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The phase behavior of aqueous dispersions of unsaturated mixtures of diacylglycerols and phospholipids

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Abstract

The phase behavior of mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycerol (1,2-POG) with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) was studied by using DSC, small-angle X-ray diffraction and ^{31}P-NMR. The results have been used to construct phase diagrams for both type of mixtures, in the 0–45°C range. It is concluded that 1,2-POG form complexes in the gel phases with both POPC and POPS. In the case of POPC, two complexes are postulated, the first one at a 1,2-POG/POPC molar ratio of 40:60, and the second one at 70:30, defining three different regions in the phase diagram. Two eutectic points are proposed to occur: one at a very low 1,2-POG concentration and the other at a 1,2-POG concentration slightly lower than 70%. In the case of the 1,2-POG/POPS mixtures, the pattern was similar, but the first complex was seen to happen at a higher concentration, about 50 mol% of 1,2-POG, whereas the second was found at 80 mol% of 1,2-POG. This indicated a bigger presence of 1,2-POG in the complexes with POPS than with POPC. In the first region of the phase diagram, i.e. at concentrations of 1,2-POG lower than that required for the formation of the first complex, and at temperatures above the phase transition, lamellar phases were seen in all the cases. In region 2 of the phase diagram, i.e. at concentrations where the first and the second complexes coexist, a mixture of lamellar and non-lamellar phases was observed. Finally, at high concentrations of 1,2-POG, non-lamellar phases were detected as predominant, these phases being of an isotropic nature, according to ^{31}P-NMR. An important conclusion of this study is that, using unsaturated lipids, similar to those found in biological membranes, it has been shown that diacylglycerols are found separated in domains, and that this process starts at very low concentrations of diacylglycerols. The formation of separated domains enriched in diacylglycerol is biologically relevant as it will allow them to have important effects on the membrane structure besides the fact that their concentration in the biomembrane is relatively low.

Keywords: Diacylglycerol; ^{31}P-Nuclear magnetic resonance; Membrane phase behavior; Differential scanning calorimetry; X-ray diffraction

Abbreviations: DAGs, diacylglycerols; 1,2-DMG, 1,2-dimyristoyl-*sn*-glycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 1,2-DPG, 1,2-dipalmitoyl-*sn*-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine; DSC, differential scanning calorimetry; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid; PC, phosphatidylcholine; PKC, protein kinase C; ^{31}P-NMR, ^{31}P-nuclear magnetic resonance; 1,2-POG, 1-palmitoyl-2-oleoyl-*sn*-glycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospholserine; SAXRD, small angle X-ray diffraction

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1. Introduction

There is a great deal of information showing that the membrane structure is modulated by the presence of DAGs. For example, it has been shown that DAGs may produce structural changes in membranes, such as lateral phase separations [1–6], non-bilayer phases [2,3,7–9] and dehydration of the membrane interface [9,10]. Significantly, this dehydration is more drastic on phosphatidylserine than on phosphatidylcholine [9]. These effects may be responsible for the facilitation of membrane fusion [11–15], and perhaps for the activation not only of PKC, but also other enzymes, such as phospholipases [16–18], CTP-phosphocholine cytidyltransferase [19] and tyrosine kinase [20].

On the other hand, several suggestions have been made on the modulation of the PKC activity by DAGs. Previously, it was suggested that the activity of PKC would increase in correlation with the tendency of lipids to form non-bilayer phases, as could be the case in regions of high bilayer curvature, produced by molecules, such as diacylglycerols or phosphatidylethanolamines, which have small polar headgroups [5,7,21,22]. It has also been pointed out that the presence of DAGs will increase the spacing between phospholipid headgroups due to the interposition of the small polar groups of DAGs [7,23–26]. It could be also possible that the effect of DAGs in the membrane surface, i.e. decrease of hydration, could facilitate the immersion of the protein in the membrane [10]. Finally, it has been recently suggested that membrane heterogeneity, i.e., coexistence of rich and poor phases in DAGs, may contribute to the activation of PKC [6].

However, most of the studies done on DAGs/phospholipids mixtures have been carried out using mixtures including saturated acyl-chains. In fact, to our knowledge, there are no detailed studies of unsaturated diacylglycerols in the literature. In this paper, we have performed a biophysical study, using DSC, ^{31}P-NMR and small angle X-ray diffraction studies, on mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycerol with 1-palmitoyl-2-oleoyl-*sn*-3-phosphocholine, and of the same diacylglycerol with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine. The aim of this work is to better understand the behavior of these unsaturated mixtures which are more closely related to biological membranes than the extensively studied saturated lipids.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycerol (POG) were purchased from Avanti Polar Lipids (Birmingham, AL). 1,2-POG was also prepared from POPC by the action of phospholipase C (*Bacillus cereus*, Boehringer-Mannheim, Barcelona) in ether/water (4:1, v/v) at 4°C for 5 h and extraction from the ether phase. The purity of the diacylglycerol was determined by thin layer chromatography on silica gel 60 plates (Macherey-Nagel, Germany) using chloroform/acetone/methanol (94.5:5.0:0.5, v/v/v) as a solvent. Water was twice distilled and deionized in a Millipore system from Millipore Ibérica (Madrid, Spain).

2.2. DSC measurements

Samples containing 3 μmol of phospholipid and the appropriate amount of POG were dried under a stream of N_2 and the last traces of organic solvent were removed by keeping the samples under vacuum for 5 h. Multilamellar vesicles were formed incubating the dried lipid on 1 ml of 20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA for 15 min at a temperature above transition with occasional and vigorous vortexing. Samples were incubated at the mentioned temperature for an additional period of 30 min more and left to cool down slowly until 20°C in a water bath at a cooling rate of approximately 0.3°C/min. Samples were centrifuged at $16\,000 \times g$ for 30 min and the pellets were transferred to small aluminum pans. Thermograms were recorded using a Perkin-Elmer (Norwalk, CT) DSC-4 calorimeter using a sample pan containing the same buffer as a reference. The DSC instrument was calibrated using indium as standard. The samples were scanned over a temperature range from −10 to 40°C, at a heating rate of 4°C/min and a sensitivity of 1 mcal/s (occasionally, a 0.5°C/min rate was used, giving no better

resolution of the thermograms). Normally, the third scan was used for transition calculations unless otherwise stated. The possible acyl chain migration in 1,2-POG to give 1,3-POG was determined, after the different experiments were concluded, as previously described [4], and it was observed that no more than 7% of 1,3-POG over total diacylglycerol was formed in any case.

