

Electroporation and electroporabilization of lipid bilayer membranes in the course of snakes' venom intoxication

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ABSTRACT

The present study was undertaken to elucidate how the plastic properties of model membranes from native lipids of different tissues of rats change in the course of *Macrovipera lebetina obtuse* (MLO), *Montivipera raddei* (MR) and *Naja kaouthia* (NK) venoms processing. The presence of viper venom in organism lead to increasing of the electrical resistance of BLMs from liver and muscle lipids approximately on a sequence, while the BLMs from brain lipids has not shown a noticeable differences of plastic properties compare the control. The same concentration of cobra venom leads to decreasing of electrical resistance of BLMs from 10^{11} Ohm till 10^8 Ohm. The low concentration of venom leads to appearance of channel activity. Especially it is noticeable in liver lipids in media of bivalent ions.

Keywords: Lipid Bilayer; Electroporation; Viper Venom; Breaking-Potential; Electroporabilization

1. INTRODUCTION

As Vipers and pit vipers (snakes of the family Viperidae) produce venoms, which contain proteins that are normally part of the coagulation cascade, the normal haemostatic system and can cause tissue repair. Nevertheless human envenomations are very often accompanied by clotting disorders, hypofibrinogenemia and local tissue necrosis. Although viperid venoms may contain well over 100 protein components, venom proteins belong to only a few major protein families, 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, cystatin and Kunitz-type proteinase inhibitors) [1,2].

The medicinal value of snake venoms has been known from ancient times [3-5]. Snake venoms are medicinally effective at low doses, while their therapeutic properties are achieved by mechanisms which are different from those of known therapies. Angiogenesis, the process by which new blood vessels are formed, is a fundamental event required for a number of physiological and pathological conditions and the crucial role of cell extracellular matrix communication in angiogenesis is well established [6,7]. Because of their unique biological effect, many types of snake's venom have been utilized as valuable pharmacological reagents for studies on the interaction of their content and organized lipid interfaces, like as BLMs, LUVs, SUVs MLVs etc. [8,9]. 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. It is generally accepted that secreted (integral) enzymes are particularly active in the presence of transient "membrane defects" which have been identified as the borders between coexisting lipid phases [10,11].

After obtaining a stable planar bilayer lipid membrane (BLM) from brain phospholipids in 1962 by Mueller & Rudin [12], the electrical properties of membranes have been intensely studied, but mainly in terms of the membrane pathology related to genetic disorders, disturbance of cholesterol-phospholipid balance in atherosclerosis, cancer-related changes in the lipid composition of membranes and etc [13-15]. Currently, there is no doubt that BLMs formed from natural lipids isolated from tissues of animals represent an adequate model of the lipid moiety of biomembranes, which makes it possible to reconstruct some features characteristic of natural bilayer membranes [16].

The present study was in part prompted by an interest in the changes of the lipid condition, which take place in native membranes under the influence of *Macrovipera lebetina obtuse* (MLO) and *Montivipera raddei* (MR)

venoms. The modeling of plastic properties of lipid bilayers in the interaction with venom could provide important information [17,18]. And as we thought, it was necessary to compare the results of these studies with analogous data from experiments with rats, processed by venom of *Elapidae* (*Naja kaouthia*, NK), containing the postsynaptic neurotoxin

2. EXPERIMENTAL PROCEDURE

2.1. Tissue Processing

First, 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 LD₅₀), by modeling of artificial membranes from native lipid content from some organs (liver, heart, brain and muscle). We tried to compare the data of *in vitro* and *in vivo* experiments. For *in vitro* experiments dried lyophilized toxin of MLO was dissolved in Tris-HCl buffer (pH 7.4) with a concentration of 3×10^{-5} M.

2.2. Phospholipid Processing

Lipids fractions were isolated from marked tissues of rats, according to the original Kates method [19]. Then a vacuum pump was used to remove the chloroform-methanol mixture. For *in vitro* experiments there 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).

2.3. BLMs Formation and Measurements

The lipid bilayer membranes were formed from the lipid fractions of rat tissues on a teflon aperture by means of the Muller method [20]. A teflon cylindrical cup having a 0.8 mm hole is 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 was determined on a device equipped with a Keithley 301 differential

feedback amplifier (United States) in a voltage-fixation mode (current-clamp mode), which let to keep up a membrane potential on any level, independent from ionic streams. The potential setting on exit of generator completely falls on membrane, the resistance of which is much higher than that 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. 0.1 M KCl, NaCl, LiCl and KJ, KBr was served as ionic media.

The breaking-potential of membrane recorded in the experiments in shielded camera is taking as the threshold value of the voltage applied. Potential of membrane rupture is a criterion for valuation of natural defects of model membranes. Under electrical potential, radius of these holes is increasing. There is a critical value of radius (r_0), and when $r_\phi > r_0$ membrane is destroying.

2.4. 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 were presented as mean \pm SEM (n = number of experiments).

