Biochimica et Biophysica Acta, 944 (1988) 465–472
Elsevier

BBA 74165

The effect of nonadec(en)ylresorcinol on the fluidity of liposome and erythrocyte membranes

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(Received 22 March 1988)
(Revised manuscript received 4 July 1988)

Key words: Resorcinolic lipid; Cereal grain; ESR; Membrane fluidity; (Erythrocyte membrane)

The effect of alk(en)ylresorcinol homologs (5-(*n*-nonadecyl)- and 5-(*n*-nonadecenyl)resorcinol) on the mobility of 5-doxyl- and 12-doxylstearate spin probes incorporated into DMPC, DMPC-cholesterol and erythrocyte membranes was studied. It was found that both homologs affect the properties of hydrophobic environment of the membranes: (1) In DMPC vesicles both homologs induce an increase in the order parameter of 5-doxylstearate at temperatures of T_c and above. (2) At higher concentrations of both homologs a decrease in mobility of the 12-doxylstearate was also observed. (3) In the presence of cholesterol in the liposome membrane the influence of alk(en)ylresorcinols on the mobility of spin probes was much greater, depending on the cholesterol content and the position of the probe in the bilayer. (4) In natural membranes (erythrocyte ghosts) both alkyl- and alkenylresorcinols induced a decrease of mobility in the region of 12-doxylstearate as well as in the region closer to the polar head groups of lipids (5-doxylstearate).

Introduction

The long-chain (13–27 carbon atoms) homologs of orcinol occurring in cereal grains are amphiphilic compounds of unknown biological role. Wieringa [1] and recently Sedlet et al. [2] showed that these compounds may play an important role in the antinutritive properties of rye grains. Among the cereals, rye was found to be the richest in these compounds [4,5]. On the other hand, recent results show remarkable antitumor activity of alkenylresorcinols in mice [3]. During the course of our studies on these resorcinolic lipids isolated from rye grains, their various activities in modulating the properties of biological membranes were shown [6]. Both natural and liposomal membranes showed enhanced permeability for water and small non-electrolytes upon interaction with alk(en)ylresorcinols [7,8]. Unsaturated aliphatic-chain homologs consisting of 15–19 carbon atoms appeared to be the most active ones [9]. These homologs at micromolar concentrations showed a strong hemolytic effect [10,11] on erythrocytes. Recent results indicate that also the properties of membrane proteins are altered upon direct interaction with resorcinolic lipids [12].

In this paper the study of the effect of two pure homologs, 5-(*n*-nonadecyl)- and 5-(*n*-nonadecenyl)resorcinols, on the mobility of fatty acid spin probes in both liposome and erythrocyte membranes is presented. The ordering effect of these

Abbreviations: DMPC, dimyristoylphosphatidylcholine; 19:0 AR, 5-(*n*-nonadecyl)resorcinol; 19:1 AR, 5-(*n*-nonadecenyl)resorcinol; 5-doxylstearate, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidimyloxyl; 12-doxylstearate, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidimyloxyl.

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natural amphiphilic compounds on cholesterol-free and cholesterol-containing membranes is described. Special attention is paid to the effect of these compounds at low (0–13%) concentrations in the membrane.

Materials and Methods

19:0 AR and 19:1 AR were isolated from rye grains as previously described [13]. For the experiments ethanolic solutions of 5 mM were used. DMPC (Avanti) and cholesterol (Merck) were used without further purification as chloroform solutions. Erythrocyte membranes were prepared from recently outdated human blood (B_1 Rh^+) as described by Dodge et al. [14]. The alk(en)ylresorcinols were determined colorimetrically with the use of Fast Blue B [15] and protein with the use of the Lowry method [16]. Spin-labelled stearic acids (5-doxylstearate and 12-doxylstearate) from Syva (Palo Alto, CA, U.S.A.) were used as ethanolic solutions.