2.3. ^{31}P-NMR

A 25 mg amount of phospholipid and the appropriate amount of 1,2-*sn*-POG were mixed in chloroform and evaporated to dryness under a stream of oxygen-free N_2. The remaining traces of solvent were removed by storage for 5 h under high vacuum. Afterwards, 0.4 ml of 20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA were added to the dry lipid mixtures and the samples were heated at a temperature above the transition temperature for 30 min with occasional and vigorous vortexing. ^{31}P-NMR spectra were recorded in the Fourier transform mode using a Bruker AC-200 spectrometer (81 MHz) interfaced with an Aspect 3000 computer (Bruker, Rheinstetten, Germany). Temperature was controlled to ± 0.5°C with a standard Bruker B-VT-1000 variable temperature control unit. All chemical shift values are quoted in parts per million (ppm) from micelles of pure lysophosphatidylcholine (0 ppm); positive values referring to low-field shifts. All spectra were obtained in the presence of a gated-broad band decoupling pulse (10 W input power during acquisition time), and accumulated free induction decays were obtained from up to 4000 transients. A spectral width of 25 kHz, a memory of 8000 data points, a 1-s interpulse time, and a 90° radio frequency pulse were the parameters used to record the spectra. Before Fourier transformation, exponential multiplication was applied resulting in a 100-Hz line broadening.

2.4. Small angle X-ray diffraction experiments

Samples for X-ray diffraction analysis were prepared similarly to those described above for DSC. Ten to 15 mg of lipid was dried and resuspended in 1 ml of buffer, and spun down in a bench microfuge. The pellet was deposited onto the diffractometer sample holder.

Nickel-filtered Cu K_α ($\lambda = 1.54$ Å) X-ray was obtained from a Philips generator, model PW1830. X-rays were focused using a flat gold-plated mirror and recorded using a linear position sensitive detector model 210 (Bio-Logic, France). Unoriented lipid dispersions were measured in aluminium holders using Mylar windows. The sample temperature was kept within ± 0.5°C, using a circulating water bath. The system was allowed to equilibrate for about 5 min at each temperature before measuring. The X-ray exposure times were 10–15 min for each sample. The system was calibrated for the spacings using crystalline cholesterol (33.6 Å spacing).

3. Results

3.1. Thermal studies

DSC of aqueous dispersions of POPC/1,2-POG have been performed, and the results are presented in Fig. 1. The depicted thermograms correspond to the third scan and were identical to other successive scans. It can be seen that the T_c transition temperature of pure POPC was -7°C. For mixtures containing 1 mol% 1,2-POG (Fig. 1A) there was a noticeable decrease in the size of the transition peak, although the temperature of the transition was not changed. At 5 mol% 1,2-POG the transition was considerable widened and a second wide peak appeared centered at about 10°C. At 10 mol% 1,2-POG the peak centered at the same temperature than pure POPC still remained although the broad peak observed at higher temperatures was centered at 17°C. The same trend was observed up to 25 mol% of 1,2-POG, with a decreasing peak centered at the same temperature of pure POPC and a second broad peak centered at progressively higher temperatures as the concentration in POG was increased. However, at 30 mol%, (Fig. 1B) this trend was changed since the peak occurring at the same temperature than the pure POPC was no longer visible, although the peak seen at a higher temperature had a tailing shoulder. This peak became quite narrow at 40 mol% and this marked the appearance of a new pattern, with a single narrow peak centered at 27°C which was observed up to 60 mol% 1,2-POG. At the last composition a shoulder appeared in the peak,

and at 70 mol% POG a second peak appeared at 8°C, which coincided in temperature with that of pure 1,2-POG. In the range of high concentrations of 1,2-POG, i.e. between 70 and 90 mol% POG, the trend was that as the concentration of the diacylglycerol was increasing, the peak appearing at high temperature was decreasing in size while the one at 8°C was growing in size.

With respect to POPS/1,2-POG systems (Fig. 2), the calorimetric results revealed a pattern qualitatively similar to that described above for POPC/1,2-POG, although some differences were appreciated. At 5 mol% 1,2-POG, the transition was considerably widened, so that whereas it still started at the same temperature as the pure POPS, the temperature of completion was considerably higher, i.e. about 9°C for the pure phospholipid and 18°C for the mixture. At 10 mol% 1,2-POG, the broad unresolved transition peak still has its onset at the same temperature as the pure POPS and its completion temperature was still higher than 20°C. At concentrations of 20 mol% and higher of 1,2-POG, the broad peak was centered at about 22°C and the onset was higher than that of pure POPS. It should be remembered that a similar effect was observed in the POPC/1,2-POG system, but at 30 mol%. From this composition and up to 40 mol% of the diacylglycerol, the broad peak suffered a progressive shifting towards higher temperatures. On the other hand, a narrow peak was found at 50 mol% for POPS/1,2-POG and this was in contrast to the POPC/1,2-POG system where this type of peak appeared already at 40 mol% of 1,2-POG. The narrow peak persisted from 50 to 70 mol% of 1,2-POG, and it was only at 80 mol% when a peak corresponding to pure 1,2-POG was observed, centered at 12°C. Again this was in contrast

