Thermal behavior of liposomes containing PCs with long and very long chain PUFAs isolated from retinal rod outer segment membranes

Silvia S. Antollini[1] and Marta I. Aveldaño

Instituto de Investigaciones Bioquímicas de Bahía Blanca, Universidad Nacional del Sur-Consejo Nacional de Investigaciones Científicas y Técnicas, Bahía Blanca, Argentina

Abstract **About one-fourth the phosphatidylcholines (PC) from retina photoreceptor rod outer segment (ROS) membranes contain docosahexaenoic acid (22:6n-3) at *sn*-2 and a very long chain polyunsaturated fatty acid (VLCPUFA) (C24 to C36) at the *sn*-1 position of the glycerol backbone. In order to study the thermotropic behavior of these PCs, subfractions and molecular species of PC (16:0/22:6, 18:0/22:6, 22:6/22:6, 32:5/22:6, 32:6/22:6, 34:5/22:6), were isolated from bovine ROS, and liposomes containing different proportions of these PCs and dimyristoyl-PC (DMPC) or dipalmitoyl PC (DPPC) were compared using the fluorescence probes Laurdan and 1,6-diphenyl-1,3,5-hexatriene (DPH). With both probes, the 22:6n-3 containing PCs from ROS, in all proportions tested, decreased the transition temperature (Tt) of both DMPC and DPPC. Below the transition temperature, coexistence of phases was evidenced in all cases. Liposomes formed with 100% of any of these PCs did not show phase transitions in the temperature range studied (8°C to 50°C). At physiological temperatures, as it is likely to be the case in ROS membranes, all of these PC species were in the liquid-crystalline state. With Laurdan, all dipolyunsaturated PCs seemed to behave similarly: despite the large number of double bonds per molecule, all of them decreased the Tt of DPPC less than did the hexaenoic PCs. With DPH, an ample difference was detected between the dipolyunsaturates, 22:6/22:6-PC and VLCPUFA/22:6-PCs, and between the latter and hexaenoic PCs throughout the temperature range studied. This difference is consistent with the interpretation that the largest "disorder" produced by PCs containing a VLCPUFA like 32:6n-3 at the *sn*-1 position occurs toward the center of the membrane.**—Antollini, S. S., and M. I. Aveldaño. **Thermal behavior of liposomes containing phosphatidylcholines with long and very long chain PUFAs isolated from retinal rod outer segment membranes. *J. Lipid Res.* 2002.** 43: **1440–1449.**

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Most of the lipid classes of the disk membranes that are tightly packed into the outer segments of bovine retinal rods contain docosahexaenoic acid (22:6n-3). About one-fourth of the molecular species of such phospholipids, which closely interact with rhodopsin, are "dipolyunsaturated" (1) or "supraenoic" (2), named that way to reflect the fact that they have PUFAs at both the *sn*-1 and the *sn*-2 positions of the glycerol backbone. Further studies showed that a considerable proportion of the dipolyunsaturated molecular species of the lipids present in rod outer segment (ROS) membranes contain 22:6n-3 as one of the acyl chains, the other one being a PUFA of the n-3 or the n-6 series with long (C20, C22) or with very long chains (C24 to C36) (3, 4). Whereas all bovine ROS phospholipid classes contain dipolyunsaturated molecular species (2), the fatty acid distribution among them differs (5). Phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine dipolyunsaturated species contain 20, 22, or 24 carbon PUFA, whereas phosphatidylcholine (PC), besides these, is made up of significant proportions of dipolyunsaturated species that contain PUFA with up to 36 carbon atoms [very long chain (VLC)PUFA] (3–5). These PCs are thus unique in that they display not only the highest degree of unsaturation but also the largest total number of carbon atoms yet reported for mammalian glycerophospholipids. Except for didocosahexaenoyl-PC, in most dipolyunsaturated PCs from ROS, having two

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; GC, gas chromatography; GP, generalized polarization; HPLC, high pressure liquid chromatography; PC, phosphatidylcholine; ROS, rod outer segments; VLCPUFA, PUFA with 24 to 36 carbon atoms. The fatty acids are designed by the convention, number of carbon atoms: number of double bonds. The notation n-3 or n-6 refer to the position of the first double bond counting from the methyl end.

[1] To whom correspondence should be addressed.
e-mail: silviant@criba.edu.ar

PUFA of different length per molecule, 22:6n-3 tends to be located at the *sn*-2 position of the glycerol backbone, while the VLCPUFA tend to locate predominantly at the *sn*-1 position (5). Thus, at least in this positional "preference", the VLCPUFA containing species of ROS PC resemble the more ubiquitous and widely known "hexaenoic" species of PC, like 16:0/22:6-PC or 18:0/22:6-PC, in which the saturated fatty acid and 22:6n-3 are generally found at *sn*-1 and at s*n*-2, respectively.

The fact that the same kind of VLCPUFA-containing molecular species occur in a glycerophospholipid specifically located in the visual cells of animal species so distant in evolution as fish, birds, and various mammals suggested that such molecules probably fulfill an important requirement for the function of photoreceptor membrane proteins including rhodopsin (3). Docosahexaenoate-containing lipids may be expected to provide a fluid bilayer, but at physiological temperatures this condition could be fulfilled with much simpler lipids, such as oleic or linoleic acid containing species. In fact, reconstitution experiments have indicated that fluidity of the lipid bilayer is a necessary, though not sufficient, condition for rhodopsin functionality. Thus, the rates of rhodopsin photochemical transformations are reduced when the protein is reconstituted in liposomes of dimyristoyl-PC, even at temperatures above the Transition midpoint temperature (Tm) (6, 7), perhaps because these PCs have "too short" acyl chains compared with those of the lipids present in the native membrane. The equilibrium concentration of metharhodopsin II formed after rhodopsin photolysis is improved in the presence of phospholipids with one or more 22:6 acyl chains (8, 9). Moreover, phospholipids with 22:6 acyl chains are required for the optimal kinetic functioning of meta II – transducin coupling (10–12). No studies have yet been designed to investigate the possible function of PCs with VLCPUFA in photoreceptor membranes. These species of PC are not the major ones, but are not negligible either: as a group, they amount to about one-third of all the PCs present in bovine ROS (5).

