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MEMBRANOTROPIC EFFECT OF *MONTIVIPERA RADDEI* AND *MACROVIPERA LEBETINA OBTUSA* VENOM WITH PHOSPHOLIPASE A2 INHIBITED BY p-BROMOPHENACYL BROMIDE

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In the article influence of *Montivipera raddei* and *Macrovipera lebetina obtusa* venom including phospholipase A2 (PLA2) inhibited by p-bromophenacyl bromide on giant unilamellar vesicles, visualized with 8-anilino-1-naphthalenesulfonic acid fluorescence probe, was investigated. The results have shown that both venoms even without the PLA2 activity have a noticeable membranotropic effect on the artificial membranes, but in different ways, so one can propose that investigated viper venoms can contain different PLA2 isomers.

Keywords: GUV, *Macrovipera lebetina obtusa*, *Montivipera raddei*, phospholipase A2, p-bromophenacyl bromide.

Introduction. The venom as medicine has interested the scientists since ancient times, but it is only in the recent years, that important achievements have been described. Snakes venom has a unique composition of biologically active proteins with specific features due to which they are widely used for scientific and medical purposes (for example, lebetox can stop bleeding, ect.). Venom proteins have multiple functions including immobilizing, paralyzing, killing and digesting prey. Venoms produced by snakes of the family Viperidae (vipers and pit vipers) contain proteins that interfere with the coagulation cascade, the normal haemostatic system and tissue repair, and human envenomations are often characterized by clotting disorders, hypofibrinogenemia and local tissue necrosis [1]. Although viperid venoms may contain well over 100 protein components, *Montivipera raddei* (*MR*) and *Macrovipera lebetina obtusa* (*MLO*) venoms proteins belong to 11 and 9 major protein families respectively, including enzymes (serine proteinases, Zn^{2+}-metalloproteinases, L-amino acid oxidase, group II PLA2) and proteins without enzymatic activity (disintegrins, C-type lectins, natriuretic peptides, myotoxins, CRISP toxins, nerve and vascular endothelium growth factors, cystein and Kunitz-type proteinase inhibitors) [2, 3]. Kunitz-type serine proteinase inhibitor and VEGF-like molecules are the unique components of *MR* venom, while the short disintegrin obtustatin is unique for *MLO* venom. A specific toxin was not

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identified in the venom of these snakes and they have no real toxins like three-finger toxins of *Elapidae*, but the enzymatic components form complexes with other non-enzymatic proteins of venom to achieve higher efficacy through synergy.

MLO and *MR* are the most important venomous snakes in Armenia. Venom yield per *MLO* snake is around 0.64 *mg* (LD50 values for mouse are 15–20 $\mu g/kg$) and respectively 0.2 *mg* (LD50 values 9–10 $\mu g/kg$) for *MR* snake venom [4].

Due to their unique biological effect, many types of snakes' venom have been used as valuable pharmacological reagents for studying the interaction of their content and organized lipid interfaces, including bilayer lipid membranes, large unilamellar vesicles, small unilamellar vesicles and multilamellar vesicles [5, 6]. But usually because of their particular characteristics (size and lamellarity) these model membrane systems are not necessarily accurate descriptions of cell membranes. The binding of proteins to lipid interfaces depends on the physicochemical and structural properties of the membrane surface. GUVs are definitely a fascinating model system, which were first described 40 years ago and they are ideal for studying lipid/lipid and lipid/protein interactions.

Many recent investigations showed the influence of factors from venom on cell membranes of mammals. Most of the studies concern the function of phosphorlipase A2 (PLA2) in venom and its action on lipids of cell membranes [7, 8]. Snake venom PLA2 consist of 120–125 amino acid residues. They are containing 7 disulfide bridges. In their amino acid sequence an aspartic acid residue at position 49 plays a critical role in catalysis. These are enzymatically active snake venom PLA2, which are referred to as D49 PLA2. However, in some PLA2 from venom of viper snakes this amino acid is substituted by lysine, serine, and the enzymes are classified as K49, S49 PLA2 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 [9, 10].

p-Bromophenacyl bromide (p-BPB) is a selective PLA2 inhibitor. It discriminate between Ca^{+2}-independent PLA2 (iPLA2) and Ca^{+2}-dependent secretory PLA2 (sPLA2), which inactivates sPLA2 by blockage of exposed His or Lys residues [11, 12]. p-BPB inhibits PLA2 from snake venom by binding covalently to a histidine residue [13] and modifies the histidine in the active center causing the inhibition of the catalytic activity.