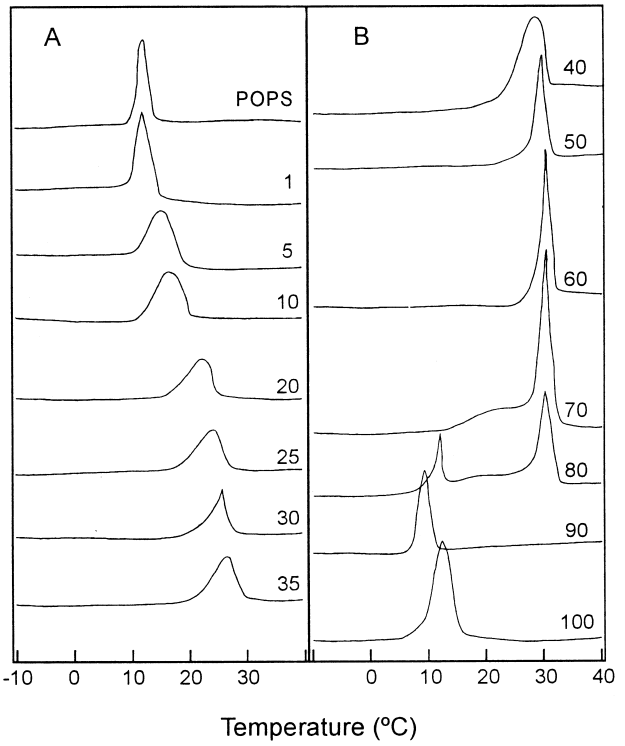

Fig. 2. Differential scanning calorimetric heating thermograms for 1,2-POG/POPS mixtures. The mol% of 1,2-POG in the mixture is indicated on each thermogram. (A) Thermograms obtained from mixtures containing 1,2-POG ranging from 0 to 30 mol%. (B) Thermograms obtained for 1,2-POG/POPS samples containing 1,2-POG ranging from 35 to 90 mol%.

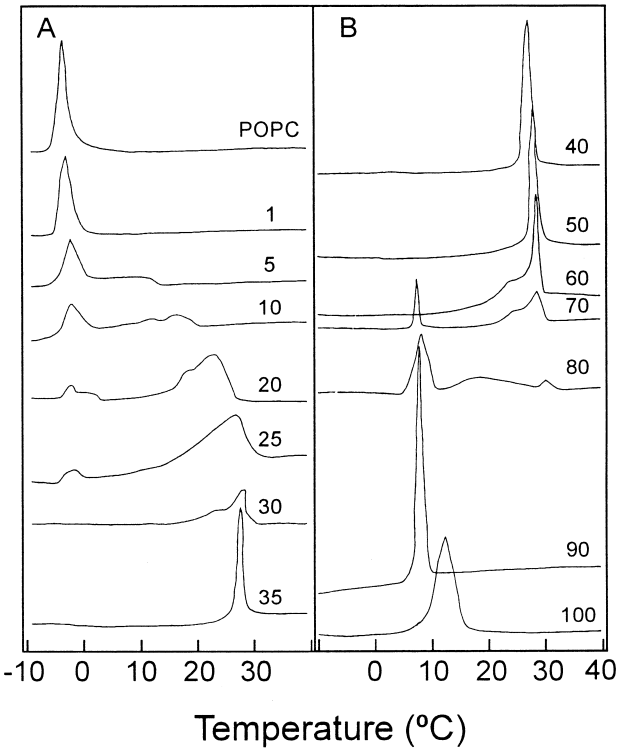

Fig. 1. DSC heating thermograms of aqueous dispersions of mixtures of 1,2-POG/POPC mixtures. The mol% of 1,2-POG in the mixture are indicated on each thermogram. (A) Thermograms obtained from mixtures containing 1,2-POG ranging from 0 to 35 mol%. (B) Thermograms obtained for 1,2-POG/POPC mixtures, containing 1,2-POG ranging from 35 to 90 mol%.

