AN INVESTIGATION OF THE INTERACTION OF COBRA VENOM CYTOTOXINS WITH LIPOSOMES BY THE FLUORESCENT-PROBE METHOD

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The interaction of cytotoxins V_c1, V_c5, and V_c6 of the venom of the Central Asian cobra with liposomes having a negative surface charge prepared from a mixture of phosphatidylcholine and palmitic acid (1:1, molar) has been investigated with the aid of a pyrene fluorescent probe. It has been shown that on interacting with liposomes a cytotoxin increases the microviscosity of the hydrophobic region of membranes. This effect depends on the phase state of the lipids. Observations on the kinetics of the transfer of energy between pyrene probes and diphenylhexatriene have shown that on the addition of a cytotoxin to samples of liposomes with a negative surface charge an aggregation of the liposomes takes place without a disturbance of their integrity.

The cytotoxins of the venom of the Central Asian cobra consist of 60 amino acids, have molecular weights of about 7000 D [1] and belong to the class of membrane-active polypeptides.

The interaction of cytotoxins with biological membranes leads to various modifications of their function, namely: to changes in permeability and selective conductivity, to a disturbance of metabolic processes, etc. [2-4]. The mechanism of these interactions includes two stages [5]: an electrostatic interaction of the positively charged amino acid residues of the toxins with the negatively charged heads of the phospholipids, and hydrophobic interactions of the lipophilic sections of the chains of the cytotoxin molecules with the acyl chains of the phospholipids when they are immersed in the hydrophobic region of the membrane, which is explained by the combination of the basic and hydrophobic properties of the cytotoxins [1].

It has been shown [6] that on the interaction of cytotoxin V_c5 with membranes, processes of complex-formation take place that are accompanied by the production of domain structures with the lipid formations investigated. The cytotoxin causes a decrease in the mobility of the fatty acid residues of the phospholipids along the whole length of their chains.

With the aim of a further study of the structural changes in biomembranes induced by the action of cytotoxins, we have investigated by the fluorescent probe method the interaction of the three toxins V_c1, V_c5, and V_c6 with the phospholipids of liposomes. Pyrene and diphenylhexatriene (DPHT) were used as fluorescent probes.

As is well known, as a hydrophobic probe, pyrene dissolves in the actual material of the membrane, and when it is introduced into liposomes in sufficient amounts a maximum of excimeric forms appears in the fluorescence spectrum [7]. In our experiments, as mentioned above, the concentration of pyrene introduced into the liposomes corresponded to a probe:lipid ratio of 1:100. At such a concentration, a fairly pronounced maximum of the excimeric forms in the spectrum of the probe was obtained. At the same time, such a concentration of the probe in the liposomes does not lead to great changes in their structure. An increase or decrease in the ratio of the intensity of the maxima of the excimers and the monomer depends on the change in the viscosity of the microenvironment of the probe.

It has been shown [5, 8] that the efficiency of the action of cytotoxins on liposomes is determined by the existence of negatively charged lipids in the biolayers. In view of this, samples that we prepared from egg phosphatidylcholine and palmitic acid in a molar ratio of 1:1 are convenient as model systems with a high negative charge. When a cytotoxin was added to samples of these liposomes at 37°C, no appreciable changes took place in the spectrum of the pyrene probe: the excimer:monomer ratio decreased by only 6-8% as compared with the initial

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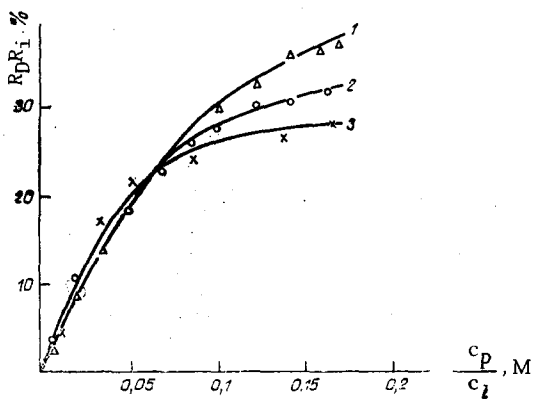