3. RESULTS

3.1. Electrical Current Measurements in Planar Bilayer Lipid Membranes (BLMs)

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. **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 0.05 ml venom stock solution (concentration 10 mg/ml in Tris/HCl buffer, pH 7.4) was added to the both sides of mem-

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	<i>Vipera lebetina</i>	<i>Montivipera raddei</i>	<i>Naja kaoutia</i>
R_m (Ohm)	$(1 \pm 0.7) \times 10^{11}$	$(6 \pm 0.8) \times 10^8$	$(1.9 \pm 0.3) \times 10^9$	$(3.3 \pm 0.2) \times 10^8$
g_m (Ohm ⁻¹)	$(2 \pm 0.2) \times 10^{-11}$	3.3×10^{-9}	4×10^{-10}	3×10^{-9}
U_r (mV)	448 \pm 12	270 \pm 8	580 \pm 10	280 \pm 9

*Average value (\pm SEM) of minimum 7 different experiments.

brane. After a few minutes of equilibration, -100 mV potential was applied in order to monitor changes in electrical properties of BLMs. Starting at 0.2 ml, 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 raised sharply the membrane conductance and witnessed about cumulative mode of venom-membrane interaction. For the next series of experiment, the lipid fraction, after removing the chloroform-methanol mixture was incubated with venom stock solution and held at a constant temperature of 37°C 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 (**Figure 1**) and venom concentration (**Figure 2**).

Collectively these results indicate that components of MLO and MR venom demonstrate rather surface activity during membrane-peptide interaction, while at least one or more components of cobra venom is definitely penetrate the bilayer. Indeed, single-channel current events induced by NK venom have been recorded in BLMs at lowest concentrations ($<8 \times 10^{-4}$ mg/ml).

3.2. Planar Lipid Bilayer Preparation 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 organism lead 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 (**Tables 2, 3**). 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 (**Table 4**). It is especially noticeable in liver lipids in media of bivalent ions.

4. CONCLUSIONS

As we can see from the experiments with planar lipid layers, that compare cobra venom with viper venom, peptides affinity is dominated by the electrostatic term. 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 lipid chain. This is in agreement with the data concerned the interaction of different peptides from snake venoms [21,22]. In such cases the binding to lipids

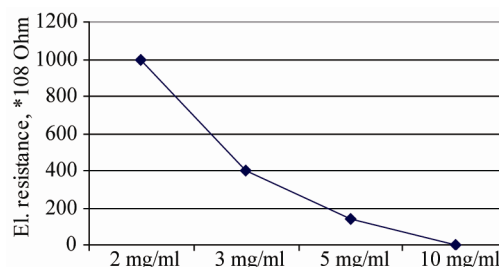


Figure 1. A dependence of electrical resistance of rat's brain lipids BLMs (incubated with cobra venom) on the processing time.

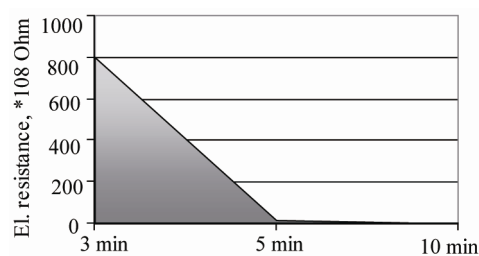


Figure 2. A dependence of electrical resistance of rat's brain lipids BLMs (incubated with cobra venom) on the venom concentration.

Table 2. Snake venoms' *in vivo* effect on the electrical properties of BLMs (resistance R_m , conductivity g_m and breaking-potential U_r) formed from different tissue's 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
<i>Vipera lebetina</i>	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
<i>Vipera raddei</i>	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
<i>Naja kaoutia</i>	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.

Table 3. *Montivipera raddei* 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's lipids in univalent ions media.

	Brain			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
K^+	7.25×10^{10}	1.3×10^{-11}	313	5.26×10^{10}	1.9×10^{-11}	239	4.62×10^{10}	2.1×10^{-11}	304
Na^+	5×10^{10}	2×10^{-11}	234	1.17×10^9	8.5×10^{-10}	162	1.94×10^{10}	5.1×10^{-11}	227
Li^+	16×10^{10}	6×10^{-12}	309	2.54×10^{10}	3.9×10^{-11}	173	1.94×10^9	5.1×10^{-10}	200
J^-	1.33×10^9	7.5×10^{-10}	355	5.98×10^8	1.6×10^{-9}	180	2.71×10^9	3.6×10^{-10}	348
Cl^-	7.25×10^{10}	1.3×10^{-11}	313	5.26×10^{10}	1.9×10^{-11}	239	4.62×10^{10}	2.1×10^{-11}	304
Br^-	10×10^{10}	1×10^{-11}	312	2.58×10^8	3.8×10^{-9}	162	3.3×10^5	3×10^{-6}	327

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

Table 4. *Naja kaoutia* 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's lipids in univalent ions medium.

	Brain			Liver		
	R_m Ohm	g_m Ohm ⁻¹	U_r mV	R_m Ohm	g_m Ohm ⁻¹	U_r mV
K^+	7.4×10^{10}	1.4×10^{-11}	407	4.2×10^{10}	2.4×10^{-11}	249
Na^+	52×10^{10}	1.9×10^{-11}	359	2.1×10^{10}	4.8×10^{-11}	233
Li^+	-	-	448	2.15×10^{10}	4.8×10^{-11}	170
J^-	-	-	427	3.7×10^9	2.7×10^{-10}	180
Cl^-	7.4×10^{10}	1.4×10^{-11}	407	4.2×10^{10}	2.4×10^{-11}	249
Br^-	1.02×10^{10}	1×10^{-10}	354	1.49×10^8	6.7×10^{-9}	157

was also strictly curvature-dependent.

Studies following the changes in the plastic properties of membranes of different tissues during snake venom envenomation are scarce. The analysis of the results obtained in the experiments with whole venom is difficult because of the complexity of ongoing processes, each of which may induce a various cascades of tissue damages. At the same time an important task of such research is to provide not only opportunities to improve tissue recovery and clinical outcome of patients, but also to adopt novel therapeutic strategies to treat a number of diseases, such as different types of aggressive cancers [23] or neurological damages [24,25].

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