Structural Changes of Liposome Phospholipid Packing Induced by Cytotoxin of the Central Asia Cobra Venom

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Abstract. Liposomes and proteoliposomes obtained from rat brain were used; structural changes induced by V_c5 cytotoxin (CT) from Central Asia cobra venom have been studied by the EPR method using spin probes (5-, 10-, or 12-doxylstearic acid). The addition of CT to liposome samples, containing spin probes resulted in the appearance of a new EPR signal in the initial spectrum (samples without CT), typical of probes with strongly retarded mobility. The presence of hydrophobic interaction between the CT molecules and spin labelled fat acids permits the assumption that CT molecules in liposomes trap both lipid probes and phospholipids localized in the reach of action of hydrophobic forces. CT may be supposed to induce formation in membranes of liposomes with domain structures. As a result of hydrophobic interaction with CT molecules both the phospholipid and lipid probe mobility in the domain is substantially less than that in liposome regions free of CT molecules. Due to this, a new signal appears in the initial EPR spectrum of the spin probes. An analysis of the dependence of the probe order parameter value on CT concentration in samples has suggested that CT act uniformly along the membrane lipid profile with a certain CT concentration range. At high concentrations CT molecules cannot penetrate the lipid region deep enough, due to mutual electrostatic repulsion and steric factors at membrane surface. As a result, structural changes involve regions adjacent to the membrane surface only.

Key words: Cytotoxin — Membrane — Phospholipid — Concentration — Probe

Introduction

Cytotoxins of snake venoms of the family *Elapidae* are membrane-active polypetides. Cytotoxins induce changes of the biomembrane structure resulting in alterations of their function. The biological effect of cytotoxin include: hemolytic activity (Khole et al. 1980a,b), increase in passive membrane permeability (Krasilnikov et al. 1976; Condrea 1974), blockade of respiration and phosphorylation in mitochondria „shadows" (Sakhibov et al. 1974; Khole et al. 1980c), interference with the ATP activity in plasmatic membranes (Mirsalikhova et al. 1973) in addition to

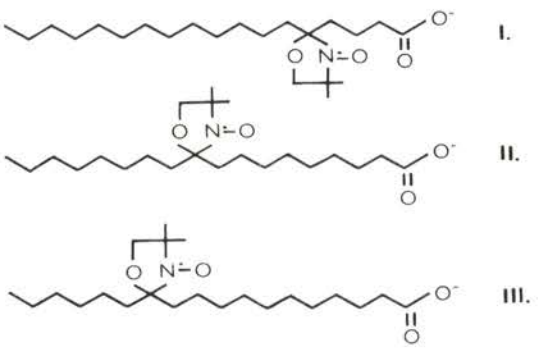

Fig. 1. Structure of spin probes used in the present work.

a number of other effects. It has recently been shown that the interaction of the cytotoxin of the Central Asia cobra venom with artificial phospholipid membranes results in the formation of conductivity channels of different amplitudes (Krasilnikov and Ternovski 1981).

Cytotoxins of different cobra venoms are proteins with comparatively small molecular weights and they are characterized by essential homology of their primary structure; this permits the assumption of a common mechanism of their action.

The purpose of the present work was to study the nature of structural changes in phospholipid liposomes using the EPR method. Changes were induced by V_c5 cytotoxin (CT) (mol. weight 68,000 D) and spin probes used were derivatives of stearic acid (Fig. 1).

CT is very active on membranes having a fixed negative surface charge (Salakhutdinov et al. 1981a,b). Therefore sonicated dispersions of phosphatidic acid were used in our experiments. They partially form lamellar structures with liposomes.

Materials and Methods

Phosphatidic acid (Serva) was used. Sonicated dispersions of phosphatidic acid were prepared according to the technique described by Salakhutdinov et al. (1981a,b). Lipid concentration was 3 mg/ml.

CT V_c5 isolated from Central Asia cobra venom (according to Yukelson et al. (1974)) was dissolved (50 mg/ml) in 10 mmol/l tris-HCl buffer (pH 7.5).

