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ASYMMETRY OF LIPIDS AND α-TOCOPHEROL DISTRIBUTION IN THE OUTER AND INNER MONOLAYER OF BILAYER LIPID MEMBRANES

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Ever-increasing attention is being paid to the targeted transport of drugs in the patient [4-6]. As a rule the agents used to carry drugs (antibiotics, corticosteroids, insulin, cytotoxic substances, etc.) to organs and target cells, are artificial phospholipid vesicles or liposomes, which are biologically inert and completely biodegradable [6]. The efficiency of delivery of the drugs depends on the duration of circulation of the liposomes and of preservation of their integrity in the blood stream, which may be disturbed as the result of interaction of the outer monolayer of the liposomes with various blood components (acyl hydrolases, acyl transferases, lipoproteins, macroglobulins, etc.) [5, 6]. Stabilization of liposomes by α-tocopherol (TP), a natural membrane protector, may be a promising method of obtaining long-term liposomal preparations. TP also is used independently as an effective therapeutic agent [4, 8, 9]. Meanwhile it has been shown that TP has limited ability to insert itself into phospholipid vesicles and a low rate of transbilayer migration [7, 9].

The aim of this investigation was to study the distribution of TP between the outer and inner monolayers of synthetic phospholipid liposomes, a matter of the utmost importance when ways of saturating biological structures with TP are contemplated.

EXPERIMENTAL METHODS

Liposomes were prepared from dioleoylphosphatidylcholine and phospholipids of the plasma membranes of rat brain synaptosomes. The liposomes were obtained by evaporation of phospholipids under argon, followed by shaking in Tris-HCl buffer (Tris-HCl 50 mM, NaCl 100 mM, pH 7.4) or bicarbonate buffer ($NaHCO_3$ 120 mM, NaCl 100 mM, pH 8.2), and treatment on a UZDN-2 ultrasonic disintegrator (22 kHz), and repeated sonication (12 × 15 sec, with intervals of 30 sec) at 0-4°C until complete clarification of the liposomal suspension. To reduce destruction of lipids, the membranes were disintegrated in a cylindrical resonator, excluding any direct contact between resonator and liposome solution. The liposomes thus obtained were centrifuged at 90,000g for 20 min, and supernatant containing monolayer liposomes was used for the measurements [10]. To incorporate TP into the outer and inner monolayers of liposomes, solutions of phospholipids and TP in chloroform were mixed in definite proportions. The mixture was evaporated to dryness in a current of argon and liposomes prepared from it. The fluorescence spectra of TP were recorded on Hitachi MPF-2A and SPF-850 spectrofluorometers (Japan) in a 1-cm cuvete using slits 5 nm wide. The TP was oxidized with potassium ferricyanide. To determine the localization of amine-containing phospholipids of phospha-

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TABLE 1. Phospholipid Composition of Plasma Membranes of Rat Brain Synaptosomes (M ± m)

Phospholipids	Relative content, % of total	Index of unsaturation of fatty acids*
Phosphatidylcholine	44,3±2,2	68,4
PEA	36,9±1,7	262,5
PS	12,5±0,7	194,0
Phosphatidylinositol	2,5±0,2	
Sphingomyelin	3,8±0,2	230.5 †

Legend. *) Index of saturation indicates number of double bonds per 100 fatty acid molecules; †) index of unsaturation of phosphatidylinositol + sphinogomyelin.

TABLE 2. Distribution of TP and of Aminophospholipids in Outer and Inner Monolayers of Liposomes Formed from Lipids of Plasma Membranes of Brain Synaptosomes (M ± m)

Component tested	Relative content, % of total	
	outer monolayer	inner monolayer
TP	21,6±1,7	78,4±6,3
PEA	31,6±2,3	68,3±4,4
Index of unsaturation	200,8	243,0
PS	11,5±0,8	88,4±6,6
Index of unsaturation	184,3	190,6

tidylethanolamine (PEA), and phosphatidylserine (PS) the monofunctional chemical modifier 2,4,6-trinitrobenzenesulfonic acid (TNBS, 2 mM) was used, as described by the writers previously [2]. Lipid phosphorus was determined by the method in [12]. The fatty acid composition of the lipids was studied on a gas-liquid chromatograph (PYE-104, England) with flame-ionization detector. Fatty acids were identified against a standard mixture of fatty acids.

EXPERIMENTAL RESULTS

For targeted delivery of drugs the most widely different phospholipid vesicles are used [5, 6]; they differ in shape, size, charge of their polar heads, degree of unsaturation of the lipid bilayer, and molecular organization of the outer and inner monolayers. Two types of liposomes were chosen for the present investigation, namely those formed: a) from one individual phospholipid (dioleoylphosphatidylcholine) and b) made from a mixture of lipids, extracted from plasma membranes of rat brain synaptosomes, mainly phosphatidylcholine, PEA, and PS (Table 1). In this way liposomes in which the same phospholipid, namely dioleoylphosphatidylcholine, or different, asymmetrically distributed phospholipids, were present in both monolayers (Table 2), could be obtained.