Fig. 1

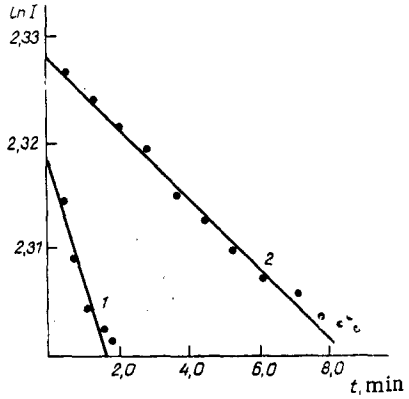

Fig. 2

Fig. 1. Change in the relative (in percentages) microviscosity of phospholipids as a function of the ratio of the concentrations of added proteins to lipids: 1) V_c5; 2) V_c1; 3) V_c6. R_o – ratio of the intensities of fluorescence of the excimeric and monomeric forms (I_e/I_m) in samples without the cytotoxin; R_i – I_e/I_m in samples with increasing concentrations of cytotoxin.

Fig. 2. Change in the intensity of fluorescence in the transfer of energy between pyrene and DPHT probes in liposomes without a cytotoxin (1) and with a cytotoxin (2).

spectrum. This is apparently connected with the fact that the palmitic acid molecule, like the molecules of a phosphatidic acid, at a temperature below the phase transition [9] form clusters and are present in the gel state. It has been shown by ESR spin-probe methods [10] that a cytotoxin does not penetrate within the hydrophobic region of a membrane at temperatures below the temperature of the gel–liquid crystal phase transition, T_p, but interacts only with the negatively charged sections of the surface of the membrane. Since palmitic acid has a phase transition temperature above 42°C, we selected a temperature of 50°C for performing the experiments, which is definitely greater than the T_p value of palmitic acid. In this case, when a cytotoxin was added to samples of liposomes a considerable decrease in the excimer:monomer ratio was observed, i.e., the immersion of the cytotoxin in the bilayer shown in [11] caused an increase in the microviscosity of the membrane. It can be seen from Fig. 1 that the curve of the dependence of the relative intensity on the ratio of the concentration of the added protein to the lipids rises linearly up to protein:lipid ratios of 1:17-1:18. Then the curve bends and rises less steeply, which is apparently connected with the effect of the saturation of the liposomes with the protein.

The experiments described were performed with cytotoxins V_c1, V_c5, and V_c6. Information is given below on the change in the microviscosity at a protein:lipid ratio of 1:10 in relation to the initial microviscosity, i.e., that without the addition of a cytotoxin to liposome samples. The errors of measurement of the relative microviscosity amounted to less than 1%.

Cytotoxin	Rel. change in microviscosity, %
V_c 1	28
V_c 5	30
V_c 6	25

The most effective interaction with the samples investigated was observed for cytotoxin V_c5, the efficiency of the interaction of cytotoxins V_c1 and V_c6 being lower. This is apparently due to the fact that the cytotoxin V_c5 molecule has a higher charge than the V_c1 and V_c6 molecules [2].

It must be mentioned that on the addition of the cytotoxins to samples of liposomes with a negative surface charge, an increase in the light scattered by them at a wavelength of 337 nm and a decrease in the total intensity of the spectrum of the pyrene probe were observed. In our experiments, the parameter of the pyrene probe was the change in the ratio of the intensi-

ties of the excimers to those of the monomer. The increase in light scattering does not affect this parameter, but the fact of this increase casts doubt on the integrity of the liposomes which may exclude the possibility of using liposomes as model systems of biomembranes in these investigations.

In order to study the integrity of the liposomes we performed an experiment on the exchange of hydrophobic probes between two systems: liposomes with a pyrene probe and with a DPHT probe. When these liposomes were mixed, an exchange of probes to a definite equilibrium took place. Then, by exciting the pyrene at a wavelength of 337 nm we followed the kinetics of the transfer of the energy of the excitation of the pyrene to the energy of the electronic excitation of the DPHT molecules.

We performed the same experiment with samples of liposomes having added cytotoxin V_c6. The concentration of cytotoxin in terms of the protein:lipid ratio was 1:30. The kinetics of the transfer of energy between the probes showed that this process was slower than in a sample without a cytotoxin. Consequently, the liposomes apparently remain whole, otherwise an acceleration of the kinetics of transfer would have been observed (Fig. 2). The retardation transfer of energy between the hydrophobic probes in samples of liposomes with a cytotoxin indicates an increase in the microviscosity of the membrane.