The liposome membranes used in this work were multilamellar dispersions of lipid (MLV) prepared in the following way: a mixture of lipid ($5 \cdot 10^{-7}$ mol), spin label ($5 \cdot 10^{-9}$ mol) with or without an appropriate amount of cholesterol, and resorcinolic lipid ($5 \cdot 10^{-9}$–$8 \cdot 10^{-8}$ mol) in chloroform was dried under a steam of nitrogen and further dried under reduced pressure (approx. 0.1 mmHg) for at least 12 h. A 40-μl aliquot of buffer (70 mM phosphate buffer (pH 7.2), 100 mM KCl, 1 mM EDTA) was added to the dried lipid film at about 20°C above the phase transition temperature of the phospholipid and the suspensions were vortexed vigorously. Obtained liposomes were transferred to 50 μl capillary micropipettes (Corning) and sealed.

Erythrocyte membranes (200 μl containing 0.6 mg of protein) were incubated at 37°C or 1 h in 2 ml of 70 mM phosphate buffer (pH 7.2), 100 mM KCl, 1 mM EDTA containing appropriate amounts of resorcinolic lipid and spin label. Prior to incubation an appropriate amount of ethanolic solution of spin label was evaporated. The final concentration of the spin labels in the samples was about $1.25 \cdot 10^{-5}$ M. After the incubation was completed the membranes were centrifuged and the pellet was sealed in capillaries. The concentrations of resorcinolic lipids in the membrane were determined in the extract of the membranes incubated first with alk(en)ylresorcinols in the same way as the samples used for ESR measurements but without spin probe. Control experiments showed that membrane lipids do not interfere with the determination of resorcinolic derivatives with Fast Blue B.

ESR spectra were made in a Radiopan spectrometer equipped with a temperature-controlling device. Temperature fluctuations during the measurements were less than 0.5 C°, this being controlled with an electronic thermometer. The field sweep was 100 G, modulation amplitude 0.2 G, time constant 0.1 s and scan time 8 min. The microwave power was kept at 40 mW and the microwave frequency measured by a JES-SH unit was 9.25 GHz. The order parameters (S) for 5-doxylstearate were calculated according to Sefton and Gaffney [17]. For 12-doxylstearate the relative correlation times (R_i) were calculated according to Ref. 18.

Results

Two spin-labelled fatty acid derivatives were used for the estimation of the mobility of hydrocarbon chains in a phospholipid bilayer. The spectra of 12-doxylstearate in liposomes and erythrocyte ghosts were characteristic of a hydrophobic core of the bilayer rather than a large degree of motional freedom so the relative correlation time ($R_i = W_{+1}\sqrt{(h+1)/(h-1)}$) was used to test the influence of resorcinolic derivatives on the membrane. As the spectra of 5-doxylstearate displayed a rather high extent of anisotropy the order parameter was used to characterize the membranes labelled with this probe [17].

The effects of increasing molar fractions of 19:0 AR and 19:1 AR in membranes on the R_i values for 12-doxylstearate in DMPC liposomes at 37°C are shown in Fig. 1A. An increase of the value of relative correlation time is observed for both homologs, indicating that the motional freedom of the probe in the membrane is restricted. Although both homologs at higher molar fractions induced an increase of R_i values their effect at low concentrations in the membrane was differ-

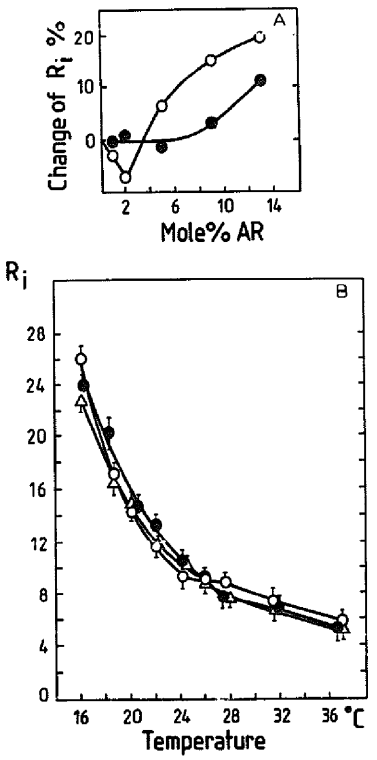

Fig. 1. Influence of alk(en)ylresorcinols on the empirical (R_i) parameter for 12-doxylstearate-labelled DMPC liposomes. (A) Dependence on alk(en)ylresorcinol concentration at 37 °C; (B) dependence on temperature at an alk(en)ylresorcinol concentration of 4 mol%. △, DMPC liposomes; ●, 19:1 AR and ○, 19:0 AR containing DMPC. Data points are means of three experiments; bars represent standard deviations.

