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INTERACTION OF DOLICHOL AND DOLICHYL PHOSPHATE WITH PHOSPHOLIPID BILAYERS

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The thermotropic phase transition of dipalmitoylphosphatidylcholine vesicles reconstituted with dolichol or dolichyl phosphate was investigated as a function of the lipid-to-polyisoprenoid ratio by means of differential scanning calorimetry and fluorescence depolarization of the embedded probe 1,6-diphenyl-1,3,5-hexatriene. At the concentrations studied, dolichol and dolichyl phosphate lowered and broadened the transition temperature of dipalmitoylphosphatidylcholine bilayers. Dolichol was found to increase the motional freedom of the bilayer both below and above the transition temperature as determined by fluorescence depolarization. In contrast, low concentrations of dolichyl phoshate decreased the bilayer motional freedom below the transition temperature while high concentrations increased the motional freedom. Above the transition temperature, dolichyl phosphate decreased bilayer 'fluidity' at all concentrations. The data suggest that these polyisoprenoids perturb the bilayer lattice, with the neutral species dolichol increasing membrane 'fluidity', while dolichyl phoshate acts to 'stiffen' the membrane.

Introduction

Thermotropic transitions of lipid molecules have been the subject of numerous studies using a variety of physical techniques [1]. At maximum hydration, phospholipids exhibit a cooperative phase transition in which the lipids pass from a relatively rigid and ordered gel phase, to a liquid-crystalline structure accompanied by an increase in the rotational motion of the hydrocarbon portion of the lipid molecule.

Pure phosphatidylcholine systems appear sensitive to foreign molecules, e.g., cholesterol, drugs and other lipids [1]. These changes are reflected in modifications in the membrane fluidity and phase transition and are accompanied by a rearrangement of the bilayer and alteration in the ability of the bilayers to fuse [1–3].

Our interests are centered on the dolichols poly-*cis*-isoprenoid lipids which function as chemical 'carriers' of saccharide units during the mebrane-directed assembly of glycoproteins. The special poly-*cis* geometry and length (100 Å) of these molecules should endow them with some unique physical properties when they interact with phospholipid bilayers.

The family of dolichols are found in a variety of tissues in both higher and lower eukaryotes [4]. Dolichyl phosphate functions as a chemical 'carrier' of saccharide units during the membrane assembly of mammalian glycoproteins [5]. Current

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Abbreviations: Dol-*P*, dolichyl phosphate; Dol, dolichol; Dol-*PP*, dolichyl pyrophosphate; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; T_c, transition temperature of the lipid gel to liquid-crystal phase transition.

models for glycoprotein synthesis require that dolichyl phosphate accepts carbohydrate moieties on the cytoplasmic face of the rough endoplasmic reticulum and donates the finished oligosaccharide to nascent polypeptide chains on the lumenal side [6]. After hydrolysis of dolichyl pyrophosphate, produced by the oligosaccharide transferase reaction, dolichyl phosphate is thought to cycle back to the cytoplasmic side to be reglycosylated. Recent studies on the membrane topology of the dolichyl phosphate-dependent glycosyl transferases, while differing somewhat in their conclusions, tend to support the above model [7–9]. Although a substantial literature concerning dolichyl phosphate has accumulated [4,10,11], little is known about its organization in the membrane, sideness, translational movements, and effects on membrane motional freedom *.

In the present work, we report on results of a study to describe the effects of dolichol and dolichyl phoshate on the motional freedom of lipid bilayers by means of differential scanning calorimetry and fluorescence depolarization.

Material and Methods

Materials

L-β-Dipalmitoylphosphatidylcholine (DPPC) was purchased from Fluka, 1,6-diphenyl-1,3,5-hexatriene from Eastman-Kodak and porcine liver dolichol from Sigma. Dolichyl phosphate, obtained from Calbiochem, was further purified by silica gel G chromatography using chloroform/methanol/acetic acid/water (50 : 10 : 3 : 1).

Preparation of DPPC/Dol and DPPC/Dol-P liposomes. DPPC/Dol and DPPC/Dol-*P* mixtures in chloroform/methanol (2 : 1) were dried under N_2, resuspended in benzene/methanol (95 : 5) and lyophilized. Multilamellar liposomes were produced by suspending the dried lipid mixtures (1 mg/ml) in water, then heating above the transition temperature of DPPC followed by rapid vortexing with a vortex mixer [12].