With the current knowledge about the organization of lipids in naturally occurring membranes, one of the most intriguing yet still unanswered questions that arises is how is it possible that phospholipid species like the described PCs (e.g., 32:6/22:6-PC), having two acyl chains at the same time so highly unsaturated and so uneven in length, accommodate in the thickness of the photoreceptor membrane. Another question that has not yet been addressed, and that we tried to approach in the present paper, is what are the physical properties of these peculiar PCs, and how they differ, if they do, from the more abundant - and much more ubiquitous - species also having 22:6n-3 at *sn*-2 but a saturate at *sn*-1. Whereas the introduction of a single *cis* double bond into a saturated acyl chain is known to result in a large decrease in molecular order in the liquid crystalline phase, the effects of higher levels of unsaturation are not as widely agreed upon, and appear to vary, depending on the specific location of the double bonds, on the location of the first double bond counting from the carboxyl ester end of the fatty acid, and on whether one or both phospholipid acyl chains contain unsaturations (13). In PCs containing two equal polyenoic chains (18:2/18:2-PC and 20:4/20:4-PC), it has been shown that double bonds in excess of two per fatty acid chain do not substantially change the transition temperature (14), but this type of measurements has not been done in dipolyunsaturated species in which one of the chains is much longer than the other. Thus, one cannot extrapolate from what is currently known what could be the behavior of dipolyunsaturated species of ROS PC, more heterogeneous as a group than previously considered.

With the above questions in mind, we explored the physical characteristics of some of the molecules isolated from subfractions of ROS PC, and the effects they produce when added to liposomes made of dimiristoyl- or dipalmitoyl-PC (DMPC, DPPC), whose thermotropic behavior is very well characterized. We have used the amphiphilic fluorescent probes Laurdan (6-dodecanoyl-2-(−dimethylamino) naphthalene) (15), and 1,6-diphenyl-1,3,6-hexatriene (DPH). Laurdan is considered to have a uniform lateral and transbilayer distribution, thus making it a good reporter molecule to sense molecular dynamics of solvent dipoles in the membrane as a whole (16). The main dipoles sensed by Laurdan in membranes are water molecules. Differences in water content in the hydrophilic/hydrophobic interface region of the membrane correlate with differences in solvent dipolar relaxation, and thus indirectly correlate with variations in membrane lipid fluidity (17). DPH has been successfully used to study lipid bilayer structure over the past three decades (18, 19). Fluorescence polarization measurements provide a measure of the rotation diffusion of fluorophores. The rate of rotation of this probe is thought to reflect the viscous hindrance imposed by its immediate environment (20). Using these tools, in this paper we describe the thermal behavior of liposomes formed with some of the 22:6n-3 containing PCs of ROS membranes, and explore how the presence of these PCs affects the thermotropic behavior of liposomes of DPPC. Despite the indirect nature of the information provided by the fluorescence probes employed, they reveal differential characteristics of the 22:6 containing PC present in photoreceptor membranes.

MATERIALS AND METHODS

Materials

Laurdan was purchased from Molecular Probes (Eugene, OR). DPH and all other drugs were obtained from Sigma Chemical Co., St. Louis, MO. All solvents used were of high pressure liquid chromatography (HPLC) grade (J. T. Baker, Phillipsburg, NJ). The gas chromatography (GC) and HPLC equipments used were from Varian, Inc.

Methods

Photoreceptor membranes. Bovine eyes were obtained from a local abattoir and placed on ice within 10 min of the animal's death. The retinas were excised on ice, under dim red light, and the rod outer segments (ROS) were isolated therefrom using a discontinuous gradient of sucrose. Briefly, retinas were removed, shaken in a 40% sucrose solution containing 1mM $MgCl_2$, 1mM

DTT, 0.1mM PMSF in 70mM sodium phosphate buffer (pH 7.2) and the retinal ROS were separated from the remains of retinas by centrifugation at 2,200 *g* for 4 min. The supernatants containing ROS were diluted with sucrose-free buffer and centrifuged at 35,000 for 30 min. The pelleted ROS were gently resuspended and purified on a discontinuous gradient of sucrose (21). The ROS band was isolated from the 0.84/1.00 M interface, washed with sucrose-free buffer, and pelleted.

Lipid preparation. Lipids were extracted from the ROS pellets according to the procedure of Bligh & Dyer (22), resolved into classes by preparative TLC on silica gel G-plates, located under UV light after spraying with dichlorofluorescein, and eluted from the support using the solvents described by Arvidson (23). For TLC, chloroform-methanol-acetic acid-0.15 M NaCl (50:25:8:2.5, v/v/v/v) (24) was used as the first developing solvent. Phosphatidylcholine tended to separate into three bands, each containing different groups of molecular species, which were separately recovered. After elution, each of these bands was subjected to a second TLC using chloroform-methanol-ammonia (65:25:5, v/v/v). The lipid content was determined by phosphorus analysis (25). The fatty acid composition was determined by gas-chromatography (5) after preparation of fatty acid methyl ester derivatives. The latter were usually purified before GC on TLC plates that had been pre-washed with a polar solvent (methanol-ether, 75:25, v/v), by means of hexane-ether (95:5, v/v).

Molecular species of PC. Molecular species of PC were isolated from the PC bands resolved by TLC by means of reverse-phase HPLC. The separation was performed at room temperature using a short column (3.5 cm × 0.4 cm ID) of stainless steel packed with spherical, 5 μm particles of silica covered with octadecylsilane (C_{18}) (Zorbax ODS, Dupont). Adequate aliquots of the extracts containing PC, previously filtered to remove traces of particulate matter, were injected. For elution, a solvent gradient was applied, consisting of aqueous 1 mM phosphate buffer, pH 7.4, (component A) and methanol (component B). Eluents were degassed before use. The columns were equilibrated with 93%B and the flowrate was 1 ml/min throughout. Gradient elution was performed in three steps: 93%B for the first 15 min, 95%B between 16 min and 40 min, and 96%B thereafter. The bands were detected with a variable-wavelength UV spectrophotometer, placed at 205 nm. The collected PC bands were recovered after evaporation of the solvent to dryness and re-dissolved in chloroform methanol (C:M) 2:1 (v/v). The separated PC molecular species were quantified by phosphorus analysis and identified by GC of their fatty acids.