ent. For the saturated homolog a slight decrease of the R_i values was first observed followed by a significant concentration-dependent increase of this parameter. The unsaturated homolog did not exhibit a marked effect up to 8 mol%; above this concentration a significant effect, although lower than in case of the saturated homolog, was observed (Fig. 1A).

The sensitivity of the R_i parameter to temperature is rather high. For DMPC below T_c, the R_i value was about 23–28 while for liquid crystalline phase this value was below 10. The effect of 4.8 mol% of resorcinolic lipids on the temperature-dependent motion of spin label in liposome membrane is shown in Fig. 1B. An increase of R_i values was observed for both homologs mainly at temperatures below the transition temperature of DMPC. Above the transition temperature the increase of R_i values was less marked and not significant for either homolog. At higher molar fractions the effect of resorcinolic lipids was accentuated (data not shown).

To determine whether the motion of the spin probes in the region closer to the polar head groups of the membrane phospholipids is also affected by resorcinolic lipids, we incorporated 5-doxylstearate into DMPC liposomes. Both alk(en)ylresorcinols studied induced a concentration-dependent increase of the order parameter of the probe (Fig. 2A). The increase of S was observed at concentrations as low as 1 mol% of 19:1 AR in the membrane. The incorporation of 19:0 AR into the membrane at levels lower than 3 mol% remained practically without effect on the order parameter. At molar fractions above 4 mol% both homologs restricted the motion of the spin label as reflected by the increase (8–12%) of the order parameter. As shown for 12-doxylstearate, 19:0 AR appeared to be the more effective homolog.

The effect of temperature on the order parameter of the DMPC liposomal membranes labelled with 5-doxylstearate containing 4.8 mol% of the studied compounds is presented in Fig. 2B. First of all the shape of the curve of the relationship between the order parameter and temperature was significantly altered in the presence of resorcinolic lipids in the membrane. The increase of the order parameter is lower at temperatures below the transition temperature of the phospholipid than at the transition temperature and above it. Although both resorcinolic lipids exhibited a similar effect the unsaturated one was slightly more effective at the region of the transition temperature.

The results presented above indicate that the studied compounds affect the mobility of both the spin probe located at the middle of the bilayer and the probe located at the region closer to the phospholipid polar head groups. The effect observed with the use of 5-doxylstearate is more pronounced, especially at and above the transition temperature of membrane phospholipid. The changes in order parameter induced by the studied

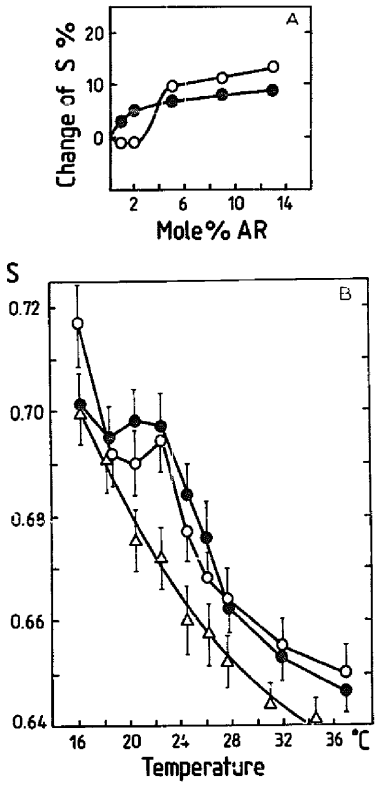

Fig. 2. Influence of alk(en)ylresorcinols on the order parameter (S) for 5-doxylstearate incorporated into DMPC liposomes. (A) Alk(en)ylresorcinol concentration dependence at 37°C; (B) temperature dependence at an alk(en)ylresorcinol concentration of 4 mol%. △, DMPC liposomes; ●, 19:1 AR and ○, 19:0 AR containing liposomes. Data points are means of three experiments, bars represent standard deviations.