Methods

Fluorescence depolarization experiments. Aliquots of stock solutions of diphenylhexatriene were added to liposome suspensions (diphenylhexatriene/phospholipid ratio was 1 : 900 (mol/mol)) in tetrahydrofuran and incubated well above the T_c for 30 min.

Fluorescence measurements were made using a SLM Model 8000 Photon-Counting Spectrofluorometer equipped with a 450-W xenon arc lamp, MC 640 excitation monochrometer and MC 320 emission monochrometer. Anisotropy measurements were done using three 10-mm ultraviolet Glan-Thompson calcite polarizers arranged in a T format. Each anisotropy measurement required two data acquisition periods of 100 s each. The first one was performed with the excitation polarizer oriented at 90° and the second with the excitation polarizer oriented at 0°. The emission polarizers were maintained at 0° and 90°. The 0°-polarized emission light was selected for wavelength of 428 nm. The 90°-polarized emission light was selected for light transmitted beyond 411 nm using a Corning CS No. 3-72 cutoff filter. The excitation wavelength was maintained at 360 nm. The steady-state fluorescence anisotropy, $\bar{r}$, is defined by:

$$\bar{r} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad (1)$$

with $I_{\parallel}$ and $I_{\perp}$ the two fluorescence intensities when the analyzer is parallel and perpendicular, respectively. Dilution of DPH over a 50-fold range is reported to have no effect on the $\bar{r}$ values [13]. Turbidity effects were very small in this system. Temperature was maintained to ±0.2 K by circulating thermostated water through the cuvette holder blocks.

Differential scanning calorimetry. Calorimetric studies were carried out using a MicroCal, MC-1 calorimeter. The sampler was hermetically sealed in Perkin-Elmer aluminum 'volatile' sample pans. The temperature and enthalpies of the various transitions were calibrated using a benzoic acid standard. Peak areas were measured by weighing paper cutouts of the peaks. Samples were heated at 1.5 K/min and before each heating the samples were maintained at the starting temperature for at least 3 min to attain thermal equilibrium. Heating and cooling was repeated several times and experimental reproducibility was judged satisfactory.

The size of the average cooperative phase tran-

* The term motional freedom is used here to indicate membrane lipid fluidity in the general sense.

sition was obtained from the sharpness of the transition [15]. N, the number of molecules in the cooperative unit, is given by the expression:

$$N = 4RT_m^2 \frac{C_{ex}(\max)}{\Delta H_{cal}^2} \quad (2)$$

where $C_{ex}(\max)$ is the maximal value of the excess heat capacity, and ΔH_{cal} is the enthalpy obtained by integration of the transition curve.

Results

Fluorescence depolarization

Steady-state fluorescence polarization of dipalmitoylphosphatidylcholine/dolichol and DPPC/Dol-*P* vesicles containing the embedded lipid fluorophore diphenylhexatriene were determined at several temperatures, both below and above the T_c of DPPC (41°C). The anisotropy parameter varies directly with the rotational relaxation time of the fluorophore and inversely with the motional freedom or 'fluidity' of the membrane lipids. The effect of dolichol on DPPC bilayers is shown in Fig. 1. Below the T_c (at 20°C) the curves plateaued with $\bar{r}$ values of 0.284 for DPPC (curve A), 0.261 for DPPC/Dol (1000:1) (curve B), 0.253 for DPPC/Dol (100:1) (curve C) and 0.216 for DPPC/Dol (10:1) (curve D). This phenomenon can be explained in terms of a dolichol-induced increase in motional freedom of the bilayer, (decrease in the order parameter) or an increase in fluorescence lifetime [13]. At the T_c, increasing amount of dolichol broadened and lowered the transition temperature of DPPC (curves B–D). Similar effect to those found below the T_c were observed above the T_c. Dolichol, at all temperatures, fluidizes the membrane.

