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CARDIOLIPIN EFFECT ON THE LIPID BILAYER STRUCTURE: PYRENE EXCIMERIZATION STUDY

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The influence of cardiolipin (CL) on the structural state of model membranes has been examined using pyrene excimerization technique. Excimer-to-monomer ratio decreased by 11 % as CL content increased from 0 to 10 mol % suggesting that CL can induce bilayer condensation. At higher CL concentrations (20 and 40 mol %) excimer-to-monomer ratio tended to increase being indicative of the reduced lipid packing density. Biological role of CL is discussed.

KEY WORDS: liposomes, pyrene excimer formation, cardiolipin, lipid bilayer structure, free volume.

The process of pyrene excimer formation is known to be sensitive to changes in the membrane physical and chemical properties. In this study pyrene excimerization technique was employed to gain insight into the CL effect on the structural state of model phospholipid membranes. CL is a unique phospholipid. Due to its chemical structure (4 fatty acyl chains and 2 phosphate groups) CL may be expected to affect the lipid bilayer structure. Approaching this problem seems to be of interest not only in physical, but also in biological and medical aspects. CL is known to play an essential role in eukaryotic energy metabolism [1], carrying protons along the polar surfaces of mitochondrial and chloroplast membranes [2] and regulating the enzyme activity [14]. Nowadays, in the age of development of gene therapy, CL is successfully used for the transduction of foreign DNA into cells [3].

MATERIALS AND METHODS

Egg yolk phosphatidylcholine (PC) and beef heart cardiolipin (CL) were purchased from Biolek (Kharkov, Ukraine). Both phospholipids gave single spots by thin layer chromatography in the solvent system chloroform:methanol:acetic acid:water, 25:15:4:2, v/v). Pyrene was from Sigma (Germany). A stock suspension of unilamellar phospholipid vesicles was prepared by the method of Batzri and Korn [4]. The ethanol lipid solution containing appropriate amounts of PC and CL was injected into 13 ml of 5 mM sodium-phosphate buffer, pH 7.4 under continuous stirring. Ethanol was then removed by dialysis. Phospholipid concentration (L) was determined according to the procedure of Bartlett [5]. Fluorescence measurements were performed at 20°C with CM 2203 spectrometer (SOLAR, Belarus). Pyrene emission spectra were excited at 337 nm. Excitation and emission slit widths were set at 2 nm. The excimer-to-monomer fluorescence intensity ratio (I_E/I_M) was determined by measuring fluorescence intensity of the monomer (at 389 nm) and excimer (at 480 nm) peaks.

RESULTS AND DISCUSSION

Pyrene excimer formation is a result of interaction between the ground-state and the excited-state (M and M^*, respectively) molecules. Although pyrene excimer formation in membranes has been extensively studied by a number of authors [6-9], the mechanism behind this phenomenon is still a matter of controversy. Existing models consider three principal possibilities: i) diffusion-controlled (collisional) excimer formation occurring during the lifetime of the excited state [6,8]; ii) pyrene aggregation in a lipid bilayer before excitation resulting in the static excimer formation between M and M^* in close proximity [6,7]; iii) membrane incorporation of pyrene dimers formed in aqueous phase [9]. Collisional pyrene excimer formation occurs when M and M^* are at a rather long distance from each other and the lifetimes of excited monomers are long enough. This process can be described by the following kinetic scheme [7,8]:

$$\begin{aligned} M^* &\xrightarrow{k_{fM}} M + h\nu_M \\ M^* &\xrightarrow{k_{NM}} M \\ M^* + M &\xrightarrow{k_{DM}c_M} D^* \\ D^* &\xrightarrow{k_{fD}} M + M + h\nu_D \\ M^* + M &\xleftarrow{k_{MD}} D^* \xrightarrow{k_{ND}} M + M \end{aligned} \qquad (1)$$