amphiphiles were as high as about 3.6% and were greatest at 22–24°C but smallest at close to 18–19°C. The differences between the effects of saturated and unsaturated homologs were not very large, reaching about 0.4% which was statistically not significant.

Natural membranes are known to contain substantial amounts of cholesterol. This compound is well known to affect the motion of hydrocarbon chains in phospholipid bilayers which results in the disappearance of phase transition (e.g., Ref. 19). Experiments presented below concerned the influence of resorcinolic lipids on the fluidity of the DMPC liposomal membranes containing two different but low concentrations of cholesterol. The spectra obtained at 37°C for DMPC-cholesterol membranes in the presence of 12- and 5-doxylstearate indicated the stabilizing effect of resorcinolic lipids on the mobility of both probes.

The resorcinolic lipid concentration dependences of the changes in the values of S for 5-doxylstearate and R_i for 12-doxylstearate are shown in Fig. 3 (A and B, respectively). The most significant effect of alk(en)ylresorcinols was observed for 12-doxylstearate (Fig. 3B). Both homologs when incorporated into phospholipid–5 mol% cholesterol membrane at 9 mol% increased the values of R_i by about 35%. A further increase in the molar fraction of the studied compounds resulted in a slight reduction of the observed effect but nevertheless the values of R_i were still significantly higher than in control membranes. When the effect of resorcinolic lipids on the phospholipid–5 mol% cholesterol membranes was studied with the use of 5-doxylstearate (Fig. 3A), for 19:1

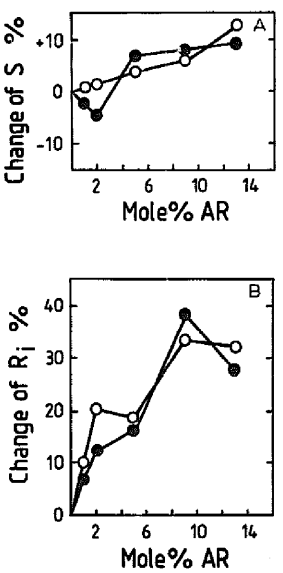

Fig. 3. Alk(en)ylresorcinol concentration dependence of changes of S and R_i for liposomes prepared from a DMPC-cholesterol mixture (95:5) labelled with (A) 5-doxylstearate and (B) 12-doxylstearate at 37°C. ○, 19:0 AR and ●, 19:1 AR containing DMPC-cholesterol liposomes. Data points are means of three experiments.

AR up to about 2 mol% a reduction of the order parameter value of 5% was observed, whereas at higher molar fractions an increase (9%) was observed. Increasing concentrations of 19:0 AR resulted in a continuous increase of the order parameter for 5-doxylstearate. A decrease in mobility of 12-doxylstearate was also observed due to the presence of both homologs in the DMPC-cholesterol membranes (Fig. 3B).