Liposome preparation. For fluorescence measurements, the isolated PC molecular species were prepared as multilamellar liposomes. Aliquots of the isolated PCs, alone or in combination with an adequate proportion of DPPC or DMPC in C:M (2:1), were mixed with an aliquot of the fluorescent probes used (Laurdan in ethanol or DPH in THF) to reach a lipid-fluorescent probe ratio of 100:1. The mixtures were evaporated in 1 h in the dark under N_2, resuspended in buffer A (20 mM HEPES buffer, 150 mM NaCl, and 0.25 mM $MgCl_2$, pH 7.4) and sonicated for 30 min. Each sample was diluted with buffer A to have a final lipid concentration of 100 μM in the (10 × 10 mm) quartz cuvettes that were used. In order to rule out the possibility of probe perturbation of the lipid bilayer and/or probe-probe interactions, we did a control experiment with a probe-lipid ratio of 1:300 and a final lipid concentration of 150 μM in the cuvettes. No significant differences were observed with respect to the present results.

Fluorescence measurements. All fluorimetric measurements were performed in a SLM model 4800 fluorimeter (SLM Instruments, Urbana, IL) using the vertically polarized light beam from a Hannovia 200 W Hg/Xe arc obtained with a Glan-Thompson polarizer (4 nm excitation and emission slits). Emission spectra were corrected for wavelength-dependent distortions. The temperature was set with a thermostatted circulating water bath.

Laurdan measurements. Excitation generalized polarization (GP) (16, 26) was calculated according to the expression

$$\text{exGP} = (I_{434} - I_{490}) / (I_{434} + I_{490}) \qquad \textit{(Eq. 1)}$$

where I_{434} and I_{490} are the emission intensities at the characteristic wavelength of the gel phase (434 nm) and the liquid-crystalline phase (490 nm), respectively. exGP values were obtained from the emission spectra at different excitation wavelengths (320–410 nm) or at only one excitation wavelength (360 nm). Emission GP was calculated according to the following formalism:

$$\text{emGP} = (I_{410} - I_{340}) / (I_{410} + I_{340}) \qquad \textit{(Eq. 2)}$$

where I_{410} and I_{340} are the excitation intensities at the wavelengths corresponding to the gel (410 nm) and the liquid-crystalline (340 nm) phases, respectively (27). The emGP values were obtained from the excitation spectra at different emission wavelengths (420–500 nm).

Measurements of steady-state polarization of DPH. The excitation and emission wavelengths used were 365 and 425 nm, respectively. Fluorescence polarization measurements were done in the T format with Schott KV418 filters in the emission channels and corrected for optical inaccuracies and for background signals. The polarization value, P, was obtained as follow (28)

$$P = [(I_v/I_h)_v - (I_v/I_h)_h]/[(I_v/I_h)_v + (I_v/I_h)_h] \qquad \textit{(Eq. 3)}$$

where $(I_v/I_h)_v$ and $(I_v/I_h)_h$ are the ratios of the emitted vertical or horizontally polarized ligth to the exciting, vertical or horizontally polarized, light, respectively. Polarization values can range between −0.33 and 0.5, the higher values denoting the higher structural lipid order.

Data analysis. The transition temperatures (Tt) were obtained by calculation of the second derivative of the experimental values (GP or polarization vs. temperature). We considered that the Tt correspond to a second derivative value of zero.

RESULTS

Partial resolution of ROS lipid classes into subfractions containing different groups of molecular species was first described by Miljanich et al. (1), who observed that the dipolyunsaturated molecular species of all ROS phospholipids including PC migrated ahead of other species by TLC or column chromatography. Previous studies from our laboratory have shown that the dipolyunsaturated molecular species of ROS PC that contained C_{24}-C_{36} polyenes, as a group, tended to migrate ahead of the rest of the PCs when subjected to TLC (5, 29). This is because the large number of carbon atoms per molecule at the acyl chains hinders or reduces interactions between the polar part of the lipid and the polar adsorbent surface silanol groups. An almost clear-cut separation of ROS PC into three groups of species can be achieved at appropriate silica support-lipid ratios (termed PC_{down}, PC_{middle}, and PC_{up} in **Table 1**), mainly made up by disaturated, hexaenoic, and dipolyunsaturated groups of species, respectively, as shown by the fatty acid composition. The data in Table 1 show that the group of species migrating ahead on TLC contain, in addition to 22:6n-3, virtually all of the VLC-PUFA of ROS PC.

TABLE 1. Typical fatty acid composition (wt%) of the three main groups of molecular species from ROS phosphatidylcholine as separated by TLC

Fatty Acid	PC_{down}	PC_{middle}	PC_{up}
14:0	1.4		
15:0	0.8	0.1	0.1
16:0	70.2	22.1	1.2
17:0	1.0	0.8	0.1
18:0	6.6	22.6	3.0
24:0	0.1	0.01	0.1
16:1	5.4	1.6	0.2
17:1	0.6	0.2	t
18:1	8.0	6.2	0.7
20:1	t	0.3	
18:2n-6	1.2	1.3	0.3
18:3n-6	0.2	0.5	0.3
20:4n-6	0.1	4.4	1.4
22:4n-6	0.1	0.4	0.2
24:4n-6	0.1	1.5	1.8
30:4n-6			0.6
32:4n-6			2.8
32:5n-6			1.0
18:3n-3	0.1	0.1	0.2
18:4n-3	0.1	0.5	0.3
20:5n-3		0.5	0.3
22:5n-3	0.1	1.9	1.1
22:6n-3	2.0	32.6	46.5
24:5n-3	0.2	1.4	2.6
24:6n-3	0.1	0.3	1.1
26:5n-3	t	0.3	1.2
26:6n-3	t	t	0.3
28:5n-3			0.6
28:6n-3			0.2
30:5n-3			2.4
30:6n-3			0.6
32:5n-3			10.6
32:6n-3			11.4
34:5n-3			3.6
34:6n-3			1.7
36:5n-3			2.3
36:6n-3			1.7

The fatty acid composition was analyzed by gas chromatography. t, percentages lower than 0.05%.