The novelty of present study is the focus on the toxicity of the whole viper venom, when one of the main components of this "orchestra" is switched off. It was undertaken to elucidate how the phospholipids GUVs from bovine brain interacted with *MR* and *MLO* venoms and purified PLA2 from viper venoms.

Materials and Methods.

Giant Unilamellar Vesicles. GUVs for fluorescence labeling and measurements were prepared according to the electroformation method, developed by Angelova and Dimitrov [14]. GUVs were formed in a temperature-controlled chamber that allowed a working temperature range from 20 to 50°*C* carrying out the following steps: ~ 20 μL of the lipid stock solution was spread on each of the two sample chamber platinum wires. Then a vacuum pump was used for ~ 1 *h* to remove any remaining trace of organic solvent. 2 μL of buffer (Tris-HCl 0.5 *M*, pH 7.4) was

added to cover the wires. Immediately after buffer addition, the platinum wires were connected to a function generator and a low-frequency alternating field (sinusoidal wave function with a frequency of 10 *Hz* and amplitude of 2 *V*) was applied for 120 *min*. The mean diameter of GUV is ~ 30 *μm*, as previously was reported by Bagatolly [15]. *MLO* and *MR* venoms were added to the sample chamber after the vesicles were formed. Venoms were dissolved in Tris-HCl buffer (pH 7.4), final concentration 30 *μM*, 1.1 *μL* of this solution was added to the sample during GUV visualization. For the inhibition of PLA2 1 *mg* p-BPB was added in venom solution and incubated for 2 *h* at 37ºC.

*Fluorescence Labeling***.** The membrane fluorescence probe 8-anilino-1--naphthalenesulfonic acid (ANS) was used to assess the state of the membrane and specifically mark phospholipid domains. Negatively charged ANS fluorescence intensity was inversely proportional to the value of membrane potential.

After formation of GUV in the electroformation chamber, one micromolar ANS was added to the samples and then incubated for 5 *min* in 25ºC [16]. Then was prepared an observation chamber for microscope study. Images were collected by an epifluorescence microscope (FM320-5M; Am Scope, USA).

Results and Discussion. At the first stage of present investigation was studied influence of *MR* venom on GUV. The GUVs were deformed after adding *MR* venom. After 30 *s* the intensity of GUV and its surrounding was growing. Then after 1.5 *min* GUV size decreased and it was deformed (so called "oval" deformation) (Fig. 1, A). At the next stage of the research was investigated how incubation with p-BPB influenced on phospholipids GUV interaction with *MR* venom. The GUVs were deformed at first 1 *min*, and then they began to recover again the same form until 2 *min*. There were no changes after 3.5 *min* (Fig. 1, B).

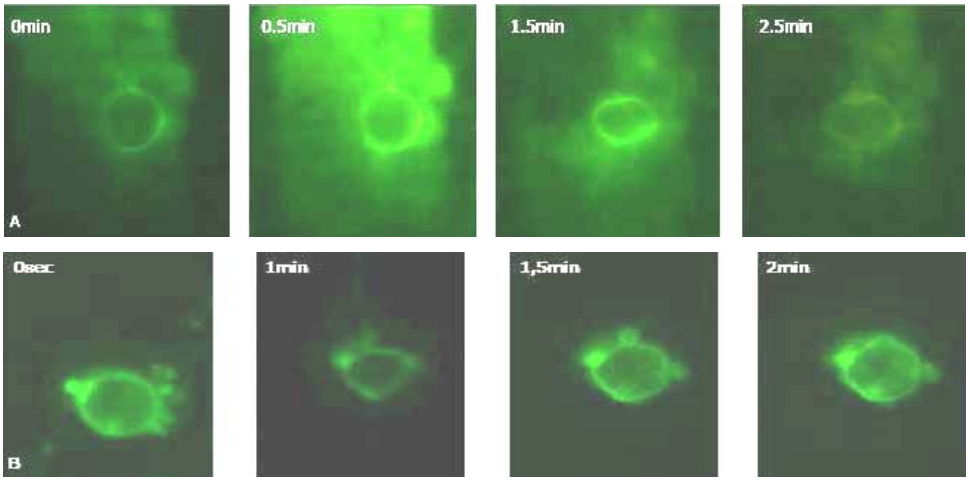

Fig. 1.