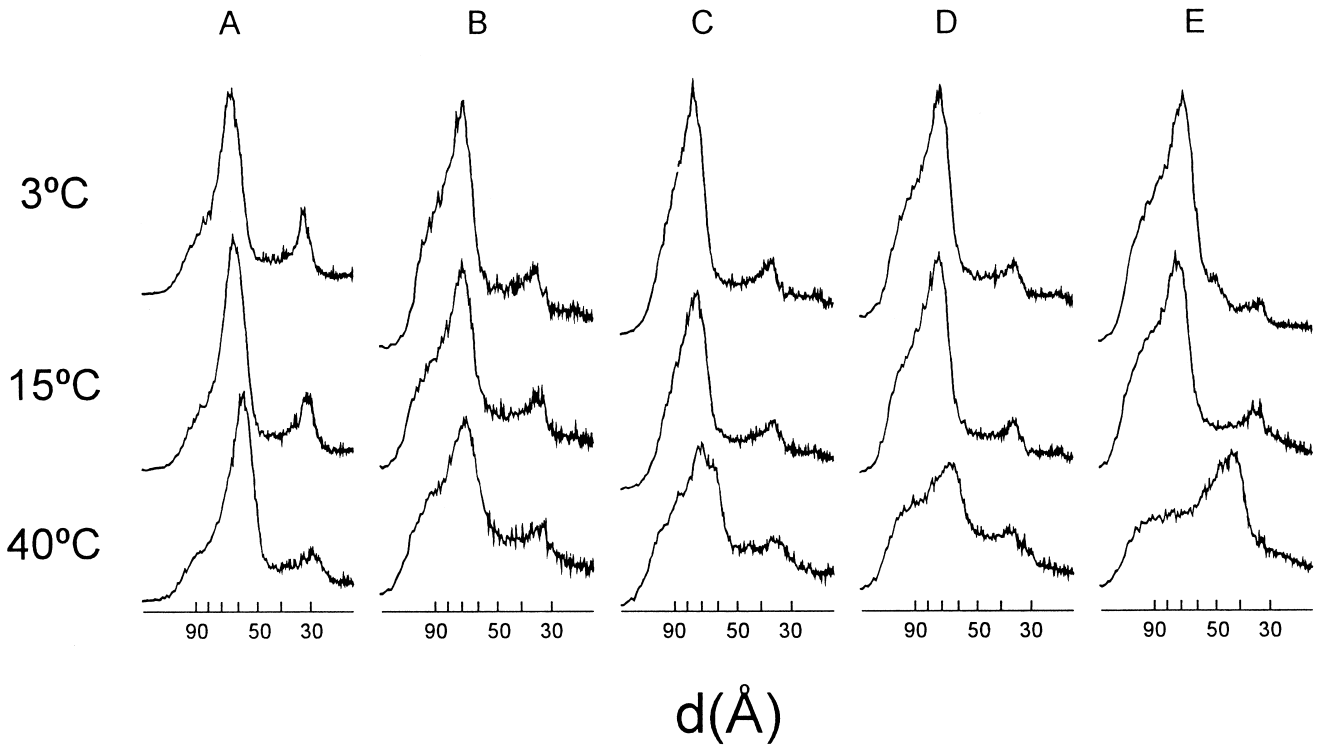

Fig. 3. Small angle X-ray diffractograms obtained from 1,2-POG/POPC mixtures, at the temperatures shown and at different mol% of 1,2-POG: (A) 0; (B) 20; (C) 30; (D) 40; (E) 80.

with the POPC/1,2-DMG where this happened at 70 mol% (Fig. 3).

3.2. Small angle X-ray diffraction studies

The sample of pure POPC (Fig. 3) gave a lamellar spacing of 6.52 nm at 3°C, and according to DSC (Fig. 1A), it will already be in fluid condition at this temperature, with a second order peak at 3.26 nm. At 15°C, the spacing decreased slightly to 6.30 nm, and at 40°C, there was a new small decrease to 5.91 nm. The sample including 20 mol% 1,2-POG was at 3°C in the middle of its transition from gel to fluid condition, according to DSC, and the spacing was 6.86 nm. This spacing was increased to 6.95 nm at 15°C and decreased to 6.69 nm at 40°C, and according to DSC, at 15°C the sample will be in the middle of the phase transition, and at 40°C the mixture will be fluid. At 30 and 40 mol% of 1,2-POG, the patterns are similar between them, being both samples supposed to be in the gel phase at 3°C, according to DSC, and it is remarkable that the spacings in these conditions are relatively high, being of 7.34 and 7.03 nm, respectively. These spacing values were similar at both compositions by increasing the temperature, being of 7.24 and 7.04 nm, respectively, for 30 and 40 mol%. It should also be remembered that this is again compatible with the DSC results which indicated that these samples were in the gel phase at these temperatures for both compositions. However the spacings went down to 6.47 and 6.46 nm at 30 and 40 mol% respectively, when the temperature was raised up to 40°C, which will make them fluid according to DSC (Fig. 1A). It should be noted that the patterns observed at 40°C for 30 and 40 mol% of 1,2-POG were not purely lamellar, so that at 30 mol%, a shoulder was observed at the 6.47 nm peak, and an additional spacing at 3.55 nm, which probably reflect the presence of a non-lamellar phase. Similarly, at 40 mol%, a second reflection was observed at 3.68 nm, so that remembering that 6.46 nm was the first one, these reflections may reflect the presence of a hexagonal H_{II} phase or a cubic one ($1:1/\sqrt{3}$). The sample containing 80 mol% of 1,2-POG, also exhibited a lamellar phase at temperatures below the phase transition, with spacings of 6.78 nm