where M denotes monomers, D denotes dimers, asterisks represent excitation, k_{fM} is the monomer fluorescence rate constant, k_{fD} is the excimer fluorescence rate constant, k_{NM} is the rate constant of monomer radiationless deactivation, k_{ND} is the rate constant of excimer radiationless deactivation, k_{DM} and k_{MD} are the excimer formation and dissociation rate constants, respectively, c_M is the monomer concentration, mol/l. Solution of the set of differential equations corresponding to scheme (1) yields the following time dependencies of the excited monomer and excimer concentrations [8]:

$$c_{M^*} = \frac{c_{M_0^*}}{\lambda_2 - \lambda_1}\left[(\lambda_2 - X)e^{-\lambda_1 t} + (X - \lambda_1)e^{-\lambda_2 t}\right]$$
$$c_{D^*} = \frac{c_{M_0^*} k_{DM} c_M}{\lambda_2 - \lambda_1}\left[e^{-\lambda_1 t} - e^{-\lambda_2 t}\right] \quad (2)$$

where $k_M = k_{fM} + k_{NM}$, $X = k_M + k_{DM}c_M$, $k_D = k_{fD} + k_{ND}$, $Y = k_D + k_{MD}$,

and $$\lambda_{1,2} = \frac{1}{2}\left[X + Y \mp \sqrt{(Y - X)^2 + 4k_{DM}k_{MD}c_M}\right]$$

Assuming photostationary conditions (*i.e.* invariance of c_{M^*} and c_{D^*} values) the steady-state solution can be obtained:

$$R_E = \frac{I_D}{I_M} = \frac{k_{fM}k_{DM}c_M}{k_{fM}(k_D + k_{MD})} \quad (3)$$

where I_M and I_D are monomer and excimer fluorescence intensities, respectively.

The static excimers originate from pyrene dimers formed before excitation. The equilibrium constant k_E can be described by the following equation:

$$k_E = \frac{c_D}{c_M^2} \quad (4)$$

where c_M stands for the monomer concentration and c_D is the dimer concentration. If c_Z denotes total pyrene concentration, then:

$$c_Z = c_M + 2c_D \quad (5)$$

By solving Eqs. (4) and (5) we can obtain the expression for c_D:

$$c_D = \frac{1 + 4k_E c_Z - \sqrt{(1 + 4k_E c_Z)^2 - 16k_E^2 c_Z^2}}{8k_E} \quad (6)$$

The extent of pyrene dimerization is determined by the probe concentration and environment polarity. Although pyrene molecules tend to aggregate in the aqueous phase, no aggregates were observed in the suspension of PC liposomes [10]. Pyrene dimerization in PC/CL liposomes seems to be hardly probable because: 1) dimer formation should manifest itself in the rather strong hypochromism and monomer fluorescence decrease. However, in our experiment no significant changes in the intensity of pyrene fluorescence were observed at increasing CL content from 0 to 40 mol %; 2) equilibrium constant of dimer formation estimated from the data presented in Fig. 2 is *ca.* 32, the value being an order of magnitude higher than that derived by Blackwell [7] from the time-resolved and steady-state fluorescence measurements (*ca.* 1.4-2.8). On the other hand, the slope of $R_E(c_{LP})$ dependence (Fig. 2) (where c_{LP} is pyrene concentration in the lipid phase), falls in the range estimated from Eq. (3) with the rate constants observed in membranes (k_{DM} ~$10^6 \div 10^9$ $s^{-1}\cdot l\cdot mol^{-1}$, k_D ~$(1 \div 5)\cdot 10^7$ s^{-1}, k_{MD} ~$10^6 \div 10^9$ s^{-1}, k_M ~$2\cdot 10^6 \div 10^7$ s^{-1}). Based on these arguments, we concluded that under experimental conditions employed here ($c_{LP}^{max} = 0.016$ M) pyrene excimerization proceeds mainly via collisional rather than a static mechanism.