The six 22:6n-3 containing molecular species of PC used in this study were isolated from the PC_{up} and PC_{middle} bands by means of reverse phase HPLC. The dipolyunsaturated species obtained from the PC_{up} fraction were didocosahexaenoyl (22:6/22:6-PC) and three species with VLCPUFA and 22:6: 32:5/22:6-PC, 32:6/22:6-PC, and 34:5/22:6-PC. From the more abundant PC_{middle} fraction two "hexaenoic" species were isolated: 16:0/22:6-PC and 18:0/22:6-PC

The thermal behavior of the three main subfractions of ROS PC obtained by the TLC separation was measured by determining the GP of Laurdan as a function of temperature. The behavior of liposomes prepared from PC_{total}, PC_{down}, PC_{middle}, and PC_{up} was compared with that of two well-characterized systems, DMPC and DPPC (**Fig. 1**). DMPC and DPPC by themselves presented a pronounced change of GP in a narrow range of temperature, characteristic of a phase transition. Both PC_{middle} and PC_{up} showed GP values lower than those of DMPC and did not exhibit a phase transition, showing that, even at the lowest temperature tested, these lipids were forming a liquid-crystalline phase. On the contrary, the curve obtained with PC_{down} was displaced to the right of that of DMPC, and rather closer to that of DPPC (Fig.1A). PC_{total} presented an intermediate behavior, quite close to that of DMPC but displaced to the left. Thus, the PC_{total} curve lay between the curve of PC_{down} (richer in saturates than PC_{total}) and those of PC_{middle} and PC_{up} (richer in polyenes than PC_{total}). When liposomes were prepared with 50% DMPC and 50% of either PC_{down}, PC_{middle} or PC_{up} (Fig. 1B), the resulting curves became closer to that of 100% DMPC.

In Fig. 1C the behavior of 100% DPPC is compared with that of combinations of 50% DPPC and 50% of the three types of lipids from ROS-PC. In all three cases, the composite liposomes presented a Tt lower than that of DPPC alone.

Figure 2 displays the values of Tt obtained from the curves in Fig. 1. Whereas the presence of PC_{down} increased the Tt of DMPC, there was almost no difference between the Tt of DPPC and that of PC_{down}, consistent with the fact that this fraction contained mostly saturated fatty acids longer than C14. The presence of both PC_{middle} and PC_{up} decreased the Tt of both DMPC and DPPC, consistent with the fact that these subfractions of PCs are polyunsaturated. The actual Tt of the 22:6 containing species studied here are all below zero. Using calorimetry, some dipolyenoic (18:2/18:2, 20:4/20:4, and 22:6/22:6) PCs exhibited endothermic transitions ranging from about −80°C to −30°C (30), and using Raman spectroscopy, the phase transition temperature calculated for 16:0/22:6-PC is approximately −3°C (31).

The thermal behavior of the 22:6-containing species of PC isolated by HPLC, as sensed by Laurdan, is shown in **Fig. 3**. Liposomes formed with 50% DPPC and 50% 22:6/22:6-PC or any of the VLCPUFA/22:6-PC showed a transition temperature quite close to that obtained with DPPC alone, whereas liposomes with 50% of any of the saturated/22:6-PC caused a somewhat larger decrease of the Tt of DPPC.

Laurdan molecules lie at ∼10 Å from the center of the bilayer, i.e., at a depth of ∼5 Å from the lipid-water interface (15). This implies that Laurdan dimethylamino group is at a relatively shallow position in the polar/hydrocarbon interface. By contrast, the fluorescence probe DPH localizes itself at the hydrocarbon core of the bilayer, aligned parallel to the phospholipid acyl chains (32). The two types of fluorescence compounds used in the present work are thus expected to yield topographically distinct yet complementary information. The shallow localization of Laurdan molecules should provide average information on the physical state of both hemilayers, whereas DPH should report on the acyl chain packing order of the inner hydrocarbon core. It was thus of interest to investigate with DPH whether the PCs under study produce local heterogeneity in the membrane at a deeper position, i.e., precisely the region where the (methylene-interrupted, *cis*) double bonds of the VLCPUFA are confined. **Figure 4A**, **B** show that in all cases a phase transition was sensed by DPH, with Tt values similar to those obtained with Laurdan. At temperatures below the Tt, DPH polarization values depended on the lipid fatty acid composition: the highest polarization value was observed with DPPC alone.

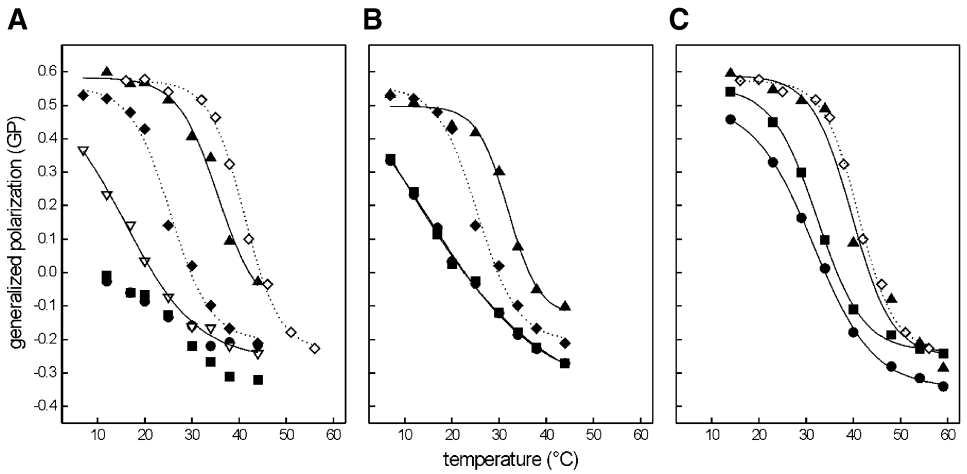

Fig. 1. Excitation general polarization (GP) of Laurdan as a function of temperature in liposomes of phosphatidylcholine (PC). A: One hundred percent dimyristoylphosphatidylcholine (DMPC) (closed diamond) and 100% dipalmitoylphosphatidylcholine (DPPC) (open diamond) in comparison with 100% PC_{total} (inverted triangle), 100% PC_{down} (closed triangle), 100% PC_{middle} (square) and 100% PC_{up} (circle) from bovine rod outer segments (ROS). B: One hundred percent DMPC (closed diamond) and liposomes prepared with 50% DMPC and 50% PC_{down} (closed triangle), PC_{middle} (square), and PC_{up} (circle). C: One hundred percent DPPC (open diamond) and liposomes of 50% DPPC and 50% PC_{down} (triangle), PC_{middle} (square) and PC_{up} (circle).