Alcoholic solutions of spin probes I, II (Syva) and III synthesized according to Berliner (1979) were added to sonicated dispersions of phosphatidic acid to obtain a final probe concentration of 10^{-4} mol/l.

EPR spectra were recorded using a Varian E-4 type radiospectrometer at modulation amplitudes not exceeding 2×10^{-4} T and at resonator input power exceeding 20 mW.

The S parameter was calculated according to the formula:

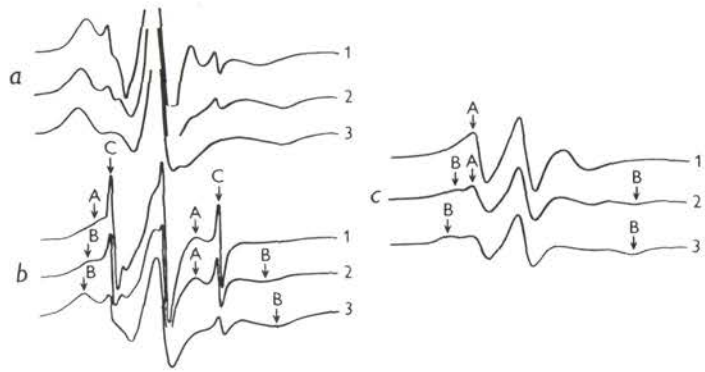

Fig. 2. EPR spectra of spin probes in lipid membranes: *a* — probe I, *b* — probe II, *c* — probe III. 1 — without CT ($C_{CT}/C_{Lip}=0$); 2,3 — under different ratios of concentrations of CT and lipid: C_{CT}/C_{Lip}; (a2)~0.075; (a3)~0.2; (b2)~0.025; (b3)~0.2; (c2)~0.06; (c3)~0.2. For probe III spectra were recorded in the presence of potassium ferricyanate 5×10^{-2} mol/l.

$$S=\frac{T_{\parallel}-T_{\perp}}{T_{zz}-\frac{1}{2}(T_{xx}+T_{yy})}\cdot\frac{a}{a'}$$

where $T_{\parallel}$ and $T_{\perp}$ are the parameters of experimental spectra determined as shown in Fig. 3. The value of a' was calculated as

$$a'=\frac{1}{3}(T_{\parallel}+2T_{\perp})$$

and that of a from

$$a=\frac{1}{3}(T_{xx}+T_{yy}+T_{zz})$$

Values of T_{xx}, T_{yy}, T_{zz} were taken from Berliner (1979).

Results

Control EPR spectra (probe spectra in samples without CT added were taken as control) showed a superposition of broad and narrow signals (Fig. 2). An analysis of probe EPR spectra in buffer solution and the narrow signal suggested that the latter was due to spin probes localized in water phase rather than to those implanted into the membrane. The addition of CT to the sample was followed by a drop in the narrow signal intensity (Fig. 2). Apparently this effect is associated with the fact that spin labelled fat acids not implanted into the membrane interact with CT molecules added to the sample and from CT-probe complexes which, in turn, are implanted into the membrane lipid region. This suggestion was checked in subsequent experiments. CT was introduced into a sample of probe I dissolved in buffer. The intensity of the EPR narrow signal (determined by probes in the solution) dropped and an EPR spectrum typical of probes bound to protein

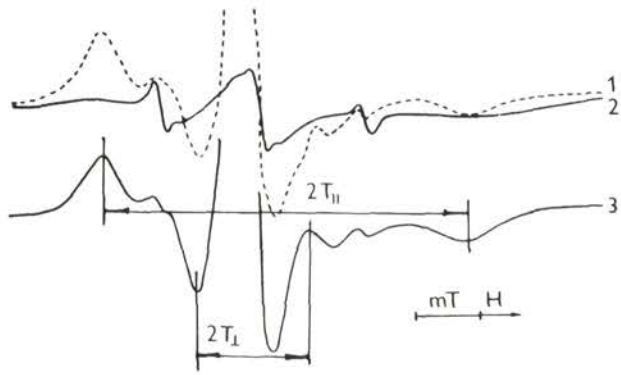

Fig. 3. EPR spectra of probe I in the presence of CT: 1 — in lipid membranes ($C_{CT} = 7.1 \times 10^{-4}$ mol/l); 2 — in Tris-HCl buffer (pH 7.5, $C_{CT} = 0.1 \times 10^{-4}$ mol/l); 3 — EPR spectrum of CT-probe complexes, added to the lipid membranes.