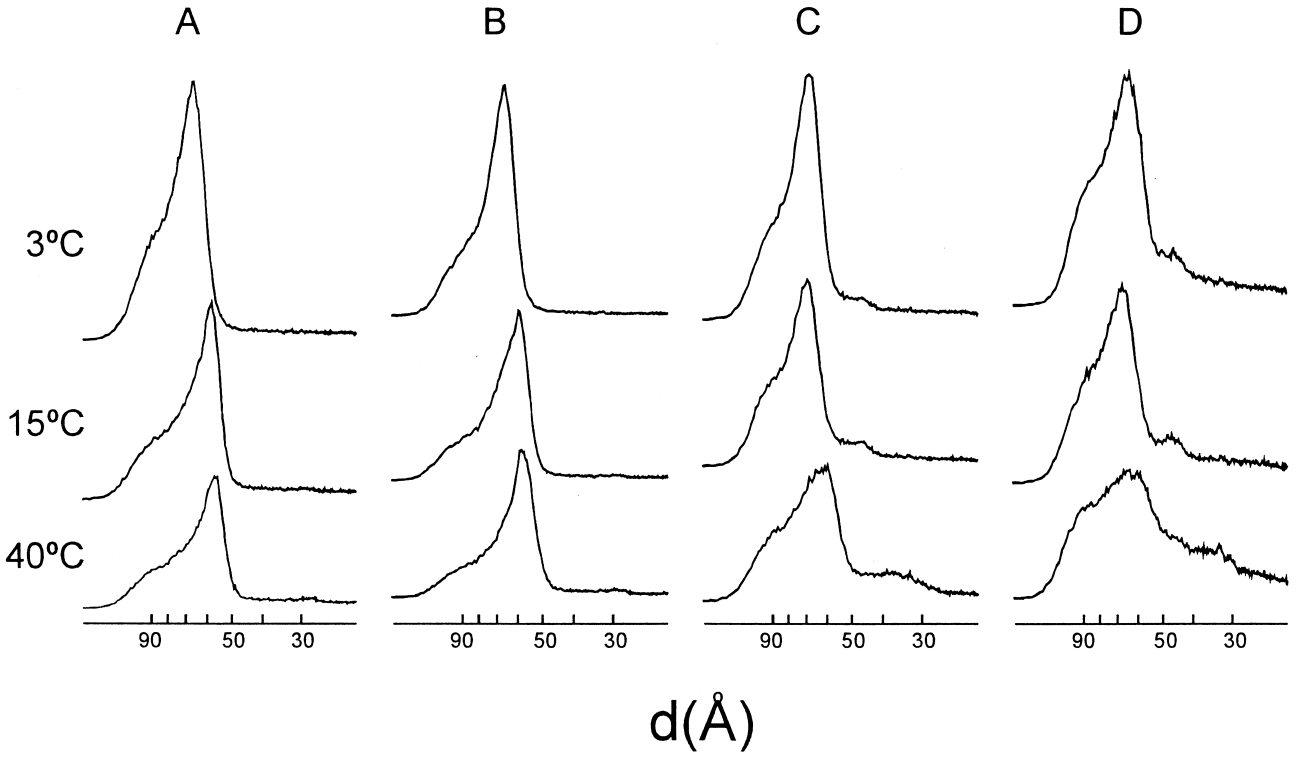

Fig. 4. Small angle X-ray diffractograms obtained from 1,2-POG/POPS mixtures, at the temperatures shown and at different mol% of 1,2-POG: (A) 0; (B) 5; (C) 50; (D) 80.

at 3°C and 7.04 nm at 15°C, but at 40°C, a non-lamellar phase was found, with a spacing of 4.22 nm.

Pure POPS (Fig. 4A), presented at 3°C, which is below its phase transition, a spacing of 6.72 nm although it was not possible to observe a second order of this reflection which must correspond to a

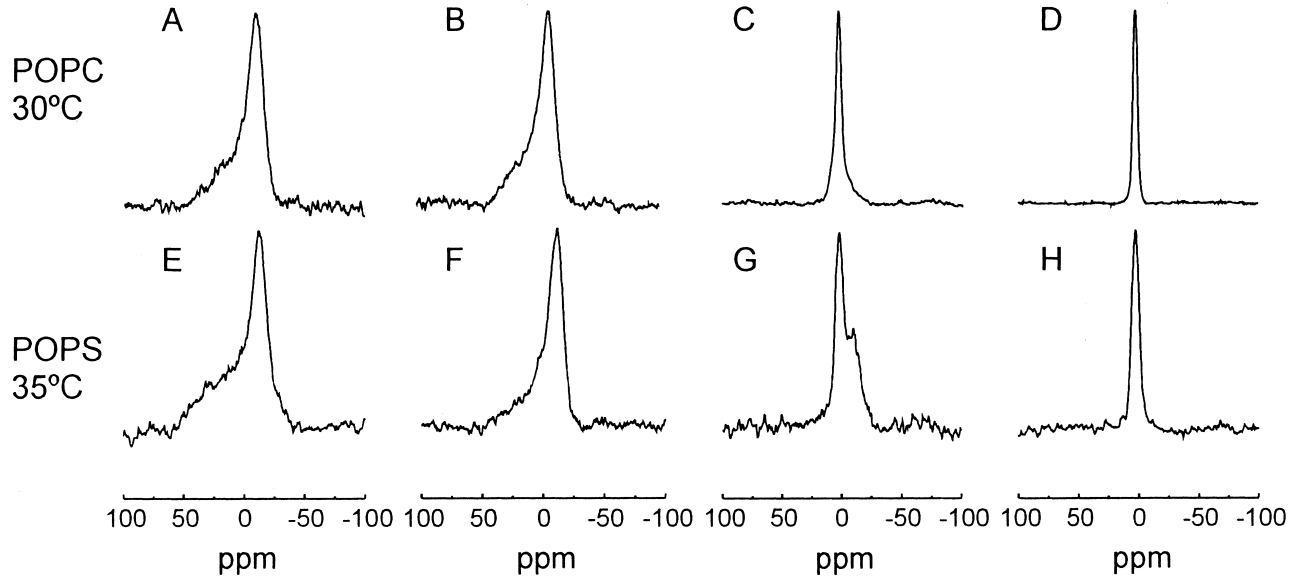

Fig. 5. ^{31}P-NMR spectra of aqueous dispersions of mixtures of 1,2-POG with POPC (top) at 30°C, and POPS (bottom) at 35°C. The mol% content of 1,2-POG in each sample were as follows: (A) 0; (B) 20; (C) 40; (D) 80; (E) 0; (F) 5; (G) 50; (H) 80.

lamellar phase according to the ^{31}P-NMR results shown below. This spacing was decreased to 5.81 nm at 15°C which was already above the phase transition and to 5.64 nm at 40°C, and a weak 2.81 nm second-order reflection could be observed at the last temperature. At 5 mol% of 1,2-POG, the pattern was very similar to that just described for the pure POPS, with spacings at 6.60, 5.97 and 5.85 nm at 3, 15 and 40°C. At 50 mol%, the pattern was that of a lamellar phase below the phase transition, but with non-lamellar components seen even below the phase transition. At 3°C the main peak revealed a spacing of 6.84 nm, and 6.93 nm at 15°C, but with second peaks at 4.64 and 4.56 nm. At 40°C, a broad peak was again seen with a maximum at 5.97 nm with a second one at 3.77 nm, probably indicating the presence of a certain proportion of the lipid in a lamellar phase and some more in a non-lamellar phase. Finally, at 80 mol% of 1,2-POG, the pattern was rather similar to that seen at 50 mol% with spacings at 3°C observed at 6.60 and 4.55 nm, and 6.72 and 4.67 at 15°C, probably indicating the presence of both lamellar and a small proportion of non-lamellar phases, below the phase transition. At 40°C the pattern clearly indicated a non-lamellar phase with spacings at 6.03 and 3.30 nm.