This polarization value decreased when 22:6/22:6-PC, 16:0/22:6-PC or 18:0/22:6-PC were present in the liposome. A further decrease was observed when either 34:5/22:6-PC or 32:5/22:6-PC were present, and the smallest value of polarization was observed when 32:6/22:6-PC was present. The range of temperatures involved in the phase transition was wider for liposomes containing these PCs than for those of DPPC alone, i.e., the mixtures exhibited considerably broader thermal melts.

Further information about the environment of the reporter Laurdan molecules can be obtained from the wavelength dependence of GP spectra (27). A wavelength-independent GP spectrum is characteristic of the gel phase, whereas the GP spectrum typically exhibits wavelength dependence in liquid-crystalline phases or in coexistence of phases conditions, that is due to solvent dipolar relaxation. In the first case, decreasing values for the excitation GP spectrum and increasing values for the emission GP spectrum are observed, whereas in the second conditions the opposite can be expected.

Laurdan-labeled liposomes of DPPC, alone and with diverse percentages of the polyunsaturated PCs of this study, were studied in order to investigate the variation of GP as a function of excitation (320–410 nm) and emission (420–

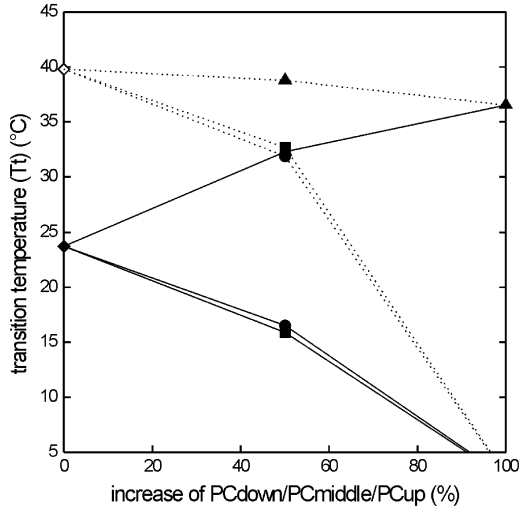

Fig. 2. Changes in the transition temperature (Tt) of DMPC (solid line) or DPPC (dotted line) by increasing the percentage of PC_{down} (triangle), PC_{middle} (square), and PC_{up} (circle) in the liposome.

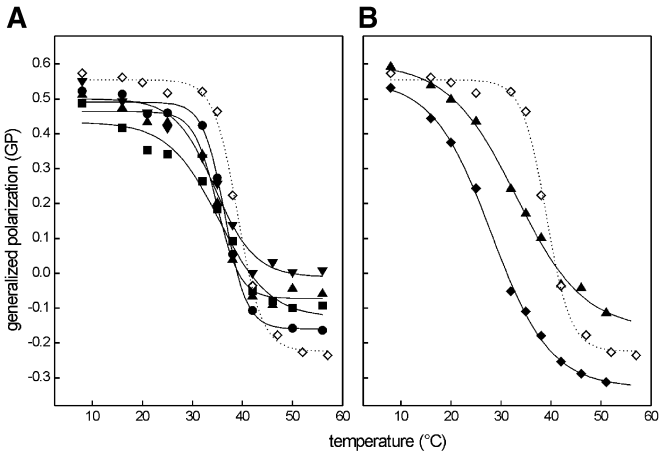

Fig. 3. Laurdan excitation GP as a function of temperature in liposomes of 100% DPPC (open diamond) and 50% DPPC + 50% ROS PCs. A: Dipolyunsaturated PCs: 22:6/22:6 (circle), 32:5/22:6 (inverted triangle), 32:6/22:6 (upright triangle) and 34:5/22:6 (square). B: Hexaenoic PCs: 16:0/22:6 (closed diamond) and 18:0/22:6 (upright triangle).

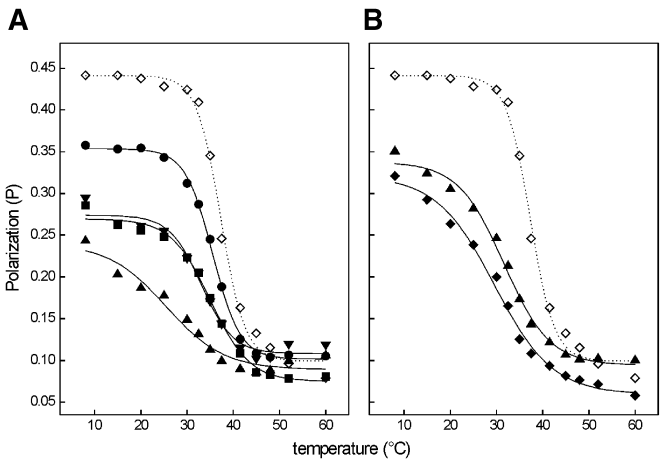

Fig. 4. Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) (P) as a function of temperature in liposomes of 100% DPPC (open diamond) and 50% DPPC + 50% ROS PCs. A: Dipolyunsaturated PCs: 22:6/22:6 (circle), 32:5/22:6 (inverted triangle), 32:6/22:6 (upright triangle), and 34:5/22:6 (square). B: Hexaenoic PCs: 16:0/22:6 (closed diamond) and 18:0/22:6 (upright triangle).

500 nm) wavelengths at different temperatures (from 8°C up to 50°C) (**Fig. 5**). Excitation and emission GP values were calculated according to eq. 1 and 2. All of the liposome conditions studied showed curves of GP as a function of wavelength that decreased GP values as the temperature increased. Also, a change of curve profile could be observed from one pattern typical of phase coexistence at low temperatures to another typical of liquid-crystalline phase at higher temperatures. When DPPC was the principal lipid in the liposome, at low temperatures the curves exhibited a slight slope; as the percentage of DPPC decreased and that of the experimental PCs increased, the slopes of the curves were increasing and getting the profile of a curve typical of phase coexistence. When liposomes prepared only with the experimental PCs were used, one single type of curve in all the range of temperatures was obtained: that corresponding to a liquid-crystalline phase.