The effects of dolichyl phosphate on anisotropy is illustrated in Fig. 2. Below the T_c, the anisotropy values at 20°C were 0.284 for DPPC (curve A), 0.324 for DPPC/Dol-*P* (1000:1) (curve B), 0.312 for DPPC/Dol-*P* (100:1) (curve C) and 0.264 for DPPC/Dol-*P* (10:1) (curve D). At low concentrations of dolichyl phosphate, we observed an increase in $\bar{r}$ values which are correlated with a decrease in motional freedom of the lipid bilayer, however, at the highest concentration used (1:10), this value dropped below the control value. Furthermore, dolichyl phosphate lowered and broadened the T_c, as observed with dolichol. Above the T_c, we observed that increasing the amount of dolichyl phosphate results in a decrease of membrane motional freedom, as shown by an increase in $\bar{r}$ values. This effect could be due to an increase in disaggregation of dolichyl phosphate, at high temperature [18], in regions in the bilayer.

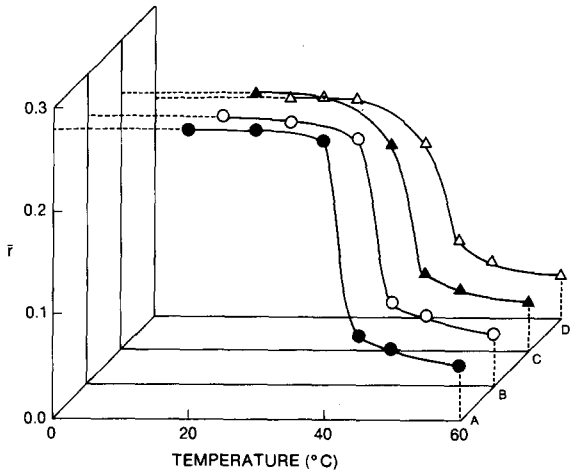

Fig. 1. Temperature dependence of the steady-state fluorescence anisotropy parameter, $\bar{r}$, of diphenylhexatriene embedded in DPPC (A) and DPPC/Dol at molar ratios of 1000:1 (B), 100:1 (C) and 10:1 (D).

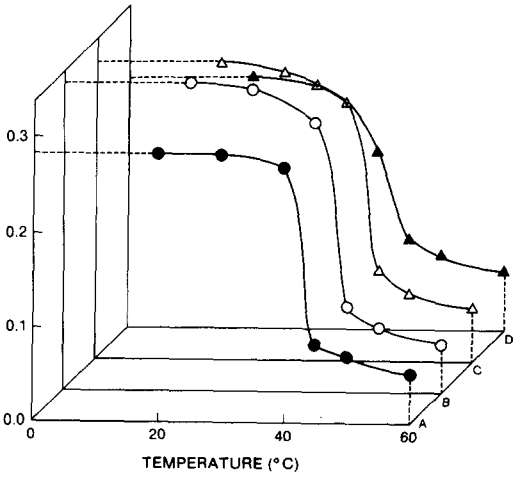

Fig. 2. Temperature dependence of the steady-state fluorescence anisotropy parameter, $\bar{r}$, of diphenylhexatriene embedded in DPPC (A) and DPPC/Dol-*P* at molar ratios of 1000:1 (B), 100:1 (C) and 10:1 (D).

Differential scanning calorimetry

Differential scanning calorimetry was performed with fully hydrated DPPC mixtures containing various concentrations of dolichol or dolichyl phosphate. Fig. 3 shows the effect of increasing concentrations of dolichol on the DPPC phase transition. Dolichol broadens and lowers the temperature of the main phase transition of DPPC. As the dolichol concentration was increased, the pretransition peak, which is believed to be associated with the appearance of a two-dimensional monoclinic lattice consisting of lipid lamellae distorted by a periodic ripple [14], was broadened and lowered. The enthalpy of the transition of DPPC (8.6 kcal/mol) was not affected by the presence of dolichol at the concentrations studied. The effect of dolichol on the DPPC phase transition as analyzed by DSC is consistent with the data obtained in the fluorescence depolarization experiments.

The effect of dolichyl phosphate on the DPPC phase transition is shown in Fig. 4. As observed for dolichol, dolichyl phosphate exhibited a broadening and lowering effect on the phase transition. The pretransition peak was absent at high concentrations of dolichyl phosphate. The enthalpy of the transition was not affected at the concentrations of dolichyl phosphate used in these experiments.