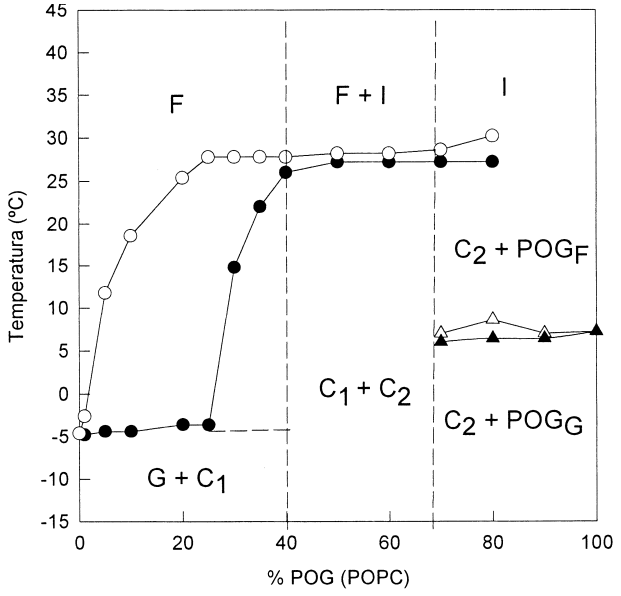

Fig. 6. Phase diagrams for aqueous dispersions of 1,2-POG/POPC, based on data derived from calorimetry, X-ray diffraction and ^{31}P-NMR. The closed and open circles were obtained from the onset and completion temperatures of the heating scans, respectively. G, a gel lamellar phase; F, a fluid lamellar phase; C_1 and C_2, the two complexes proposed.

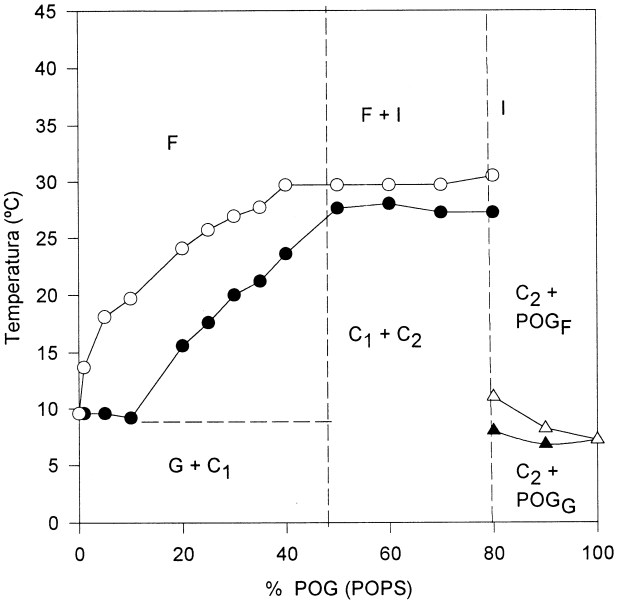

Fig. 7. Phase diagrams for aqueous dispersions of 1,2-POG/POPS, based on data derived from calorimetry, X-ray diffraction and ^{31}P-NMR. The closed and open circles were obtained from the onset and completion temperatures of the heating scans, respectively. G, a gel lamellar phase; F, a fluid lamellar phase; C_1 and C_2, two complexes proposed.

3.3. ^{31}P-NMR spectroscopic studies

The effect of 1,2-POG on the phase polymorphism of POPC and POPS was also investigated by ^{31}P-NMR spectroscopy. The pure POPC, at 30°C, i.e. in the fluid phase, presents an asymmetric lineshape with a high field peak and a low field shoulder characteristic of an axially symmetrical shift tensor and consistent with the arrangement of the phospholipids in a bilayer configuration (Fig. 5). The lineshape of the spectrum corresponding to the same temperature, but in the presence of 20 mol% of 1,2-POG was also characteristic of a bilayer structure.

At 40 mol% of 1,2-POG in POPC, the pattern observed was predominantly isotropic (Fig. 5), although some of the phospholipids might still be in a bilayer configuration given the shoulder present at the high field. This is compatible with the results just described obtained with X-ray diffraction. On the other hand, the sample containing 80 mol% of

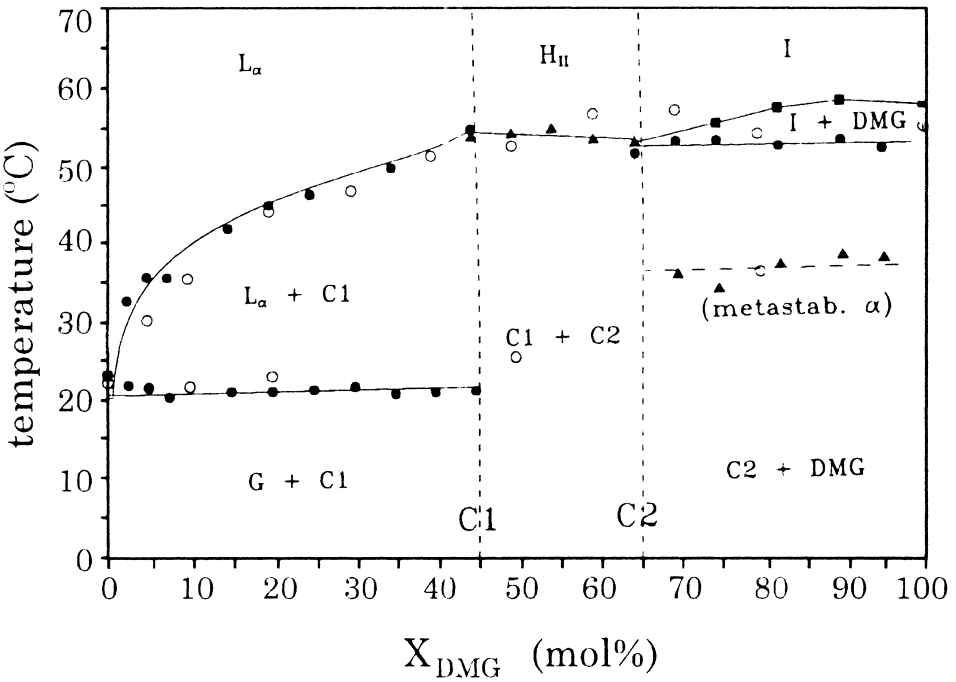

Fig. 8. Phase diagram reprinted with permission from [3]. This phase diagram is for binary dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/1,2-*sn*-DMG mixtures hydrated in 0.1 M KCl, 10 mM Tris, 10 mM EDTA (pH 7.0) deduced from the phase boundaries indicated by the DSC scans (filled symbols), and from electron spin resonance temperature scans (open circles). C1 and C2 denote compounds with DMPC:1,2-*sn*-DMG stoichiometries of approximately 1:1 and 1:2 mol/mol, respectively. DMG represents crystalline 1,2-*sn*-DMG; G is the gel phase consisting primarily of DMPC; and L_α, H_{II}, and I are fluid lamellar, inverted hexagonal, and isotropic phases, respectively. 'metastab. α' denotes the metastable transition of 1,2-*sn*-DMG (α-form).