The GP values in Laurdan-labeled liposomes of DPPC, alone and with diverse percentages of 22:6-containing PCs (i.e., each of the conditions presented in the examples given in Fig. 5) were also studied as a function of temperature (**Fig. 6**). With respect to DPPC alone, as the percentage of the insaturated PCs in the liposome increased, the absolute GP values decreased at high temperatures and increased at low temperatures in all cases. At up to 50% PC, all liposomes presented a clear phase transition. However, when the experimental PCs were the only lipid in the liposome, the curves presented a single, constant slope throughout all the temperature range. The transition temperatures of the mixed liposomes are shown in **Table 2**. Comparable Tt values were obtained in previous work using polarization of DPH (namely 39°, 37°, 33°, and 32°C for vesicles containing DPPC and 10, 20, 30, and 40% 16:0/22:6-PC, respectively) (33). A phase diagram of DPPC and 16:0/22:6-PC constructed from data obtained with *cis*- and *trans*-parinaric acids (34) gave "fluidus" temperatures also consistent to the Tt shown here for liposomes containing this lipid.

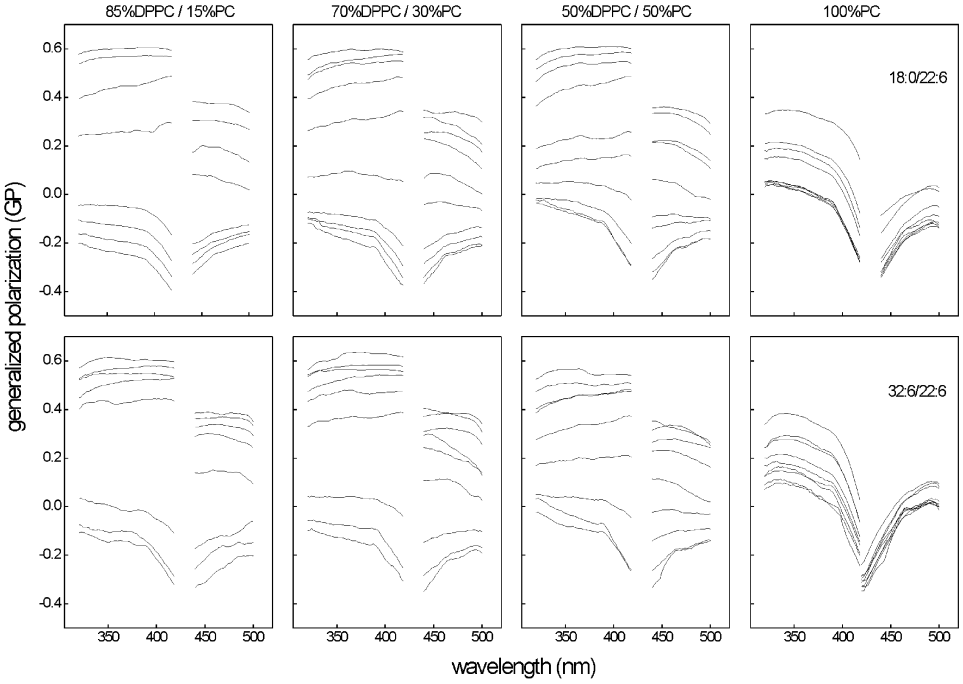

Fig. 5. Laurdan GP as a function of excitation (320–410 nm) and emission (420–500 nm) wavelengths in liposomes of DPPC with increasing percentages of 18:0/22:6-PC (upper row) and 32:6/22:6-PC (lower row). Plots from left to right: 15%, 30%, 50%, and 100% of the PCs. Within each plot, the different curves correspond to measurements done at different temperatures (from top to bottom: 8°C to 50°C).

