virtually remains of the vesicle (instead of the usual vesicles formed from phospholipids (Ayvazyan et al. 2012). It is worth noting that p-GUVs with high concentration of proteins are more stable and there is little increase in size unlike the p-GUVs with low concentration of proteins.

The other series of experiments was done with a visualization of p-GUVs modified with MLO venom in the course of GUVs formation. This was done to ensure the involvement of venom components in the membrane and investigation of the properties and morphological characteristics of p-GUVs with a damaged lipid bilayer. The p-GUVs with high concentration of proteins are again stable instead of vesicles with low concentration of proteins, where there were no structured p-GUVs of the media of low venom concentration. We also tried to form p-GUVs in the media of high venom concentration. Our preliminary results have shown that some p-GUVs formed in the media of high venom concentration, have some shape deformation and a very short life period so we suppose that it is a reason for lipid package violation (**Fig. 9**).

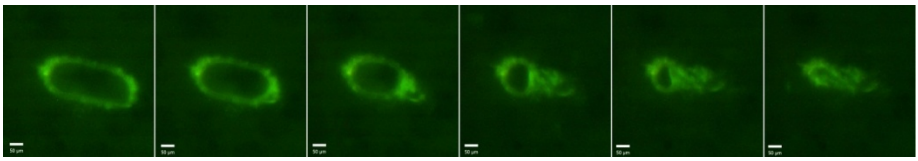


Figure 9. Rapid changes of the size of the ANS-containing p-GUVs formed in *MLO* venom solution (58s). We tried to prepare p-GUVs according to the electroformation method in a temperature controlled chamber containing Tris-HCl buffer (pH 7.4), final concentration of *MLO* 10^{-3}M.

We have also shown that PLA_2 amino acid sequence replacement with Lys 49 (K49) leads to PLA_2 enzyme activity loss which can be seen from the absence of morphological changes during the interaction with GUVs.

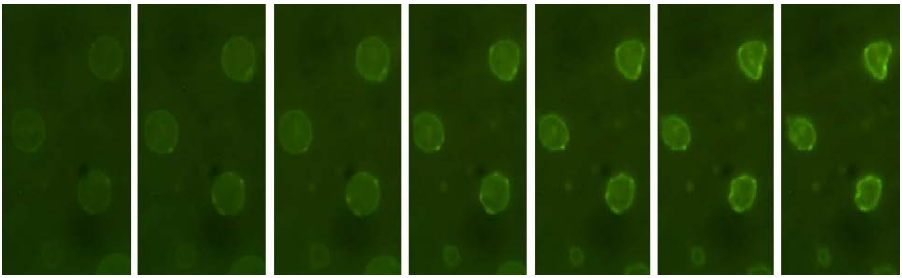


Figure 10. ANS-containing GUVs deformation in the course of *MLO* venom addition (PLA_2 inhibited). Dried lyophilized toxin of MLO was dissolved in Tris-HCl buffer (pH 7.4), to which we added $BaCl_2$ (0.1 M) final concentration $3*10^{-5}$M, 1.1 μl of this solution were added to the fluorescent microscope sample (5 minute).

Ba^{2+} ions inhibit the PLA_2 activity in the complex venom and influence GUVs which is expressed with the morphological changes of the GUVs and lets us conclude that the other components of the venom also participate in the interaction with the lipid membrane (**Fig. 10**).

CONCLUDING REMARKS

The binding of venom components to lipid interfaces is dependent on many properties of the membrane surface. It has been a generally 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 (Burack et al., 1997; Maloney and Grainger, 1993; Sanchez et al., 2002). 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 to answer this question by direct visualization of the peptides on unsupported lipid bilayers (GUVs).

As we can see from the experiments with planar lipid layers, the hydrophobic effect is thus not sufficient to maintain a deep embedding of the peptides within the lipids. So the components of venom are very probably adsorbed at such a lipid interface with a few non-polar residues at the contact with the lipid chain. This is in agreement with the data concerning the interaction of different peptides from snake venoms (Ohkura et al., 1997; Jan et al., 2002). In such cases the binding to lipids was also strictly curvature-dependent.

We used ANS, pyrene, PRODAN and LAURDAN as fluorescence probes, which makes it possible to estimate the state of the membrane, the degree of water molecule penetration in the bilayer. the change in polarity during the interaction with the protein as well as gel liquid crystal phase transition degree.

The studies following the changes in the plastic properties of membranes of different tissues during snake venom envenomation are scarce (Ayvazyan et al., 2012). The analysis of the results obtained in the experiments with the whole venom is difficult because of the complexity of ongoing processes, each of which may induce various cascades of tissue damages.