1,2-POG, an isotropic component was found (Fig. 5). This is again in close agreement with X-ray diffraction which showed a non-lamellar structure for this sample at temperatures above the phase transition. All these samples presented spectra characteristic of lamellar phases at 15 and 5°C, i.e. below the phase transition, but the samples containing 80 mol% at 5 and 10°C, presented a small isotropic component 5°C (not shown). With respect to these isotropic components observed at both 5 and 10°C for this 80 mol% sample, it is difficult to identify their origin, since it could be the existence of small particles or regions of the bilayer surface with a relatively high curvature. Something similar has been observed previously by other authors examining analog mixtures, such as dimyristoylphosphatidylcholine and 1,2-dimyristoylglycerol [3] and dipalmitoylphosphatidylcholine and 1,2-dipalmitoylglycerol [4]. But note that the X-ray diffraction results did not indicate, for these samples, the presence of non-lamellar phases below the phase transition.

Finally, at 80 mol% and 30°C, the POPS/1,2-POG system behaved similarly to the 1,2-POG/POPC just described. Again, below the phase transition (5 and 15°C), all the samples examined showed spectra characteristic of lamellar phases, with anisotopic lineshapes. At 35°C, however, and according to DSC, all the samples should be above the phase transition, and spectra characteristic of fluid lamellar phase were observed for pure POPS and 5 mol% of 1,2-POG, (Fig. 5). The sample containing 50 mol% of 1,2-POG, presented, however, a predominant isotropic component, although the maximum of an anisotropic peak appeared at high field, indicating that some of the phospholipids were in a lamellar organization (Fig. 5). At 80 mol%, however, the spectrum was isotropic, similar to the POPC/1,2-POG sample with the same composition. These results for 50 and

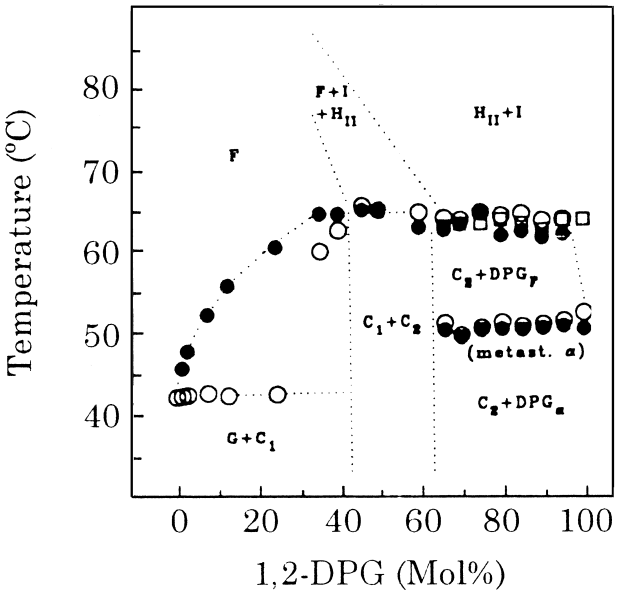

Fig. 9. Phase diagram of aqueous dispersions of DPPC/1,2-*sn*-DPG constructed from data derived from DSC and dynamic X-ray diffraction. Phase assignments were confirmed by freeze-fracture electron microscopy, FT-IR and ^{31}P-NMR. The open and closed circles were obtained from T_c of the heating and cooling scans, respectively. C_1 and C_2 denote DPPC/1,2-*sn*-DPG complexes of about 1:1 and 1:2 stoichiometries respectively. G and F denotes DPPC in the gel and fluid lamellar state, respectively. H_{II} and I are inverted hexagonal and isotropic phases, respectively. 'metastab. α' represents the metastable transition of the α-form of 1,2-*sn*-DPG. DPG_α denotes the crystalline form (α-form) of 1,2-*sn*-DPG and DPG_F represents the melted form of 1,2-*sn*-DPG. Reprinted with permission from [4].

80 mol% at 35°C, closely agree with the X-ray diffraction results (Fig. 4B).

4. Discussion

Diacylglycerols are very hydrophobic molecules which will remain in biomembranes after their formation from phospholipids as a consequence of the action of phospholipase C. Since they may act as second messengers, they are a very interesting case of bioactive lipid which have an action exclusively localized in the membrane. Since a possibility is that their biological action is exercised through changes in membrane structure and organization, as suggested by many authors (see [27,28], for reviews), a considerable number of biophysical studies have been carried out using mixtures of phospholipids and diacylglycerols. In this paper, we have addressed our attention to the study of unsaturated phospholipids, such as POPC and POPS which are of more physiological interest than the saturated phospholipids which have been the main object of previous studies. Together with these phospholipids, we have used 1,2-POG, i.e. the diacylglycerol which bears identical acyl chains than the phospholipids. It should be said here that whereas detailed studies on the phase behavior of saturated diacylglycerols are abundantly found in the literature, unsaturated diacylglycerols have been almost totally neglected so far.

Using the data obtained through DSC, SAXRD and ^{31}P-NMR, phase diagrams were constructed for both the 1,2-POG/POPC system (Fig. 6) and the 1,2-POG/POPS one (Fig. 7). The phase boundaries of the solidus and fluidus lines have been established from the respective onset and completion temperatures of the scans of the different mixtures with variable percentages of 1,2-POG. SAXRD and ^{31}P-NMR data were used to characterize the phase organization of samples at the different temperatures.