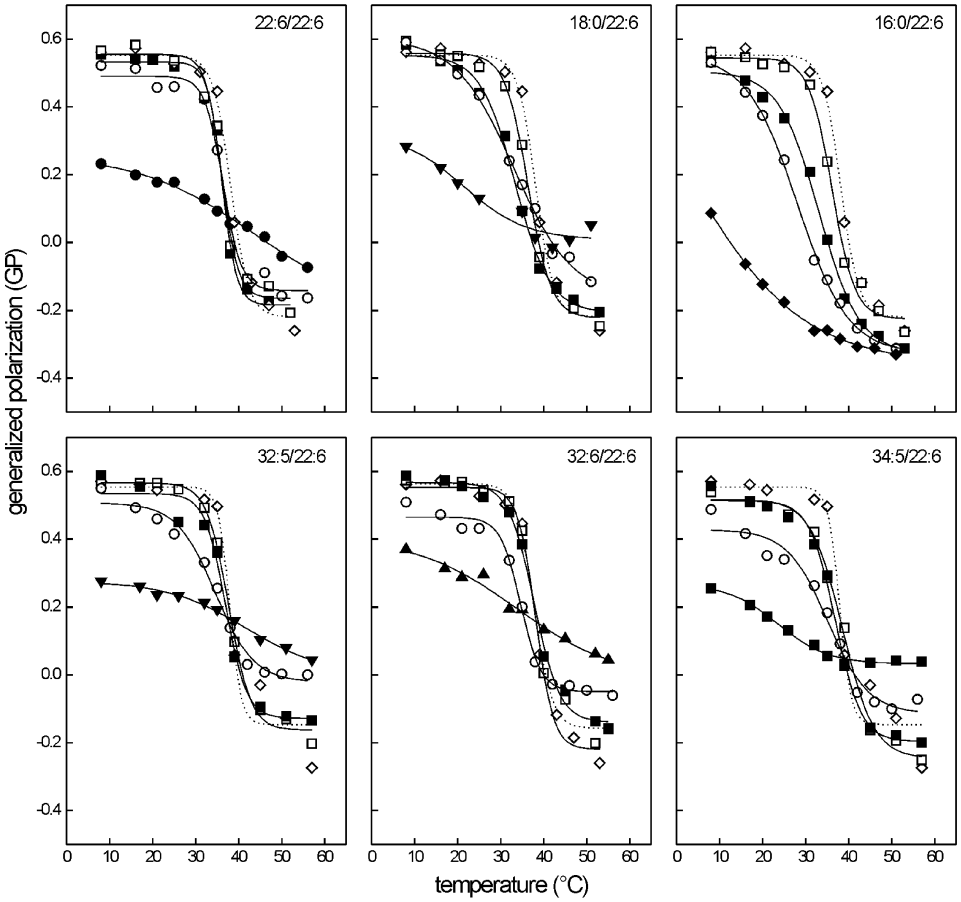

Fig. 6. Laurdan excitation GP as a function of temperature in liposomes of DPPC (open diamond) and of DPPC with increasing percentages of the PC species studied in this work, one depicted on each panel. The percentages are represented as follows: 0% (open diamond), 15% (open square), 30% (closed square), and 50% (open circle). The values for 100% 22:6/22:6 (closed circle); 100% 18:0/22:6 (inverted triangle); 100% 16:0/22:6 (closed diamond); 100% 32:5/22:6 (inverted triangle); 32:6/22:6 (upright triangle); and 34:5/22:6 (closed square) are also included in the corresponding panels.

From the data in Fig. 6 and Table 2 it is clearly apparent that the behavior of the PCs containing 22:6n-3 at *sn*-2 falls into two distinct groups: the dipolyunsaturated PCs, containing either a VLCPUFA or 22:6n-3 at the *sn*-1 position, and the PCs having a saturated fatty acid at the *sn*-1 position. Figure 7 summarizes this observation, averaging the individual values of the changes induced to the Tt of DPPC by the increase of the experimental PCs, grouped as indicated. The standard deviations obtained for the Tt within each group were small and the difference between averages was statistically significant. The only difference between these PCs is the fatty acid at *sn*-1, so the diverse behavior of these lipids depends mainly on the characteristics of the fatty acid located at that position.

TABLE 2. Transition temperatures (°C) of DPPC liposomes with increasing percentages of PC species isolated from retinal ROS

PCs	16:0/22:6	18:0/22:6	22:6/22:6	32:5/22:6	32:6/22:6	34:5/22:6
0%	38.4	38.4	38.4	38.4	38.4	38.4
15%	36.0	36.6	36.7	37.6	38.1	37.9
30%	34.0	34.0	36.7	37.5	37.8	37.6
50%	32.6	33.6	36.5	36.7	35.9	37.3
100%	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected.

DISCUSSION

The strategy used in this work to study the characteristics of VLCPUFA-containing molecular species of ROS-PC was to compare the physicochemical properties of lipid bilayers formed with PCs that differ only in the length and unsaturation of the fatty acid at *sn*-1 position, all having in common docosahexaenoic acid as the fatty acid at the *sn*-2 position of the glycerol backbone. For their isolation, advantage was taken of the property these species display of separating on adsorption chromatographic supports into subfractions, the one with the lowest migration containing mostly disaturated PC, the one at the middle being rich in hexaenoic molecular species, and the one migrating ahead containing most of the VLCPUFA. For the recovery of individual species within each of these subfractions, advan-

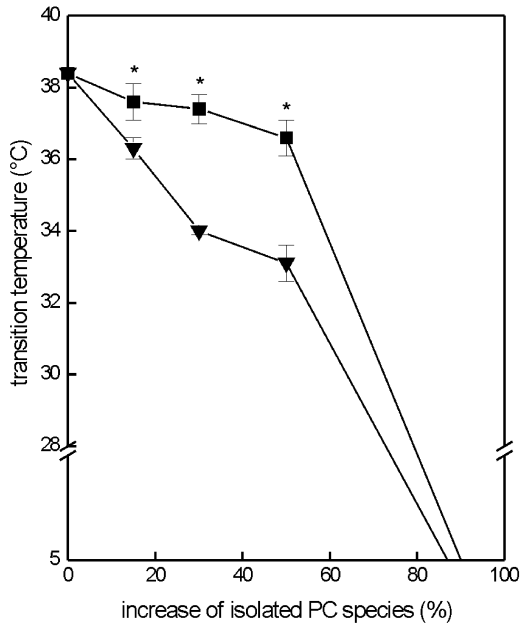

Fig. 7. Modification of the Tt of DPPC by increasing percentages of 22:6-containing PC species from ROS. From the data in Fig. 6, the species were assembled into two main groups and the results were averaged: closed square, dipolyunsaturated PCs; inverted triangle, hexaenoic PCs. * Statistically significant differences among groups by using a paired *t*-test (a value of less than 0.05 was considered significant): 15%, $P < 0.025$; 30%, $P < 0.001$; and 50% $P < 0.005$.

tage was taken of the large number of double bonds per molecule these PCs possess, which allows good detection with a standard HPLC spectrophotometer and with not too stringent HPLC conditions.

Liposomes prepared with the subfractions containing mostly hexaenoic and dipolyunsaturated molecular species (PC_{middle} and PC_{up}, respectively), as well as liposomes made of any of the isolated 22:6n-3 containing PCs, formed a liquid-crystalline phase in all the range of temperatures tested in this investigation, as sensed by Laurdan. Concerning PC subfractions from ROS, the present results are consistent with a previous study recording the fluorescence polarization of *trans*-parinaric acid as a function of temperature with aqueous dispersions of ROS-PC fractions that had been separated by silicic acid column chromatography (1). With both fluorophores, the behavior as a function of temperature of all ROS-PC subfractions gave curves (polarization or GP) localized to the left of that of DPPC. In the above-mentioned work (1), a curve produced by liposomes of (synthetic) 16:0/22:6-PC was to the left than that produced by the ROS-PC subfraction containing the dipolyunsaturated species. This is also consistent with the present results with individual molecular species of PC, since Laurdan GP measurements with liposomes made of 100% 16:0/22:6-PC gave curves that were located to the left of those produced by liposomes of dipolyunsaturated PC species, including 22:6/22:6-PC and VLCPUFA/22:6-PC.