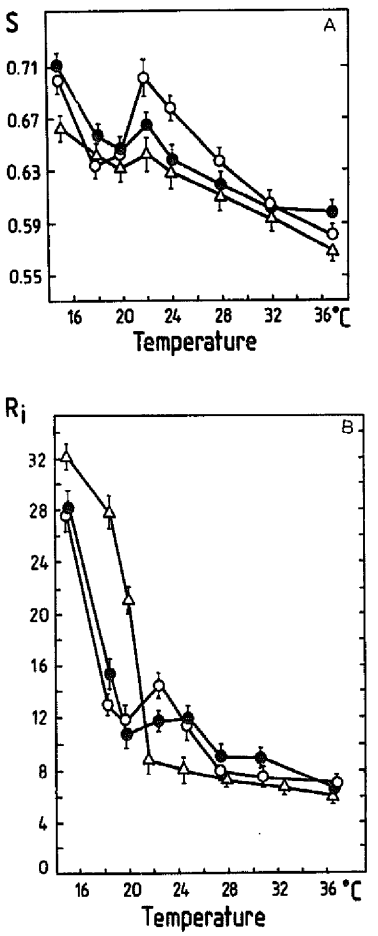

Fig. 4. Temperature dependence of R_i and S for liposomes prepared from a DMPC-cholesterol (95:5) mixture labelled with (A) 5-doxylstearate and (B) 12-doxylstearate. ○, 19:0 AR and ●, 19:1 AR containing DMPC-cholesterol liposomes; △, DMPC-cholesterol liposomes. Data points and bars represent means ± S.D. of three experiments.

The temperature dependence of the effect of 4.8 mol% of resorcinolic lipids on the R_i and S parameters describing the motional freedom of the two probes used are shown in Fig. 4. The effect of alk(en)ylresorcinols on phospholipid–5 mol% cholesterol membrane followed with the use of 5-doxylstearate (Fig. 4A) is most significant between 21°C and 28°C and below 18°C. Between 21°C and 18°C the effect of the saturated homolog is significantly higher than that of the unsaturated one. The motion of 12-doxylstearate in phospholipid–5 mol% cholesterol membrane is significantly restricted at the transition temperature and below it (Fig. 4B) in comparison to the mobility of probe in the membranes comprised only of phospholipid (Fig. 1B). Incorporation of 4.8 mol% of resorcinolic lipids into phospholipid–5 mol% cholesterol membranes results in a significant change of the R_i values in relation to temperature. This effect however, is more pronounced below the transition temperature. In the presence of resorcinolic lipids a significant decrease of R_i values below 20°C was observed whereas at the transition temperature and above it an increase of R_i was detected. This increase of R_i values, however, was more pronounced at the transition temperature.

When 13 mol% of cholesterol was incorporated into DMPC membrane, an ordering effect of resorcinolic lipids on the motion of 12-doxylstearate and 5-doxylstearate was also observed (Fig. 5). Increasing the mole fractions of both homologs above 2 mol% in the membrane induced an increase of the order parameter for 5-doxylstearate of about 15% (Fig. 5A). Resorcinolic lipids, when studied with the use of 12-doxylstearate, also showed a rigidifying effect as indicated by an increase of R_i values (Fig. 5B). Up to 2 mol% of 19:0 AR a minute increase (by 1%) of the R_i values was observed. A further increase in the concentration of this lipid in the membrane resulted in a significant increase (by 20%) of the R_i values. When 19:1 AR was incorporated an increase of motional restriction of the probe was observed at concentrations as low as 1 mol% of the compound in the membrane. The maximal effect of this homolog was observed at 9 mol% of the compound in the membrane, at which the R_i parameter value was more than 22% greater than

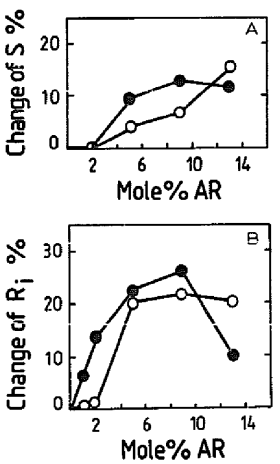

Fig. 5. Alk(en)ylresorcinol concentration dependence of S and R_i of liposomes prepared from a DMPC-cholesterol (87:13) mixture labelled with (A) 5-doxylstearate and (B) 12-doxylstearate at 37°C. ○, 19:0 AR and ●, 19:1 AR containing DMPC-cholesterol liposomes. Data points are means of three experiments.

that observed for a DMPC–13 mol% cholesterol membrane. A similar, though smaller effect could be observed in the case of the order parameter measured using 5-doxylstearate (Fig. 5A).

In order to test whether the observed changes can be detected also in natural membranes, the effect of both alk(en)ylresorcinol homologs on erythrocyte membrane was examined. A high cholesterol content, a wide range of polar lipid species and the presence of membrane proteins make the effect of alk(en)ylresorcinols on the fluidity of this complex membrane interesting.