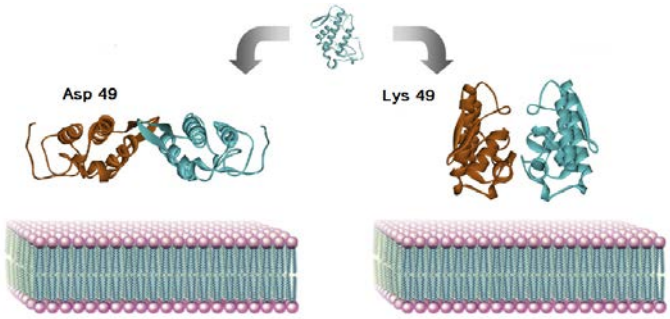
Biochemically, snake venoms are a complex mixture of pharmacologically active proteins and polypeptides (Sanz et al., 2008). All of these components act in concert. The synergistic action of venom proteins may enhance their activities or contribute to the spread of toxins (Calvete et al., 2009). The synergy is especially important for the sub-family Vipera, because they don't have real toxins in the venom (like three-finger toxins of Elapidae or cytotoxins of pit vipers). In most cases, snake venom enzymes act as monomers and exhibit optimal pharmacological properties and contribute to toxicity. But in case of Vipera sub-family snake venoms, they form complexes with other non-enzymatic proteins to achieve higher efficiency through synergy. By the injection of venom we are launching out the cascade of processes and the results obtained here allow us to conclude that this 10-min treatment with the snake venom leads to harmful changes even in the tissues which are rather far from the bite which accounts for the so called spreading effect in organisms, i.e. the rapid spread of the venom which is due not only to the venom components but to the secondary products of their interaction with the tissues (lisophospholipids and so on) as well.

The data obtained during our research enable us to suppose that being a complex of biologically active substances the snake venom interacts with GUVs.

The hypothesis that there may be profound effects on the vesicle structure because of PLA_2 is not new and has been discussed by Biltonen et al. (Sanchez et al., 2002; Bagatolli and Gratton, 2000), but never for the whole venom. While many toxicological studies have been conducted with purified components of venom, all previous observations of $sPLA_2$-dependent GUV morphology changes have been the shrinking response to $sPLA_2$.

Our results show that addition of snake venom on GUV results in serious morphological changes, both GUV membrane damage and size increase.

The experimental methods of K49 type with no PLA_2 activity and those of the whole venom with inhibited activity make it possible to make conclusions about the role of the different molecule points and other low molecular proteins in the venom during interaction with PLA_2 dimer membrane on intoxication.



The approximate orientation by which dimers of PLA_2 approach a membrane.

CONCLUSIONS

The following conclusions were made based on experimentally obtained data:

1. It has been shown *in vivo*, that the existence of *Macrovipera lebetina obtusa* venom (10 min, 0.35mg/kg) in the organism resulted in the increase of electrical resistance of liver and muscle BLM, while the brain lipid BLMs didn't demonstrate a significant difference as compared with the control.
2. It has been shown with the help of fluorescence probes (ANS, pyrene, PRODAN and LAURDAN) that modification with the venom leads to the membrane fluidity decrease of the liposomes formed from heterogeneous lipids in the protein/lipid contact point, while in contrast the lipid bilayer fluidity increases, and the membrane fluidity of liposomes formed from pure proteolipids decreases both in the protein/lipid contact point and in the lipid bilayer.
3. It has been discovered that the liposomes restored with the above mentioned venom facilitate the input of venom components into the lipid bilayer interacting with the BLMs and as a result the bilayer demonstrates channel like activity which is periodical in case of high voltage (200mV).
4. Interacting with the BLMs proteolipid liposomes are inserted into the lipid bilayer and demonstrate channel like activity (50mV) with no particular periodicity. In both cases the venom is a blockader for the formed channels and it reduces channel conductivity.
5. It has been shown that modification with the venom leads to the liposome membrane damage and liposome lysis both in phospholipid liposomes and in liposomes formed from proteolipids. Neither phospholipid liposomes nor proteolipid liposomes are formed in the media with high venom concentration, but some proteolipid liposomes that are formed have a short life span (a few seconds) and are deformed.
6. It has been shown that PLA_2 amino acid sequence replacement with Lys 49 leads to PLA_2 enzyme activity loss which can be seen from the absence of morphological changes during the interaction with GUVs. At the same time the studies with the fluorescence probes (PRODAN and LAURDAN) show that PLA_2 changes bilayer fluidity and lipid packaging.
7. As a result of Ba^{2+} ion inhibition the complex venom influence on GUVs is expressed with the morphological changes of the latter and lets us conclude that the other components of the venom also participate in the interaction with the lipid membrane.