The distribution and localization of TP in the monolayers of the lipid bilayer can be conveniently studied by recording its characteristic fluorescence [7]. On the addition of a nonpenetrating agent (potassium ferricyanide) to liposomes, the intensity of fluorescence of TP falls due to selective oxidation of that part of the TP molecule which is located in the outer monolayer and is accessible for potassium ferricyanide (Fig. 1). It will be clear from the data in Fig. 1 that incorporation of 5 mole % of TP into liposomes formed from dioleoylphosphatidylcholine, in which there is no asymmetry of distribution of phospholipids by charge of both polar heads and fatty acids (the index of unsaturation is 100), present leads to distribution of TP, so that about 54% of the TP lies in the outer monolayer and about 46% in the inner (due to differences in the volume of the hydrophobic phase of the inner and outer monolayers of the liposomes). Another picture is observed during insertion of TP into liposomes prepared from a mixture of phospholipids. Table 1 gives data on the composition

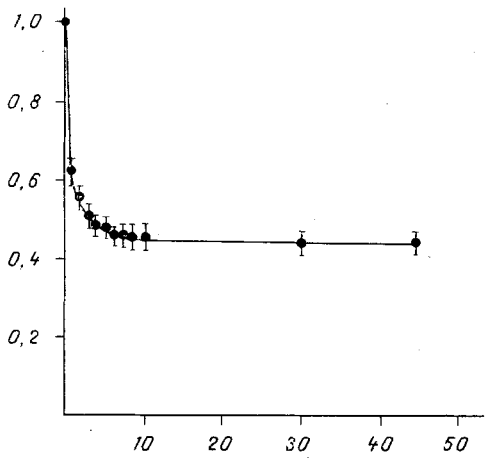

Fig. 1. Changes in intensity of fluorescence of TP in liposomes from dioleoylphosphatidylcholine on the addition of potassium ferricyanide. Abscissa) incubation time (in min); ordinate) normalized intensity of fluorescence of TP (F/F_0). Concentration of lipids $4.2 \cdot 10^{-4}$ M, TP concentration $2.2 \cdot 10^{-5}$ M, potassium ferricyanide concentration $7.0 \cdot 10^{-5}$ M.

of phospholipids and values of the index of unsaturation of fatty acids of plasma membrane lipids of brain synaptosomes, from which the liposomes were formed. Clearly, amine-containing phospholipids (PEA, PS) are characterized by higher values of the index of unsaturation than phosphatidylcholine, and they account for about half of the total of all lipids. The study of the distribution of amine-containing phospholipids in monolayers of liposomes shows that PEA (68.3%) and PS (88.4%) are present mainly in the inner monolayer of the membrane (Table 2), i.e., they are distributed asymmetrically. Incorporation of TP (5 mole %) into these liposomes leads to its uneven distribution among the monolayers; moreover, the natural antioxidant is found to be localized mainly (in the ratio of 4:1) in the inner monolayer (see Table 2). What is the cause of the asymmetrical distribution of TP in liposomes from a mixture of lipids?

The writers showed previously [3] that not only the phytol chain of the TP molecule, but also its chromane ring are located in the hydrophobic zone of the lipid bilayer. This suggests that the uneven distribution of TP is based on interaction with hydrophobic acyl groups of phospholipids. Considering the possibility of formation of a complex of TP with polyunsaturated fatty acids, established previously [1], it can be postulated that one factor regulating the level of distribution of TP between the monolayers may be the content of polyunsaturated fatty acids (PUFA) in the monolayer. The use of the method of chemical modification of amine-containing phospholipids by means of TNBS enabled the fatty-acid composition of the PEA and PS in the inner and outer monolayers to be studied. It follows from the data in Table 2 that the outer monolayer contains PEA and PS with more saturated fatty acids than the inner monolayer. The results are evidence that the outer monolayer is much more saturated in the composition of its fatty acids than the inner layer due to predominance of PEA and PS in the inner monolayer and the higher index of unsaturation of PEA and PS in the inner monolayer. These differences will be even more marked if the contribution of phosphatidylcholine, located chiefly in the outer monolayer [6], and which is characterized by a low PUFA content, is taken into account.

Thus TP is incorporated mainly into the monolayer of liposomes in which phospholipids with a high content of PUFA are concentrated; this asymmetrical distribution both of phospholipids and of TP, moreover, is realized spontaneously and does not necessarily require the participation of specific enzymic mechanisms or of asymmetrically localized protein carriers.

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CHANGES IN PLASMA HORMONE AND PEPTIDE CONCENTRATIONS IN RATS WITH EXPERIMENTAL EPILEPSY

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The mechanisms and pathogenetic importance of changes in blood hormone levels during epileptic activity (EpA) remain largely unexplained [2, 3, 6]. In the study of this problem, attention must be paid to the effect of secondary nonspecific factors (stress, anoxia, etc., for example) during the seizure process on changes in blood hormone levels. From this aspect it is important to determine hormone levels at different stages of development of the epileptic process, starting with the latent period and until the onset of convulsions.

In this investigation plasma levels of seven hormones were studied in rats during the development of generalized convulsive EpA induced by metrazol.

EXPERIMENTAL METHODS

Experiments were carried out on noninbred male rats weighing 200-220 g, kept under standard animal house conditions (temperature 22 ± 2°C, alternation of 12 h daylight and 12 h darkness, standard diet). The experiments were carried out in the spring and summer. An experimental epileptic syndrome was induced by intraperitoneal injection of a 10% solution of metrazol in a dose of 75 mg/kg; control animals received the same volume of physiological saline [4].

Blood was taken during the latent period, namely 30 sec after injection of metrazol in the absence of any clinical signs of seizure activity or electrocorticographic manifestations of EPA, 90-150 sec after injection of metrazol during the development of clonico-tonic convulsions with the animals falling on their side and with a marked tonic extension phase, and 5-10 and 30 min after injection of metrazol, during continuing EpA. The duration of EpA in live animals lasted 9-11 hours. Blood was taken at the same time of day in order to exclude

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