The number of DPPC molecules undergoing a cooperative phase transition was calculated from

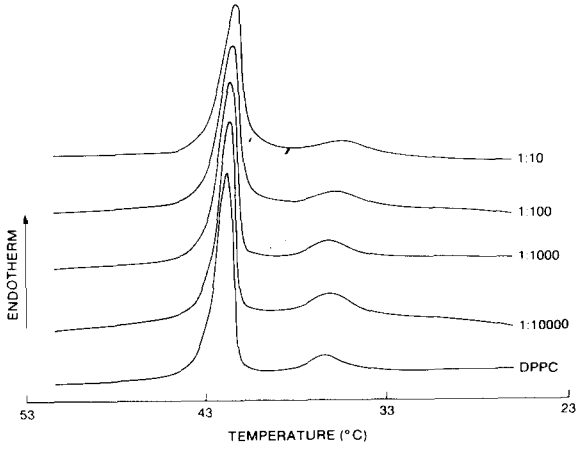

Fig. 3. The DSC calorimetric heating curves for DPPC and Dol/DPPC systems. Molar lipid/dolichol ratios are indicated on the curves. Heating rate, 1.5 K/min.

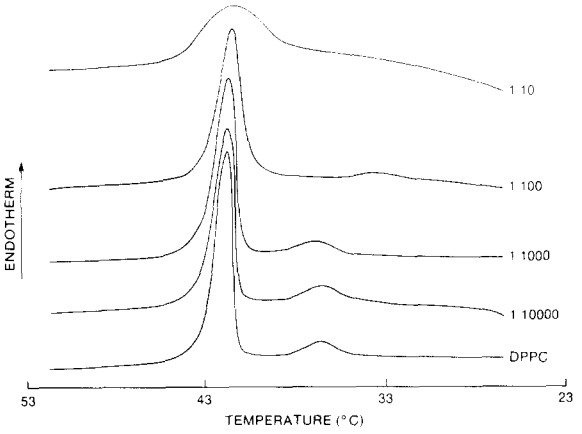

Fig. 4. The DSC calorimetric heating curves for DPPC and Dol-*P*/DPPC systems. Molar lipid/dolichyl phosphate ratios are indicated on the curves. Heating rate, 1.5 K/min.

Eqn. 2 [15]. The number (N) of DPPC molecules which undergo a cooperative phase transition was calculated to be 68. N was reduced to 64 for DPPC/Dol (10000:1), $N = 58$ for DPPC/Dol (1000:1), $N = 51$ for DPPC/Dol (100:1) and $N = 48$ for DPPC/Dol (10:1). The trend demonstrates that the presence of dolichol reduces the number of molecules undergoing a cooperative phase transition. This number was even further decreased in the presence of dolichyl phosphate. DPPC/Dol-*P* (10000:1), $N = 57$; DPPC/Dol-*P* (1000:1) $N = 57$; DPPC/Dol-*P* (100:1), $N = 50$; DPPC/Dol-*P* (10:1), $N = 18$.

Discussion

A remarkable feature of dolichols is their length, approx. 100 Å when fully extended, consequently, dolichol might easily span the entire thickness (40–60 Å) of the bilayer. This property may endow them with unique physical properties which could be important to their biological functions.

Previous analyses of polyisoprenoids in liposomes used ESR techniques or fluorescent probes [16–19]. The results suggest that phosphorylated polyisoprenoids are localized in bilayers with the phosphate head groups at or near the surface of the bilayer. Neutral polyisoprenoids, however, are located in the interior of the bilayer, where they may undergo self-aggregation at molar ratios of polyisoprenoid/phospholipid of 1:200 or greater at temperatures above the T_c. Our stud-

ies, using fluorescence depolarization and differential scanning calorimetry complement and extend these observations.

The experimental results demonstrate that dolichol and dolichyl phosphate interact with the bilayer disrupting the chain packing which results in a lower and broader lipid phase transition and in a reduction of the size of the cooperative unit at the phase transition. Similar conclusions were reported in a study of undecaprenol in DPPC liposomes using fluorescence and ESR probes [16–19].

In the present studies, it is noteworthy that the fluorescence-depolarization curves obtained are in good agreement with those obtained from DSC over the entire range of concentrations of polyisoprenoids studied. This suggests that the fluorescent probe correctly senses the changes in the thermodynamic parameters of the lipid in its environment.

Despite the polyisoprenoid-induced changes in the DPPC phase transition, no significant effects were observed in the enthalpy of this transition at the concentrations used for these experiments. Similar effects at the T_c have been reported for ubiquinones [20], where significant effects on the enthalpy occurred only at a molar ratios of 2 : 1.