The data presented in Table I indicate that alk(en)ylresorcinols at low concentrations in the membrane (2.5–14%) also affect markedly the mobility of the spin probes in the membrane phospholipid bilayer. Similarly to the effects shown for liposomal membranes, alk(en)ylresorcinols incorporated into erythrocyte ghost membrane induced an increase in both R_i and S values, indicating decreased mobility of the probes in the hydrophobic core region as well as in the region closer to the membrane surface.

The changes of R_i and S in alk(en)ylresorcinol-modified erythrocyte ghost membranes were more pronounced for 12-doxylstearate than for 5-doxylstearate. At above 5% of 19:0 AR and 10% of 19:0 AR in the membrane increases of the R_i values of over 30% were observed. The increase of the order parameter was lower and showed considerable scatter, indicating the possibility of differential interaction of resorcinolic lipids with various membrane components.

Discussion

Our previous results from calorimetric studies [20] on mixtures of phosphatidylcholines and alk(en)ylresorcinols suggested localization of alk(en)ylresorcinol molecules in a bilayer similar to those proposed for other amphiphilic mole-

TABLE I

INFLUENCE OF THE INCREASING CONCENTRATIONS OF 5-(*n*-NONADEC(EN)YL)RESORCINOL ON THE S AND R_i PARAMETERS OF 5-DOXYL- AND 12-DOXYLSTEARATES INCORPORATED INTO THE ERYTHROCYTE MEMBRANE

Spectra were taken at 37°C. The concentration of resorcinolic lipids in the membrane was calculated based on protein content and the assumption of a 50:50 ratio of membrane lipid to protein (w/w). The values represent means ± S.D. from three experiments. Asterix indicates statistically significant differences ($P < 0.05$) as evaluated with the use of a t-test.

Concentration of resorcinolic lipid in the membrane (%)	R_i		S	
	19:0 AR	19:1 AR	19:0 AR	19:1 AR
0	16.96 ± 0.9	16.96 ± 0.9	0.703 ± 0.010	0.703 ± 0.010
2.5	16.00 ± 1.1	17.65 ± 1.6	0.726 ± 0.006 *	0.699 ± 0.009
5.2	23.10 ± 1.4 *	17.30 ± 1.3	0.723 ± 0.007 *	0.732 ± 0.011 *
10.2	20.68 ± 1.1 *	20.83 ± 1.2 *	0.712 ± 0.010	0.766 ± 0.008 *
14.0	22.28 ± 0.8 *	22.98 ± 1.1 *	0.728 ± 0.011 *	0.716 ± 0.010

cules, such as *n*-alkanols, fatty acids or nonionic surfactants [21], namely in the vicinity of the C-1 to C-9 carbons of phospholipid acyl chains. However, addition of alk(en)ylresorcinols to phosphatidylcholine bilayers resulted in a certain degree of immiscibility between resorcinolic lipids and phospholipid [20].