A. Changes of the size of ANS-containing GUV in the course of *MR* venom addition. *MR* venom was dissolved in 0.5 *M* Tris-HCl buffer (pH 7.4), with a final concentration of 30 *μM*, and 1.1 *μL* of this solution were added to the fluorescence microscope sample.

B. Changes of the size of ANS-containing GUV in the course of *MR* venom with p-BPB addition.

During the other series of experiments we also have investigated how *MLO* venom with p-BPB influenced on phospholipids GUV. It was described earlier [17], that phospholipids vesicles showed noticeable increasing of size in course of modification with low concentrations of *MLO* venom at 21°*C* in the liquid phase (Figures were kindly provided by Prof. N.M. Ayvazyan, Fig. 2, A). But after adding *MLO* venom incubated with p-BPB solution GUV did not change significantly, just increased fluorescence intensity of GUV (Fig 2, B).

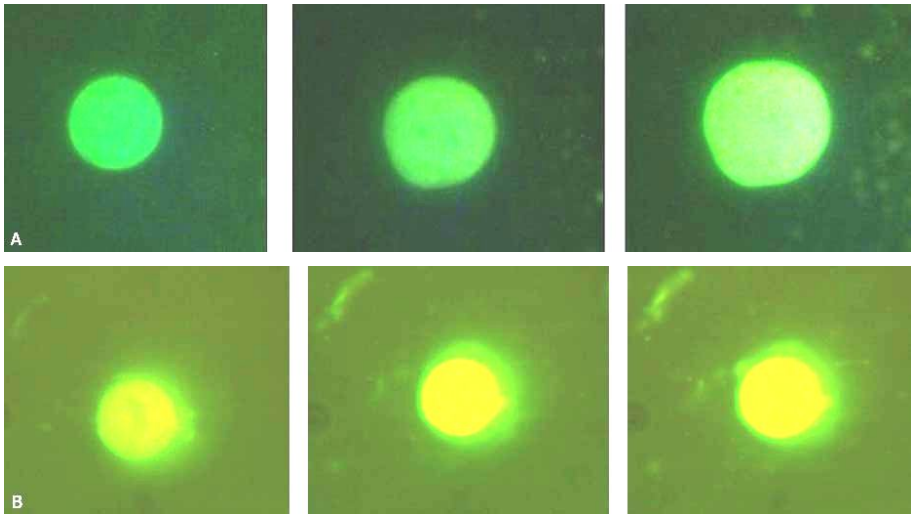

Fig. 2.

A. Changes of the size of ANS-containing GUV in the course of *MLO* venom addition. *MLO* venom was dissolved in 0.5 *M* Tris-HCl buffer (pH 7.4), with a final concentration of 30 μM, and 1.1 μL of this solution was added to the fluorescence microscope sample.
B. Changes of the fluorescence intensity of ANS-containing GUV in the course of *MLO* venom with p-BPB addition.

In the next series of our investigation we have studied the influence of purified PLA2 (enzymatic D49 and non-enzymatic S49) on the GUV (Fig. 3, A) and p-BPB inhibitor effect on the synergistic action of these PLA2 (Fig 3, B). It is necessary to mention, that studying the membranotropic effect of these PLA2 separately and together was done by our scientific group previously [18, 19]. We have already known, that D49 cause the oval deformation of GUV, while S49 could not change either shape, or size of the vesicles. On the contrary, these two isoforms of PLA2 jointly lead to crucial decrease of GUV in the presence of Ca^{2+} mainly characteristic for sublethal doses of entire *MLO*. In present series of experiments we have studied how p-BPB incubation with D49 and S49 influences on phospholipids GUV. After adding of this solution there are no essential changes in GUV size. There is only noticeable increasing of fluorescence intensity (Fig 3, B).