Fig. 1 illustrates typical pyrene fluorescence spectra in PC and PC/CL liposomes. These spectra are featured by a well-defined vibronic structure characteristic of pyrene monomer emission and excimer fluorescence band (λ_{max}~480 nm). Excimer-to-monomer fluorescence ratio (R_E) reflecting the extent of pyrene excimerization depends mainly on the monomer lateral distribution in the lipid bilayer. As shown in Fig. 3, R_E dependence on CL content exhibits a dip (decrease by *ca.* 11 %) at 10 mol % CL. This finding can be explained

in terms of the free volume model of diffusion in lipid bilayer [11]. The membrane free volume characterizes the difference between the effective and van der Waals volumes of lipid molecules. Packing constraints and thermal motion may result in the enhanced trans-gauche isomerization of hydrocarbon chains and appearance of dynamic defects in the membrane interior. A local free volume arises from the lateral displacement of the hydrocarbon chain following the kink formation. The free volume of lipid bilayer depends on its composition, degree of acyl chain saturation, extent of hydration, temperature, etc. [12]. The free volume model considers diffusion of membrane constituents or guest molecules as a three-step process: 1) opening of a gap in a lipid monolayer due to formation of kinks in the hydrocarbon chains; 2) jump of the diffusing molecule into a gap leading to the creation of a void; 3) filling the void by another guest molecule.

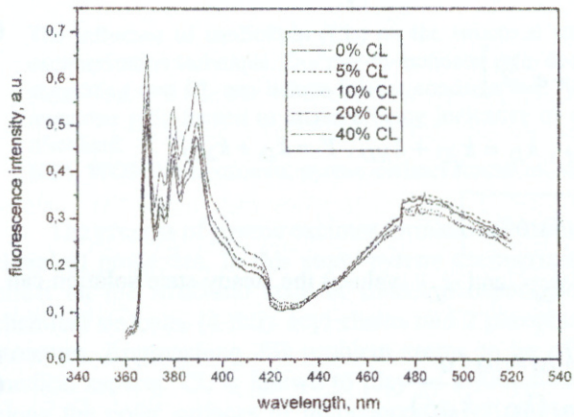

Fig.1. Pyrene fluorescence spectra in the dispersion of PC and PC/CL liposomes

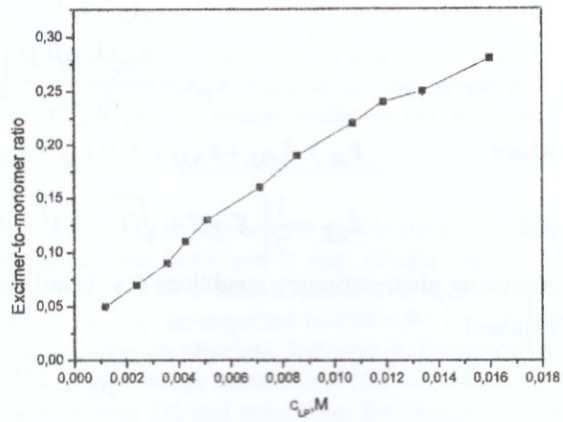

Fig.2. Dependence of excimer-to-monomer ratio on pyrene concentration

Decrease of excimerization extent recovered at varying CL content from 0 to 10 mol % suggests that CL brings about reduction of the lipid bilayer free volume. This observation is consistent with the data of Shibata *et al.* [13] indicating that CL is capable of reducing liposome water permeability because of the bilayer stabilization. Increase of R_E value observed at higher CL content (exceeding 10 mol %) is most likely a consequence of the free volume increase caused by the repulsion of the negatively charged CL head groups. CL incorporation into the vesicles can lead to a change in the membrane surface state, namely in the lipid polar head group conformation [13]. Homogeneous distribution of CL in PC bilayers results in the change of membrane zeta potential – it becomes more negative. Such changes are decisive for molecular orientation in lipid bilayers. Negative charge of CL moves the N^+-end of P-N dipole parallel the surface of the membrane thereby causing the rearrangement of water bridges at lipid bilayer surface and stabilization of the intramolecular hydrogen bonds including the water molecules of hydration layer. Perturbation of the antisymmetric PO_2^- stretching vibrations of PC polar groups caused by CL confirms the above mentioned mechanism of CL influence on the molecular organization of a lipid bilayer [2, 13]. CL content of 50 mol % may induce some changes in the phase behavior of lipid bilayer [13].