The present results indicate that in a pure phospholipid bilayer the presence of alk(en)ylresorcinols affect the motion of the probes both at the C-5 region and at the C-12 region of the acyl chains. It should be realized, however, that using the ESR methodology, discrimination between real changes in phospholipid bilayer fluidity and the effect of an additive on the spin-labelled probe properties within the bilayer is rather difficult. The results obtained should be considered more adequately as the effect of the studied amphiphiles on the perturbation of the local environment of the probes. Nevertheless the results may be recognized as an indication of changes appearing in the bilayer environment of the probe upon incorporation of the resorcinolic lipids into the phospholipid–spin-labelled probe membranes. The presence of a flat dihydroxybenzoic ring in a resorcinolic lipid molecule would serve to explain the effect observed at the C-5 region for both saturated and unsaturated homologs. The incorporation into the membranes of such a structure with two free OH groups may result in direct interactions (e.g., formation of hydrogen bonds) between alk(en)ylresorcinol and adjacent phospholipid molecules. In consequence, a restriction of molecular mobility and membrane stabilization would occur. Recent studies indicate that the membrane-stabilizing effect of α-tocopherol, a compound in which a flat OH-bearing ring also appears, is due to the formation of hydrogen bonds between phospholipids and the vitamin E OH group [22,23]. The use of DMPC bilayers in which C_{19} resorcinolic lipids were incorporated may have introduced an additional complication. It should be noted that a similar situation would arise during the interaction of cell membrane with a naturally occurring mixture of alk(en)ylresorcinols in which the average chain length is well above 19 carbon atoms. When the phenolic head of alk(en)ylresorcinol is located at the C-1 to C-9 region of the phospholipid acyl chain then the tail would affect also the acyl chain order in the adjacent leaflet of the bilayer (Fig. 6A). The decrease in fluidity in the C-5 region is rather inconsistent with the arrangement presented in Fig. 6A. The packing presented in Fig. 6B could explain the ordering effect of the studied compounds in the DMPC membranes. Such an arrangement would affect the bilayer thickness and would induce formation of resorcinol-rich and resorcinol-poor domains.

When cholesterol was present in bilayers an enhancement of the resorcinolic lipid effect was observed, suggesting also a possible role of the interactions between cholesterol and the phenolic ring of the resorcinolic lipid molecules in their effect on the membrane. Therefore the observed effects will be stronger than in a cholesterol-free bilayer. The significance of cholesterol for the effect of resorcinolic lipids is especially evident at a low molar fraction of alk(en)ylresorcinols in the membrane and with 12-doxylstearate as a probe (see, e.g., Fig. 1B and Fig. 4B). Since resorcinolic lipids have high octanol/water partition coefficients [6] an association between these molecules and biological membranes will be biologically significant. Previous study evidenced a remarkable increase in membrane permeability for various solutes upon interaction with different alk(en)ylresorcinols [6–8]. It was also shown that alk(en)ylresorcinols were able to destabilize bilayer structure and induced the hexagonal and isotropic phases [20].

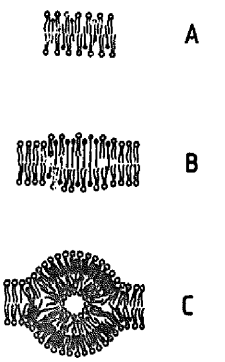

Fig. 6. Possible localization and the effect of resorcinolic lipids on the molecular packing in the phospholipid bilayer. For details see the text (Discussion).

A possible relationship between the phenomena observed in alk(en)ylresorcinol–membrane interaction may be considered as presented in Fig. 6B and C. The incorporation of an amphiphile in the membrane will lead to the formation of alk(en)ylresorcinol-rich domains which are of greater thickness and packing than the surrounding pure phospholipid domains (Fig. 6B). In such domains the nonlamellar phase structures (hexagonal, micellar) would be formed upon rearrangement of molecules (Fig. 6C).

Cholesterol in phosphatidylethanolamine-phosphatidylcholine systems, which can be considered as models of natural membrane, is also known to destabilize bilayer structure [24,25], therefore the strong effect of alk(en)ylresorcinols upon natural membrane may result from a specific synergistic action of cholesterol and the studied amphiphiles.

The intriguing observation is the difference between the effect of resorcinolic lipids and other natural amphiphiles such as fatty acids. Fatty acids induce membranous effects similar to those observed for alk(en)ylresorcinols [26,27]. However the effect of fatty acids on the mobility of phospholipid acyl chains is opposite to that observed for alk(en)ylresorcinols [21,24,28]. On the other hand, fatty acids also induce H_{II} phase structure in erythrocyte membranes [29].

The similar actions of these different compounds (cholesterol, fatty acid, resorcinolic lipid) exhibiting different effects on the motional freedom of phospholipid acyl chains in the bilayer would suggest a difference in importance of the perturbation of molecular structure of the membrane and the acyl chain mobility observed in biological events induced by amphiphiles.

Acknowledgement

This work was supported by grant No. RP II.11.4.9.

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