molecules appeared (Berliner 1979). Subsequent addition of this solution, containing CT-probe complexes, to the liposomes resulted in a transformation of the EPR complex spectrum yielding a spectrum that was typical of membrane implanted spin labelled fat acid (Fig. 3). Similar results were obtained with probes II and III, respectively. It may thus be concluded that they were the probes localized in the water phase that were responsible for the narrow EPR signals in the control spectra.

The broad signal in the control spectra represents probes implanted in the hydrophobic region of the membrane. This conclusion is based on the fact that the addition of a broadening agent, potassium ferricyanate $K_3[Fe(CN)_6]$, the ions of which do not penetrate the lipid phase, to the liposomes induces no changes of parameters of the broad signal, and results in a broadening of the narrow signal (Fig. 4) (Kuznetsov et al. 1976). In contrast to probe I, the spectra of membrane localized probes II and III do not show bilateral extremes (Fig. 2); suggesting a different degree of their mobility in the lipid phase. The difference has been explained by the fact that iminoxyl fragments of the probe I localize in the membrane in the region of dense packing of phospholipids, while those of the probes II and III localize in the regions characterized by a higher mobility of phospholipid chains (Ruuge et al. 1977). CT added to the samples induced changes of the primary spectra. A new signal characteristic of probes with a markedly retarded mobility appears in the spectra of both the probe II and III. Within a CT /lipid concentration ratio, C_{CT}/C_{Lip}, below 0.1, the spectra show a superposition of signals of two different groups of probes: those retarded weakely (responsible for the primary spectrum) and those retarded strongly (responsible for the new signal). At C_{CT}/C_{Lip} exceeding 0.1, the spectra are mainly determined by strongly retarded

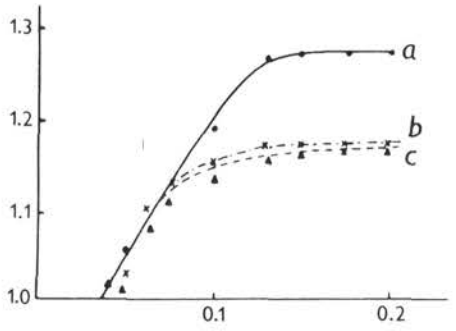

Fig. 4. Relative change of parameter S in dependence on membrane CT concentration; membranes prepared from phosphatidic acid. Abscissa: C_{CT}/C_{Lip}. Ordinate: $S_n/S_{\frac{C_{CT}}{C_{Lip}}=3.75\times10^{-2}}$

probes II and III. In the spectrum of the probe I, the new signal is not distinct; however an increase in CT concentration in the sample up to $C_{CT}/C_{Lip} \sim 0.1$ is followed by an increase in the value of the $2T_{\parallel}$ parameter which is indicative of a diminshed mobility retardation in the group of the probes I (Fig. 2). Further increasing CT concentration results in no change in the shape or the parameters of the probe I spectrum.

To estimate CT-induced structural changes in a liposome quantitively the parameter of the order of the spin probe, characterizing the probe orientation in relation to the liposome surface, was used. At $S = 1$, the probe molecules are oriented perpendicular to the surface, and at $S = 0$, they move fast and isotropically (Berliner 1979).

An increase in membrane CT concentration up to $C_{CT}/C_{Lip} \sim 0.075$ results in an equal increase in the value of S for all the probes. Within a concentration ratio range of 0.075 and 0.1 the value for probe I keeps increasing while those for probe II and III, respectively increase much slower (Fig. 4). AT C_{CT}/C_{Lip} ratios exeeding 0.1 the parameter value remains unchanged for all the probes.