We should point out that the results of the steady-state polarization analysis reported here do not distinguish between limiting rotational diffusion (r_∞) of diphenylhexatriene and its rotational correlation time (Υ_c). These components are related by:

$$\bar{r} = r_\infty + (r_0 - r_\infty)\frac{\Upsilon_c}{\Upsilon_c + \Upsilon_F} \qquad (3)$$

where r_0 is the fundamental anisotropy and Υ_F the fluorescence lifetime. The limiting anisotropy, when not equal to zero, indicates that the distribution of orientations at equilibrium is anisotropic. Heyn et al. [26] have discussed the significance of both parameters with respect to lipid dynamics and steric effects and caution against over-interpretation of steady-state anisotropy measurements in the absence of decay studies. One such dynamic depolarization analysis [27] demonstrates that membrane additives have little effect on Υ_c, the value of $\bar{r}$ being predominantly influenced by changes in r_∞.

Consequently, our use of the term motional freedom should be taken to mean changes attributable to the order parameter, changes in fluorophore lifetime or a combination of both factors.

Dolichol was found to increase the overall motional freedom of the bilayer both below and above the T_c at all concentrations studied. McCloskey and Troy [18] noted the aggregation of neutral polyisoprenoids at molar ratios greater than 1 : 200 and above the T_c of the DPPC liposomes in which they were embedded. Our data are consistent with these findings but since we are not directly measuring dolichol-dolichol interactions, an exact interpretation of the results cannot be provided.

The polar head group of dolichol phosphate interacts with the DPPC polar head groups, thus conferring different physical properties from those found with dolichol. Below the T_c, dolichyl phosphate at a molar ratio of 1 : 1000 considerably increased the anisotropy of diphenylhexatriene, which suggests that dolichyl phosphate acts as an 'anchor', restricting the movement of DPPC molecules or making a more compact bilayer. As the molar ratio of dolichyl phosphate was increased to 100 : 1, this anchoring or rigidifying effect decreased and at a molar ratio of 1 : 10, the anisotropy was lower than that of control. This phenomena might be explained by segregation of dolichyl phosphate from the rest of the lipids forming aggregates in the bilayer. This phenomenon has been previously observed with glycolipids [21]. Our results suggest that as the concentration of dolichyl phosphate increases, larger aggregate areas are formed, thereby reducing the number of dolichyl phosphate molecules in contact with DPPC molecules. When these areas reach a certain size (as at a molar ratio of 1 : 10), clear separate domains of DPPC and dolichyl phosphate may occur, creating irregularities in the packing at the border of the domains where DPPC molecules can move more freely. The bilayer would become more 'fluid' and the anisotropy parameter would fall below control values. The increase in the number of dolichyl phosphate molecules also reduced the size of the cooperative unit in the phase change. A drastic effect was observed at molar ratio 10 : 1. Another possible explanation for this pronounced effect may include a conformation rearrangement of the

long isoprenoid chain of dolichyl phosphate.

Apparently, the effect of dolichyl phosphate in the bilayer is greater than that of dolichol, perhaps attributable to the interaction of the dolichyl phosphate polar head region with the bilayer, an association which does not occur with dolichol. At a DPPC/Dol-*P* ratio of 10:1, a drastic change in the number of molecules undergoing a cooperative phase transition is consistent with the pronounced changes observed both by DSC and microviscosimetry.

High levels of dolichol in biological systems have been reported by Rip et al. [22], who estimated 300 ng dolichol/mg protein in plasma membranes. In human testes, values as high as 300 mg/dolichol per g testes have been reported [23], perhaps reflecting the importance of dolichol not only in modifying membrane physical properties, but also in modulating dolichol kinase enzyme activity and other membrane-bound enzymes.

Lower amounts of these isoprenoids have been reported in rat liver subcellar fractions, 70 ng/mg protein in the nuclear fraction and 338 ng/ml in mitochondrial and lysosomal preparations [24]. Dolichyl phosphate levels in these membranes are 1/20 of that of dolichol [25]. It is doubtful that these isoprenoids play an important role as modulators of the physical properties of such subcellular membrane, unless they are concentrated on specific regions where protein glycosylation is more active. In that case they could also modulate specific membrane-bound enzymes such dolichol kinase and the physical properties of the membrane.

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