The analysis of the results obtained in the experiments with whole venom is difficult because of the complexity of ongoing processes, due to induction of damage tissues. Biochemically, snake venoms are complex mixture of pharmacologically active proteins and polypeptides [3]. There is a synergic mechanism between all of venom components. The synergistic action of venom proteins may

enhance their activities [20]. Especially the synergy is important for the sub-family Vipers, because they have no real toxins in the venom.

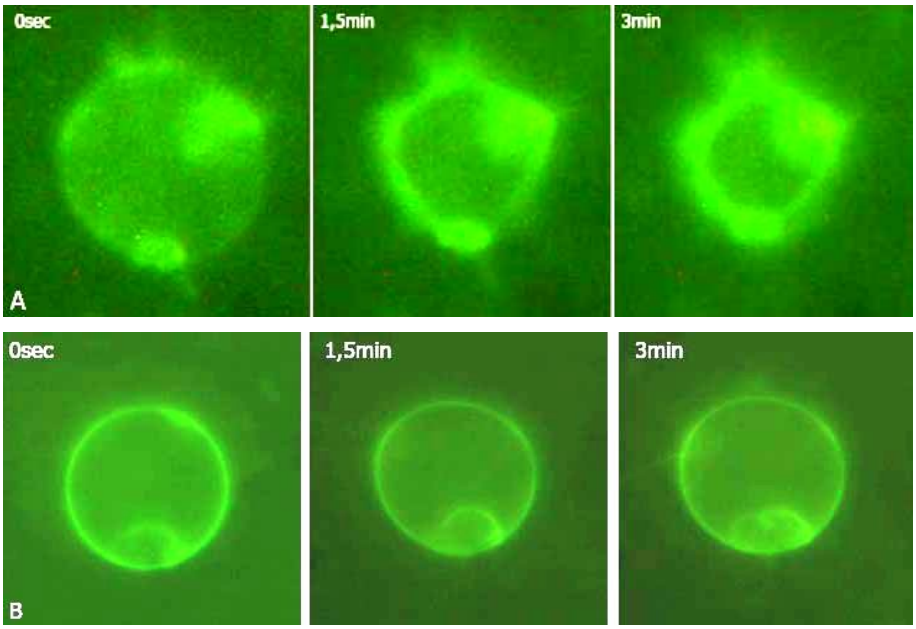

Fig. 3.

A. GUV interaction with a mixture of D49 and S49 PLA2 in the presence of Ca^{2+} ions. The mixture was dissolved in Tris-HCl buffer (pH 7.4) final concentration of overall PLA2 mix is 30 μM, and 1.1 μL of this solution was added to the fluorescence microscope sample (3 *min*).

B. Changes of the fluorescence intensity of ANS-containing GUV in the course of p-BPB with D49 and S49 PLA2 in the presence of Ca^{2+} ions addition.

Our results demonstrate that the membranotropic properties of PLA2 are amplified in the course of synergistic effect of D49 and S49 proteins in the presence of Ca^{2+} ions. The addition of *MLO* venom to GUV of mixed-lipid shows gross morphological changes, and increasing of the vesicle size. The addition of *MR* venom to GUV besides morphological changes, demonstrates decreasing of the vesicle size or even shrinking of GUV. Comparing with *MLO* venom, *MR* venom contains different PLA2 isoforms and many other membrane-acting agents. The changing of fluorescence intensity witnesses that ANS charge has a vital impact on PLA2 interaction with the lipid bilayer which can be seen from the absence of morphological changes during the interaction of MLO venom with GUVs. At the same time the studies with this classical fluorescence probe shows that both viper venoms studied changes bilayer fluidity and lipid packaging. These effects will likely disturb the local packing structure of the lipids, potentially affecting the binding and hydrolysis of the surface phospholipids by venom components. p-BPB is a selective PLA2 inhibitor, so, PLA2 inhibition should lead to the same effect of venom influence, because these enzymes are accepted as the main membranolitic components of venom, but in the case of *MR* venom the mechanism of entire venom with "switched off" PLA2 seems more complicated and let us suggest the membranotropic effect also for some other components of toxic cocktail.

The deciphering of this question will be in the focus of our interests for next series of investigations.

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