LIST OF PUBLICATIONS AS A PART OF DISSERTATION TOPIC

1. Ghazaryan N.A., Ghulikyan L.A., Ayvazyan N.M. (2013) Morphological changes of proteolipid giant unilamellar vesicles affected by Macrovipera lebetina obtusa venom visualized with fluorescence microscope. **Journal of Membrane Biology**. DOI 10.1007/s00232-013-9576-1**.** Accepted.
2. Ghazaryan N.A., Ghulikyan L.A., Ayvazyan N.M. (2012) Macrovipera lebetina obtusa venom action on giant unilamellar vesicles from brain proteolipids. International Young Scientists Conference "Perspectives for development of molecular and cellular biology", Yerevan, Armenia, pp.77-81.
3. Ayvazyan N.M., Ghazaryan N.A., Zaqarian N.A. (2012) Electroporation and electropermeabilization of lipid bilayer membranes in the course of snakes' venom intoxication. **Journal of Biophysical Chemistry**, 3(1):44-48.
4. Ayvazyan N.M., Zaqarian N.A., Ghazaryan N.A. (2012) Molecular events associated with Macrovipera lebetina obtusa and Montivipera raddei venom intoxication and condition of biomembranes. **BBA-Biomembranes**, 1818(5):1359-1364.
5. Ayvazyan N.M., Ghazaryan N.A. (2012) Lipid bilayer condition abnormalities following Macrovipera lebetina obtusa snake envenomation. **TOXICON**, 60 (4):607-613.
6. Ghazaryan N.A. (2011) Electroporation and electropermeabilization of lipid bilayer membranes in the course of snakes' venom intoxication. **Biological Journal of Armenia**, 63 (3):99-104.
7. Ayvazyan N.M., Zaqarian A.E., Ghazaryan N.A. (2011) Free radical oxidation and condition of membranes from brain lipids of vertebrates in the course of Vipera Lebetina Obtusa venom interaction. **The new Armenian Medical journal**, 5 (3):4-10.
8. Ghazaryan N.A., Ghulikyan L.A., Ayvazyan N.M. (2013) Behavior of proteolipid GUVs formed in Macrovipera lebetina obtusa venom solution. Biophysical Society 57th Annual Meeting, Philadelphia, Pennsylvania, USA, p. 35.
9. Ghazaryan N.A., Ghulikyan L.A., Ayvazyan N.M. (2012) Molecular mechanisms of Macrovipera lebetina obtusa venom action on giant unilamellar vesicles from high density lipoproteins. Second European Symposium on Microbial Lipids, Microbial Lipids: Diversity in Structure and Function, Bern, Switzerland, p.49.
10. Ayvazian N.M., Ghazaryan N.A., Ghulikyan L.A. (2012) Lipid Bilayer Condition Abnormalities Following Macrovipera lebetina obtusa and Montivipera raddei Snake Envenomation. Toxicon Special Issue: 17th World Congress of the International Society on Toxinology and Venom Week 2012, 4th International Scientific Symposium on All Things Venomous, Honolulu, Hawaii, 60 (2):167.
11. Ghazaryan N.A., Ghulikyan L.A., Ayvazyan N.M. (2012) Molecular mechanisms of GUVs from high density lipoproteins under the influence of MLO venom. 11th Greta Pifat-Mrzljak International School of Biophysics, Primosten, Croatia, p. 67.
12. Ayvazian N.M., Zaqaryan N.A., Ghazaryan N.A. (2011) Snake venom and chemical engineering of artificial lipid vesicles and bilayer membranes: theory to experiment. 17th Congress of the European Section of the International Society on Toxinology. Valencia, Spain, p.201.