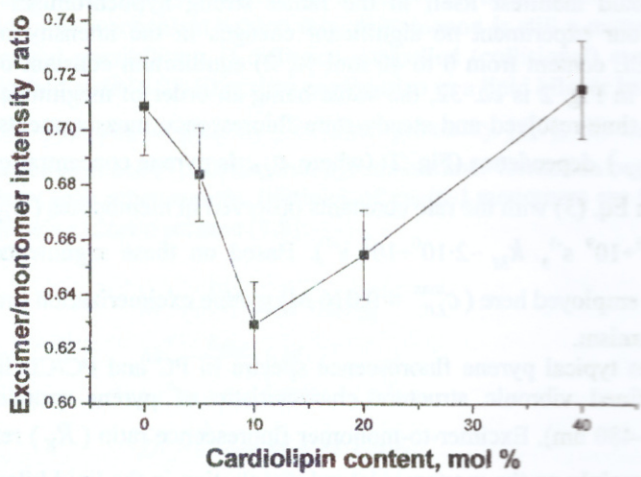

Fig. 3. Excimer-to-monomer ratio in PC/CL liposomes as a function of CL content

It is important to note that CL effect on the membrane physical properties may have essential physiological implications. As CL is an integral component of mitochondrial membranes it is known to play an essential role in energy metabolism and electron transport through the mitochondria. Several lines of evidence indicate that CL plays a great role in maintaining optimal activity of such membrane proteins as NADH dehydrogenase, cytochrome bc_1 complex, ATP synthase, cytochrome *c* oxidase [14]. This is a characteristic feature of cardiolipin. For instance, the affinity of cytochrome oxidase for this lipid is approximately 5 times greater than for PC [15]. Another physiological function of this lipid is CL tendency to form an inverted hexagonal (H_{II}) phase under certain conditions [13,16]. In lipid bilayers such phase may be both caused and suppressed by divalent metal ions or different kinds of proteins. In the cell membrane such unique ability of CL is supposed to be involved in the Ca^{2+} translocation across the membrane [16].

CL content in membranes may be decreased by aging, radiation, nitric oxide and by some diseases such as cardiac ischemia. Radical oxygen species can induce the modification of phospholipid structure [1].

It is also noteworthy that CL is successfully used in gene therapy, in the treatment of a variety of pathologies by incorporation of the genes into the cells with a purpose of directed change of gene defects or for infusion of new functions to the cells. Use of CL which forms both bilayer membranes and inverted micellar structures is very perspective in such therapy. In the presence of Ca^{2+} or Mg^{2+} DNA interaction with this lipid becomes stronger, liposomes tend to aggregate and DNA internalization into the cell takes place [3].

CONCLUSIONS

Examination of the process of pyrene excimerization in PC/CL model membranes revealed that at the concentrations lower than 10 mol % CL exerts condensing effect on the lipid bilayer structure. At higher CL content membrane free volume was found to increase. These findings suggest that variations in CL content can modulate structure-function relationships in biological membranes.

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ВПЛИВ КАРДІОЛІПІНА НА СТРУКТУРУ ЛІПІДНОГО БІШАРУ: ДОСЛІДЖЕННЯ ЕКСИМЕРІЗАЦІЇ ПІРЕНА

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Досліджено вплив кардіоліпіна на структурний стан модельних мембран методом ексимерізації пірена. Відношення інтенсивності флуоресценції ексимерів до інтенсивності мономерів зменшилось на 11 % при підвищенні концентрації кардіоліпіну від 0 до 10 %, що можна пояснити конденсацією ліпідного бішару. При збільшенні концентрації кардіоліпіну до 20 та 40 % відношення інтенсивності ексимерів до інтенсивності мономерів зростало, що свідчить про зменшення щільності пакування ліпідних молекул. Обговорюється біологічна роль кардіоліпіна.