In a previous investigation [8] using liposomes prepared from natural lipids it was shown that the lipophilic sections of a cytotoxin molecule are immersed in the hydrophobic region of the liposomes, while the charged part of the molecule is exposed to the aqueous medium under these conditions. The formation in this way of a membrane coated with the positively charged sections of the cytotoxin molecule may lead to aggregation through electrostatic interaction with the negatively charged sections of other liposomes. This fact may explain the turbidity of a sample of liposomes on the addition of a cytotoxin.

EXPERIMENTAL

Egg phosphatidylcholine (Khar'kov) and palmitic acid (Sigma) were used. The liposomes were obtained by the sonication in a current of helium at 3-5°C for 10 minutes of a suspension of lipids with a concentration of 2 mg/ml in 0.05 M Tris-HCl buffer, pH 7.5, in a UZDN-1 disperser. Before an experiment, the liposomes were diluted with buffer solution to a lipid concentration of 0.1 mg/ml.

The pyrene and DPHT probes (both from Serva) were dissolved in absolute ethanol in a concentration of $2 \cdot 10^{-3}$ M. The probes were introduced into the liposomes in a probe:lipid ratio of 1:100 at the rate of 1 µl/min with continuous stirring. The DPHT probe was incubated further at 37°C for 40 min to improve its penetration into the liposomes.

Cytotoxins V_c1, V_c5, and V_c6 were isolated from the whole venom of the Central Asian cobra by the method described in [12]. Before use they were dissolved in 0.05 M Tris-HCl buffer, pH 7.5, in a concentration of $7 \cdot 10^{-5}$ M and were added to the liposomes to a protein:lipid concentration of 1:5.

Fluorescence spectra were recorded on an Aminco SPF-500 instrument. The slit widths of the monochromator was 2 mm, the time constant 1 sec, and the rate of recording the spectra 100 nm/min. The samples were thermostated at temperatures of 37 and 50°C. Quartz cells with dimensions of 1 × 1 cm were used.

SUMMARY

Analysis of the results of our work and of literature information permits the following conclusions to be drawn:

1. The interaction of a cytotoxin with liposomes depends on the phase state of the phospholipids forming the liposomes.

2. On interacting with liposomes, a cytotoxin is inserted into the bilayer, causing an increase in the microviscosity of the membrane.

3. The interaction of a cytotoxin with negatively charged liposomes may lead to the aggregation of the latter.

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SYNTHESIS OF A HEPTAPEPTIDE FORMING A MODIFIED ACTH 4-10 FRAGMENT

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A scheme is given for the synthesis of a heptapeptide representing a modified ACTH 4-10 fragment on the basis of which it is possible to create a preparation that is an effective adaptogen of peptide nature. A proposed variant of the synthesis permits a peptide with an adequate degree of purity to be obtained comparatively simply on a larger scale. The intermediate compounds and the final products were obtained with good yields and were distinguished by chromatographic homogeneity. The heptapeptide synthesized did not differ with respect to its physicochemical characteristics and biological action from the analogous compound obtained previously. Some physicochemical characteristics of the compound obtained (angles of optical rotation, chromatographic mobilities) are given.

One of the promising directions in the field of peptide chemistry is the synthesis of a new medicinal preparation of peptide nature fulfilling the role of regulators of the functions of the central nervous system (CNS). In particular, it has been shown that fragments of ACTH in small doses act on the CNS and affect learning processes in animals and Man [1]. The main disadvantage of these compounds is the briefness of their action which is apparently due to their rapid degradation by enzymes and makes it difficult to use them as drugs.

A heptapeptide consisting of a modified ACTH 4-10 fragment has recently been obtained in the Institute of Molecular Genetics of the USSR Academy of Sciences from which a preparation has been created which is an effective adaptogen of peptide nature [2]. The laboratory scheme of the synthesis of this compound has a number of disadvantages making it difficult to prepare the heptapeptide in large quantities. In the first place, it presupposes the use in the main stages of N,N'-dicyclohexylcarbodiimide as condensing agent, which is a powerful allergen and is being produced in limited amounts. In the second place, in a number of stages in the course of synthesis the intermediate products are treated repeatedly with trifluoroacetic acid to remove protective BOC groups. Nevertheless, according to the literature [3] and to the results of our own investigations, under these conditions the tripeptide (5-7) present

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