13. Ayvazian N.M., Zaqaryan N.A., Ghazaryan N.A. (2011) Ionic Permeability of Mixed-Lipid Giant Unilamellar Vesicles Modified with Vipera lebetina obtusa Venom. “Solvation and Ionic Effects in Biomolecular Recognition: Theory to Experiment”, Tsakhkadzor, Armenia, p.51.
14. Ayvazian N.M., Zaqaryan N.A., Ghazaryan N.A. (2010) Fluorescence microscopy of the viper venom’s interaction with Mixed-Lipid Giant Unilamellar Vesicles from bovine brain. Journal of Neurochemistry, Supplment: 4th ISN Special Conference “Membrane domains in CNS Physiology and Pathology”, Sicily, Italy, 113:2.
15. Ayvazian N.M., Zaqaryan N.A., Ghazaryan N.A. (2010) Chemical engineering of artificial lipid vesicles and bilayer membrane in toxicological studying: theory to experiment. Joint EUROMAR 2010 and 17th ISMAR Conference “WWMR2010”, Florence, Italy, p.143.
16. Ayvazian N.M., Zaqaryan N.A., Ghazaryan N.A. (2009) Single Mixed-Lipid GUV Method Reveals Interaction of Viper venom with Lipid Membranes. FEBS Journal 276 (Suppl. 1): 34th FEBS Congress, Prague, Czech Republic, p.177.

ՂԱԶԱՐՅԱՆ ՆԱՐԻՆԵ ԱԼԲԵՐՏԻ

MACROVIPERA LEBETINA OBTUSA ՕՁԻ ԹՈՒՅՆԻ ԱԶԴԵՑՈՒԹՅԱՆ ՄԵԽԱՆԻԶՄՆԵՐԸ ԱՐՀԵՍՏԱԿԱՆ ԵՐԿՇԵՐՏ ԼԻՊԻԴԱՅԻՆ ԹԱՂԱՆԹՆԵՐԻ ՎՐԱ

Ամփոփագիր

Հանգուցային բառեր՝ *Macrovipera lebetina obtusa*, թաղանթային մոդելներ, էլեկտրապորացիա, խզման պոտենցիալ, PLA_2, K49, էլեկտրաթափանցելիություն, ֆլուորեսցենտային զոնդեր:

Թույնի բաղադրիչների կապումը լիպիդի հետ կախված է թաղանթի մակերեսի մի շարք հատկություններից: Լիպիդային երկշերտի նկատմանբ PLA_2-ի ակտիվությունը շատ հետազոտողներ բացատրում էին երկշերտում կարճատև “մակերեսային դեֆեկտների” առկայությամբ (Burack et al., 1997; Maloney and Grainger, 1993; Sanchez et al., 2002): Ֆոսֆոլիպազները նախընտրում են կազմակերպված լիպիդային սուբստրատները լիպիդների ֆազային անցումների մոտ ջերմաստիճանում և մասնավորապես մեծ ակտիվություն են ցուցաբերում PLA_2-ի գրոհների նկատմամբ հենց այդ ջերմաստիճանում: Այս տեսակետից կարևորագույն հարցերից հանդիսանում է այս կամ օձի թույնի այլ սպիտակուցային բաղադրիչների փոխազդեցությունը լիպիդի տարբեր հատվածների հետ: Մեր փորձարարական մոտեցումը կարող է օգնել պատասխանելու այս հարցին՝ ուղղակի վիզուալիզացնելով սպիտակուցները առանց հենարանի լիպիդային երկշետում (ՀՄՎ):

Ինչպես երևում է ԵԼԹ-րի վրա կատարած հետազոտությունից, ի տարբերություն կոբրայի թույնի, իժի թույնի էլեկտրաստատիկ հատկությունները պայմանավորված են լիպիդային երկշերտում սպիտակուցային բաղադրամասերի խնամակցությամբ: Այսպիսով՝ հիդրոֆոբ էֆեկտը բավարար չէ լիպիդային երկշերտում սպիտակուցների խորը ներդրման համար, քանի որ կարևոր է նաև լիպիդային թաղանթի կորությունը, որը սկզբունքային դեր է կատարում որոշ սպիտակուցների թաղանթի մեջ ներդրման համար (Ohkura et al., 1997, Jan et al., 2002):

Այսպիսով, մեր կողմից ընտրված ֆլուորեսցենտային զոնդերը (ԱՆՍ, պիրեն, ԼԱՈւՐԴԱՆ, ՊՌՈԴԱՆ), շնորհիվ երշերտի հետ իրենց կապման տոպոլոգիայի, հնարավորություն են տալիս գնահատելու թաղանթի վիճակը, երկշերտի մեջ ջրի մոլեկուլների ներթափանցման աստիճանը, սպիտակուցի հետ փոխազդելիս բևեռականության փոփոխությունը, ինչպես նաև գել/հեղուկ բյուրեղ փուլային անցման աստիճանը:

Քիչ են օձի թույնով թունավորման ժամանակ տարբեր հյուսվածքների մեմբրանների պլաստիկ հատկությունների փոփոխությունների վերաբերյալ ուսումնասիրությունները (Ayvazyan et al., 2012): Ամբողջական բաղադրիչներով թույնի ազդեցության արդյունքների վերլուծությունը բավական դժվար է ընթացող գործընթացների բարդության պատճառով, քանի որ նրանցից յուրաքանչյուրը կարող է

առաջացնել հյուսվածքի՝ իրար հաջորդող վնասվածքներ: Բացի այդ առանձին բաղադրամասերի տոքսիկությունը հայաստանյան օձերի տեսակների թույնի դեպքում չի գերազանցում 1%-ը: Ըստ բաղադրության օձի թույնը ֆարմակոլոգիական ակտիվ սպիտակուցների և պոլիպեպտիդների խառնուրդ է (Sanz et al., 2008): Լինելով ֆերմենտների և ցածրամոլեկուլյար միացությունների հավաք՝ այն օժտված է որոշակի սիներգիզմով, և առանձին բաղադրամասերի փոխազդեցությունը թաղանթային կառույցների հետ տարբերվում է թույնի ընդհանուր ազդեցության մեխանիզմներից: Թույնի սպիտակուցային բաղադրամասերի սիներգիկ ազդեցությունը կարող է ուժեղացնել թույնի ազդեցությունը, ինչպես նաև կարող է նպաստել տոքսինի տարածմանը (Calvete et al., 2009): Հատկապես սիներգիզմը շատ կարևոր է *Vipera* ենթաընտանիքում, քանի որ նրանք չունեն իրական տոքսին (ինչպես օրինակ three-finger տոքսինը *Elapidae*-ում) և թույնի ֆերմենտային ակտիվություն ունեցող բաղադրիչները ոչ ֆերմենտային ակտիվություն ունեցող սպիտակուցների հետ ձևավորում են կոմպլեքսներ, որպեսզի հասնեն ավելի մեծ արդյունավետության: Մեր հետազոտություններում թույնի միջմկանային ներարկման միջոցով մենք սկիզբ ենք դնում մի շարք կասկադային պրոցեսների, և ստացված արդյունքները թույլ են տալիս եզրակացնելու, որ օձի թույնով 10 րոպեանոց մշակումը բերում է նույնիսկ խայթոցից բավականին հեռու գտնվող հյուսվածքների վնասակար փոխակերպմանը, ինչով և արտահայտվում է կենդանի օրգանիզմներում այսպես կոչված “spreading” էֆեկտը, այսինքն թույնի արագ տարածումը, որի համար պատասխանատու են ոչ միայն թույնի բաղադրամասերը, այլ դրանց հյուսվածքների հետ փոխազդեցության երկրորդային պրոդուկտները (լիզոֆոսֆոլիպիդներ և այլն):

Հետազոտությունների արդյունքում ստացված տվյալները թույլ են տալիս եզրակացնել, որ օձի թույնը, լինելով կենսաբանորեն ակտիվ նյութերի մի բարդ ամողջություն, փոխազդում է ՀՄՎ-րի հետ: Այն հիպոթեզը, որ ՀՄՎ-րի վրա խոր ազդեցություն կարող է ունենալ PLA_2-ը, նոր չէ և քննարկվել է Բիլտոնի կողմից (Sanchez et al., 2002; Bagatolli and Gratton, 2000), բայց երբեք դա ցույց չի տրվել ամբողջական թույնի դեպքում: Մեր արդյունքները ցույց են տալիս, որ ՀՄՎ-րի վրա թույնի ավելացումը բերում է լուրջ մորֆոլոգիական փոփոխություններ, ինչպես ՀՄՎ-րի թաղանթի վնասում, այնպես էլ չափերի մեծացում:

PLA_2-ի ակտիվություն չունեցող K49 տարատեսակի (Lomonte and Rangel, 2012) և PLA_2-ի ճնշված ակտիվությամբ ընդհանուր թույնի, փորձարարական մոտեցումները թույլ են տալիս անել եզրահանգումներ PLA_2-ի դիմերի թաղանթի հետ փոխազդելու ընթացքում մոլեկուլի տարբեր տեղամասերի և թույնում առկա այլ ցածրամոլեկուլյար սպիտակուցների դերի մասին ինտոքսիկացիայի ժամանակ:

КАЗАРЯН НАРИНЕ АЛЬБЕРТОВНА

МЕХАНИЗМЫ ДЕЙСТВИЯ ЗМЕИНОГО ЯДА *MACROVIPERA LEBETINA OBTUSA* НА ИСКУССТВЕННЫЕ БИСЛОЙНЫЕ ЛИПИДНЫЕ МЕМБРАНЫ

РЕЗЮМЕ

Ключевые слова: *Macrovipera lebetina obtusa*, мембранные модели, электропорация, потенциал пробоя, PLA_2, K49, электропроницаемость, флюресцентные зонды.