The shift to the left of the GP curve of 16:0/22:6 with respect to the dipolyunsaturates may be explained by taking into account the contribution of three structural characteristics of the fatty acids of the latter: overall chain length, overall degree of unsaturation, and the disparity in length and unsaturation of the chains at *sn*-1 and *sn*-2. Concerning chain length, it is known that the transition temperature of phospholipids increases monotonically with the chain length of their fatty acids (35). Regarding the degree of unsaturation, it is also known that the introduction of just a single double bond in the hydrocarbon chain of a fatty acid has dramatic effects on the chain-melting transition (Tt may be decreased between 1°C and −60°C, depending on the position of the double bonds in the chains) (36–38). The third factor, unequal length of the two acyl chains at the *sn*-1 and *sn*-2 positions, is known to affect interchain attraction and to decrease the chain packing density at the region of the hydrocarbon terminus (13, 39). Thus, the large total number of carbons in dipolyunsaturated PCs is the dominating factor, shifting the curves to the right with respect to that of 16:0/22:6-PC.

Of the six individual 22:6n-3 containing PCs of this study, all broadened the melting transition of DPPC, but only the hexaenoic species, 16:0/22:6 and 18:0/22:6, induced a significant decrease in the Tt of DPPC. However, all of them caused a distortion of phases, as evidenced by the decrease of the values of GP (Laurdan) or polarization (DPH) in the gel phase and by their increase in the liquid-crystalline phase (Figs. 4 and 6). It is noteworthy that the distortions of the phases evidenced with GP values were lower than those evidenced with polarization values. These distortions may be related to the fact that the spectrum of Laurdan in the gel phase is a factor of three more intense than the spectrum in the liquid-crystalline phase (16). Thus, the Laurdan molecules located in the liquid-crystalline phase contribute less to the total intensity than the Laurdan molecules localized in the gel phase. With DPH, the differences of the polarization curves at temperatures below Tt (gel phase) are quite informative of the structural differences between the studied PCs. There is an ample difference between 22:6/22:6-PC and the VLCPUFA/22:6-PCs, the largest "disorder" toward the center of the membrane, reported by this probe, being produced by the PC containing 32:6n-3 at the *sn*-1 position.

The fact that the dipolyunsaturated PCs virtually induced no change in the Tt of DPPC while the hexaenoic PCs decreased it (Figs. 6 and 7) does not seem to agree with the notion that the effect of acyl chain unsaturation on the Tt is magnified when both acyl chains are highly polyunsaturated (40). One possible explanation could be that, for the unusual dipolyunsaturated lipids of this study, the effect of the large number of carbon atoms imposes on the effect induced by the double bonds. A previous work using ^{2}H-NMR to study the reorientational motions of two deuterated PCs (CD_3-16:0/22:6-PC and CD_3-16:0/16:1-PC) agrees with this interpretation, since the phase behavior of 16:0/22:6-PC resembles better that of a fully saturated phospholipid than that of 16:0-16:1-PC (41). Another possible explanation could stem from the different interaction between lipids due to the disparate length of their acyl chains. The PC species that have a VLCPUFA at

sn-1, have a long stretch of CH_2 groups toward the carboxyl end (as it is the case in saturated fatty acids), and a bulky group of (four to six) methylene-interrupted *cis* double bonds toward their methyl tails. This last part is absent from 18:0/22:6-PC. Using computer modeling of 18:0/22:6-PC, it was shown that in this lipid, the angle configuration of 22:6 fits well the 18:0 conformation not only in shape but in length (assuming that the saturated chain in an all-*trans* conformation), thus obtaining similar end-to-end distance ratio for both acyl chains (18:0/22:6 ≅ 2.163/2.184) (42). This good match in the length of both acyl chains allows for optimum chain-chain interaction in hexaenoic molecular species of PC (36). Thus, 16:0/22:6-PC or 18:0/22:6-PC may be expected to behave as a unit, and therefore manifest their characteristics when they are in a mixed phase (i.e., like in the present case, changing the Tt of another lipid). In the case of dipolyunsaturated species, such a chain-chain matching is likely to be possible for 22:6/22:6-PC, but not for VLCPUFA/22:6-PCs. In order to maximize van der Waals contacts in the gel phase, and prevent the occurrence of voids toward the region of the terminal methyl groups (39), phospholipids with such a dissimilar length of their acyl chains like the ones studied here may be expected to undergo some degree of interdigitation of the supernumerary carbons across the central region of the bilayer. This possibility has been proposed even in the case of phospholipids with only one or two carbons of difference between chains (43).

Mixed liposomes made with DPPC and up to 50% 22:6-containing PCs exhibited phase coexistence, i.e., there was lateral separation of the minor lipid at low temperatures, whereas only one liquid crystalline phase was observed at temperatures higher than the Tt (Fig. 5). This interpretation agrees with previous work where a phase diagram of DPPC and 16:0/22:6-PC constructed from fluorescence data exhibited solid phase immiscibility, presumably due to the large differences in the transition temperatures of these two components (34). In the present work, pure liposomes (100%) of all 22:6-containing ROS PC species were in the liquid-crystalline phase in all the range of temperatures used. Thus, when these PCs are alone they tend to form a disordered bilayer, i.e., they form a liquid-crystalline phase, but when they are in a minority proportion in liposomes, the latter tend to maintain the characteristics of the predominant lipid.

In ROS membranes at physiological temperatures, the PCs with a VLCPUFA at the s*n*-1 and 22:6 at the *sn*-2 positions may be envisaged as interacting with rhodopsin at the protein-lipid interface, in the depth of the membrane hydrophobic matrix. The present results support, or at least do not exclude, the previous suggestion that the unusual fatty acids these PCs have at *sn*-1 could partially surround intramembranous segments of the protein part of the time (5). A recent report demonstrated that helical and angle-iron conformations are the preferred ones for polyunsaturated chains in liquid-crystalline bilayers, because they favor chain extension while maintaining bilayer flexibility (44). This lends support to the idea that the presence of relatively long, extended fatty acyl chains may play a role in the solvation of the hydrophobic surfaces of integral membrane proteins, such as rhodopsin. The presence of this protein is likely to impose a kind of "disordered order" to these peculiar lipids in the native bilayer of ROS, and the lipids in turn may provide a mechanically optimum environment to support the important conformational changes the protein undergoes after photon absorption.

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