In the case of the 1,2-POG/POPC system, the resulting phase diagram was similar to those previously published for DMPC/DMG [3] and for DPPC/DPG [4]. The diagram has three well-differentiated regions. The first one corresponds to concentrations of 1,2-POG lower than 40 mol%. This region can be approached by an eutectic model, so that the eutectic point will be located at a very low concentration of 1,2-POG. The immiscibility in the gel phase is presumed to occur between the phospholipid molecules without diacylglycerol and a compound that can be called C_1, with a POPC/1,2-POG molar ratio at about 40:60. There are, nevertheless, some deviations from total immiscibility in the gel phase, which probably correspond to limited solubility of the phospholipid in the compound C_1. Similar situations have been previously observed in other systems [4,29]. The gel phase immiscibility, will lead to a phase containing pure phospholipid and another formed by phospholipid and diacylglycerol. Above the phase transition temperature, a good miscibility seems to take place between phospholipids and diacylglycerols. Both small angle X-ray diffraction and ^{31}P-NMR experiments corresponding to this region of the phase diagram show that the mixture adopts a lamellar structure at temperatures below that of the

phase transition, and at temperatures above the phase transition as well.

In the second region of the phase diagram (between 40 and 70% of 1,2-POG), immiscibility occurs both above and below the phase transition. It is postulated that two compounds, C_1 and C_2, will coexist here, in the gel phase. C_2 will have a composition of about 70 mol% of 1,2-POG and 30 mol% of phospholipid. A second eutectic point is probably present at a composition very close to that of compound C_2. SAXRD and ^{31}P-NMR results indicate that below the phase transition, lamellar phase is the only one observed, but above the phase transition, coexist both a fluid lamellar phase (L_α) and a non-lamellar phase, and the latter gives place to an isotropic component (I) in the corresponding ^{31}P-NMR spectrum.

In the third region of the diagram, phase separated diacylglycerol coexists with C_2 compound. Below the phase transition, the C_2 compound adopts a lamellar structure, but above it, it has an isotropic structure, according to SAXRD and ^{31}P-NMR results. The non-lamellar structure detected by SAXRD and which can be qualified as isotropic according to ^{31}P-NMR, in both regions 2 and 3 of the diagram, is presumably cubic, according to several detailed studies which have characterized different phospholipid/diacylglycerol mixtures with a high diacylglycerol content [7,30–32]. A difference of these unsaturated lipids is that at variance, with respect to previously studied saturated phospholipid/diacylglycerol systems [3,4], hexagonal H_{II} phases have not been detected in these unsaturated lipidic mixtures.

The general pattern of the phase diagram corresponding to the POPS/1,2-POG system (Fig. 7) was similar to that of the POPC/1,2-POG just discussed, but some differences were appreciated. These differences were related to the compositions of the complexes, so that those formed by POPS had higher concentrations of diacylglycerol than those formed by POPC: with POPS, C_1 had a 50:50 molar composition, and the C_2 80 mol% of 1,2-POG and only 20 mol% of POPS. This indicated a better solubility of 1,2-POG in POPS than in POPC. Another aspect which can be mentioned is the clear deviation from total immiscibility of the complex C_1 and the gel form of POPS. This deviation was accentuated with respect to the POPC system, and might be explained by a certain solubilility of the C_1 complex in the gel phase of POPS.

The increase in the phase transition temperatures, observed after the inclusion of 1,2-POG in either POPC or POPS, could be explained by a closer packing of the hydrocarbon chains resulting from reduced repulsion between the phospholipid head groups of the phospholipid and the consequent increase in van der Waals's cohesive forces between the hydrocarbon chains.

We found that the behavior of the 1,2-POG/POPC and 1,2-POPS systems, are qualitatively similar to binary systems containing a PC and a DAG, bearing both of them identical and saturated acyl chains, such as DMPC/DMG [3], as depicted in Fig. 8, and DPPC/DPG [4], as shown in Fig. 9. These two previously published phase diagrams, were based on eutectic models, and the formation of two complexes was proposed in both cases, as we have done here. It should be stressed that the systems studied here were closer to these phosphatidylcholine systems than to the DPPS/DPG system previously described [9].

A difference with what was found in saturated systems, such as 1,2-DPG/DPPC, using small angle X-ray diffraction [4], was that the low-angle repeats values were not so high in the unsaturated systems reported here. These high spacing-values were observed in the gel phase of the saturated samples and they were attributed to ripple phases which do not seem to be present in the unsaturated systems.

It is biologically relevant that 1,2-POG was able to segregate into domains forming complexes with phospholipids, even at very low concentrations of the diacylglycerol, these domains being immiscible with pure phospholipid, and this was observed in mixtures with both POPC and POPS. This was also the case in other diacylglycerol/phospholipid systems, previously studied [1,4,23,32]. The formation of these domains may be important to explain why diacylglycerols may produce their biological effects although they are present in relatively low concentrations in biological membranes, so that only in exceptional circumstances may they go up to 2–10 mol% [33,34]. Their segregation to domains formed by diacylglycerol/phospholipid complexes may allow them to be in high concentrations in certain points, and this may have important consequences for their mode of action. For example, protein kinase C which

is one of the main targets of diacylglycerols, has been suggested to be modulated by physical perturbations of the membrane [2,7,21,25–27,35,36]. It is clear that the formation of domains rich in diacylglycerols could facilitate the perturbation of the membrane in these local areas. Among these perturbations of the membrane, could be the formation of non-lamellar phases at physiological temperatures, at which biomembranes will be fluid, and this has been shown to be very effective to activate protein kinase C [5,24,35,37]. The induction of non-bilayer phases by diacylglycerols was also invoked as one of the reason for their activating capacity on other enzymes, such as phospholipases [16,18,38] and also on CTP-phosphocholine cytidyltransferase by lipids [39].

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