Связывание составляющих яда с липидами зависит от ряда особенностей мембранны. Активность PLA_2 по отношению к липидному бислою многие исследователи объясняют наличием в них поверхностных дефектов (Burack et al., 1997; Maloney and Grainger, 1993; Sanchez et al., 2002). Фосфолипазы предпочитают липидные субстраты в темпереатурах близких к фазовым переходам липидов, в частности активность к атакам PLA_2 повышается именно в условиях температур фазового перехода. С этой точки зрения важнейшим вопросом является взаимодействие белковых компонентов яда змеи с липидными фрагментами. Наш эксперименталный подход может способствовать в решении данной проблемы посредством прямой визуализации белков в липидном бислое без подложки.

Как выяснилось из исследований на БЛМ, по сравнению с ядом кобры, электростатические свойства яда гадюк обусловлены сродством белковых компонентов к липидному бислою. Таким образом только гидрофобный эффект недостаточен для глубокого внедрения белков в липидный бислой, что соответсвует данным по взаимодействию отдельных белков из яда змей с липидными бислоями (Ohkura et al., 1997, Jan et al., 2002). В этих случаях очень важна кривизна липидной мембраны, которая играет ключевую роль во внедрении некоторых белков в бислой.

Таким образом выбранные нами флюресцентные зонды (АНС, пирен, ЛАУРДАН, ПРОДАН), благодаря топологии связывания с бислоем, позволяют оценить состояние мембраны, степень проницаемости бислоя для молекул воды, изменение полярности при взаимодействии с белками, а также степень фазового перехода гель – жидкий кристалл.

На данный момент нет достаточных исследований относительно изменений пластических свойств мембран разных тканей при отравлении ядом змеи (Ayvazyan et al., 2012). Анализ результатов действия целостных компонентов яда достаточно проблематичен из-за сложности процессов, так как каждый из них может вызывать последующие повреждения тканей. Однако, наряду с вышеуказанным, токсичность отдельных компонентов ядов разных видов армянских змей, не превышает 1%. Яд змеи представляет собой смесь фармакологически активных белков и полипептидов (Sanz et al., 2008). Являясь совокупностью ферментов и низкомолекулярных соединений, ему свойствен резко выраженный синергизм, и взаимодействие отдельных компонентов яда со структурами мембраны отличается от механизов действия цельного яда. Синергичность белковых компонентов яда может усилить его эффект, а также способствовать быстрому распространению токсина (Calvete et al., 2009). Синергизм особенно важен в роду *Vipera*, так как эти гадюковые, как правило, не имеют в составе яда истинных токсинов (как, например, three-finger токсин у *Elapidae*) и ферментативно-активные компоненты яда с

неэнзиматическими компонентами могут образовать комплексы. В наших исследованиях посредством внутримышечной инъекции дается начало ряду каскадных процессов и получанные результаты позволяют заключить, что 10-минутное воздействие яда гюрзы приводит к повреждению даже отдаленных от укуса тканей, демонстрируя так называемый “spreading” эффект в живом организме, то есть быстрое распространение яда, которое обусловлено не только его компонентами, но и вторичными продуктами взаимодействия данных компонентов с тканями (лизофосфолипиды и т.п.).

Данные наших исследований дают возможность заключить, что яд змеи, являясь сложным единством биологически активных веществ, взаимодействует с липосомами. Гипотеза, что PLA_2 может иметь глубокое воздействие на липидный бислой не является новой и описана Билтоном (Sanchez et al., 2002; Bagatolli and Gratton, 2000), но такое взаимодействие не было описано для цельного яда с липосомами.

Наши результаты показывают, что добавление яда к гигантским униламеллярным везикулам приводит к глубоким морфологическим изменениям, наблюдается повреждение мембраны липосом и увеличение их размеров.

Экспериментальные подходы с использованием токсина K49 (Lomonte and Rangel, 2012) не имеющего фосфолипазной активности и общего яда с блокированным ферментом PLA_2 позволяют делать выводы относительно роли разных низкомолекулярных белковых компонентов яда и разных пептидных доменов молекулы при интоксикации: во время взаимодействия димера PLA_2 с мембраной.