Discussion

The presence of hydrophobic interaction between the molecules of CT and the spin labelled stearic acid allows a suggestion that CT molecules in the membrane trap both lipid probes and phospholipids localized in the reach of action of hydrophobic forces. Thus CT-free sections and those without CT-lipid complexes occur in the membrane, and the proportions of those section depend on the number of CT molecules implanted into the lipid region. Probes localized in both the CT-free

sections of liposomes and domain areas underly the superposition signals of probes with strongly or weakly immobilized mobility in CT sample spectra. Data obtained on the formation of regions of CT-lipid complexes in the membrane were confirmed by experiments of Ksenzhek et al. (1978). These authors assumed that a mosaic structure, i. e. bilayer sections are formed under the action of V_c5 CT on the phospholipid membrane. These sections are separated by cytotoxin-lipid complexes. An analysis of the change of parameter S and its dependence on relative concentration of CT in the sample (Fig. 4) showed that, at C_{CT}/C_{Lip} below 0.075, the CT-induced changes in the mobility of acyl chains of phospholipids are indentical in all sections where iminoxyl fragments of the probes I, II and III are localized. As a result the mobility of the probes becomes changed. At C_{CT}/C_{Lip} exceeding 0.075 differences in changes of the membrane phospholipid mobility occur along its profile. This different sensitivity of phospholipid mobility to CT concentration in liposomes can be explained by the fact that, at CT concentrations up to $C_{CT}/C_{Lip} \sim 0.075$ the CT molecules penetrate the membrane deeply enough to bind the entire molecule of the phospholipid; in other words, fat tails of phospholipid molecules become agglutinated with CT molecules. Due to this the mobility retardation degree of phospholipids is uniform along the entire length of their acyl chains. A further increase in CT concentration up to $C_{CT}/C_{Lip} \sim 0.1$ (stoichiometry of toxin and lipid binding 1 : 10), its molecules cannot penetrate so deeply into the hydrophobic region of the membrane due to mutual electrostatic repulsion and steric factors at the membrane surface. In this case, the bond of the phospholipid with a CT molecule is a hinged nature with a strong limitation to the mobility of the polar part of the phospholipid and a rather weak one in the region of the methyl ends. Within a range of $C_{CT}/C_{Lip} > 0.1$, CT molecules are not bound to the membrane. Immobilization of acyl chains of the membrane phospholipid under the action of another membrane-active polypeptide, melittine, from the bee venom was shown by Williams and Bell (1972).

On the basis of our data and the results of Ksenzhek et al. (1978) and Williams and Bell (1972) it may be concluded that CT molecules form areas of CT-lipid complexes in the membrane. The packing, density of phospholipids in the complexes is apparently higher than that in CT-free regions of the membrane. At certain concentrations, the effect of CT on the membrane is uniform along its lipid profile. At higher concentrations CT molecules cannot penetrate the lipid region deeply enough, due to mutual electrostatic repulsion of CT molecules and steric factors at the membrane surface. As a result, it is only the region adjacent to the membrane surface that undergoes structural changes.

It was suggested earlier (Krasilnikov et al. 1976) that the cytotoxin action of the protein studied is due to the induction of ion conductivity of cell membranes. It was found later that the appearance of integral conductivity of bilayer membranes

in the presence of CT at one side of the membrane is a result of the formation of ion channels (Krasilnikov et al. 1981).

Data on the structure of the boundary protein-lipid complexes presented in this paper may provide an additional information on the organization of aqueous pores in bilayer phospholipid membranes. A substantial decrease in the mobility of fat-acid residues of phospholipids as a result of hydrophobic interaction with CT points to the fact that a local destruction of the bilayer order occurs at the site of CT penetration, consisting of aqueous protrusions and membrane thinning. Due to this low voltage application may result in breakdown and channel conductivity.

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