Jordan, P. M., & Warren, M. J. (1987) *FEBS Lett. 225*, 87–92.
Jordan, P. M., Burton, G., Nordlov, H., Schneider, M. M., Pryde, L. M., & Scott, A. I. (1979) *J. Chem. Soc., Chem. Commun.*, 204–205.
Jordan, P. M., Thomas, S. D., & Warren, M. J. (1988a) *Biochem. J. 254*, 427–435.
Jordan, P. M., Warren, M. J., Williams, H. J., Stolowich, N. J., Roessner, C. A., Grant, S. K., & Scott, A. I. (1988b) *FEBS Lett. 235*, 189–193.
Mauzerall, D. (1960) *J. Am. Chem. Soc. 82*, 2605–2609.
Pluscec, J., & Bogorad, L. (1970) *Biochemistry 9*, 4736–4743.
Radmer, R., & Bogarad, L. (1972) *Biochemistry 11*, 904–910.
Russell, C. S., & Rockwell, P. (1980) *FEBS Lett. 116*, 199–202.
Seehra, J. S., & Jordan, P. M. (1980) *J. Am. Chem. Soc. 102*, 6841–6847.
Smith, A., Hill, S., & Anthony, C. (1988) *J. Gen. Microbiol. 134*, 1499–1507.
Thomas, S. D., & Jordan, P. M. (1986) *Nucleic Acids Res. 14*, 6215–6226.
Warren, M. J., & Jordan, P. M. (1988) *Biochem. Soc. Trans. 16*, 963–965.

Interaction of Diacylglycerols with Phosphatidylcholine Vesicles As Studied by Differential Scanning Calorimetry and Fluorescence Probe Depolarization†

Antonio Ortiz, José Villalaín, and Juan C. Gómez-Fernández*
Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, E-30071 Murcia, Spain
Received January 25, 1988; Revised Manuscript Received May 16, 1988

ABSTRACT: Mixtures of 1,2-dipalmitoylglycerol (1,2-DPG), 1,2-dioleylglycerol (1,2-DOG), 1,2-dicapryloylglycerol (1,2-DCG), 1,3-dioleylglycerol (1,3-DOG), and 1,3-dicapryloylglycerol (1,3-DCG) with dipalmitoylphosphatidylcholine (DPPC) have been studied by means of differential scanning calorimetry (DSC) and fluorescence depolarization of the probe diphenylhexatriene (DPH). DSC measurements showed that the tested diacylglycerols (DG's) modified both the pretransition and the main transition of DPPC, but whereas increasing concentrations of 1,2-DPG tended to produce mixtures with transition temperatures higher than that of pure DPPC, all the other diacylglycerols tested tended to decrease this temperature. This is interpreted as a preferential partitioning of 1,2-DPG into rigid domains whereas all the other DG's preferentially partition into fluid domains. Lateral phase separation was detected in all the mixtures, so that the presence of diacylglycerols produced lipid immiscibilities. The phase diagrams constructed from the calorimetric data showed that 1,2-DPG induced solid-phase immiscibility from 0 to 12.5 mol %, whereas 1,2-DCG produced fluid-phase immiscibility at low concentrations, with an eutectic point at 0.64 mol %. 1,2-DOG also showed fluid-phase immiscibility. 1,3-DCG behaved differently than 1,2-DCG, but 1,3-DOG was rather similar in its effects to 1,2-DOG. Fluorescence depolarization of DPH included in these lipid mixtures was measured at different temperatures, so that phase transitions and the order of the bilayer were monitored. The phase transitions observed by the fluorescence technique were in general in agreement with those monitored by calorimetry. 1,2-DPG did not change the anisotropy value, as referenced to pure DPPC, neither above nor below the phase transition interval, but 1,2-DCG and 1,2-DOG decreased the anisotropy below the phase transition and increased it above this transition. 1,3-DCG decreased the anisotropy at all temperatures, and 1,3-DOG behaved similarly to 1,2-DOG. The physiological importance of the preferential partition of diacylglycerols into domains of different fluidity and their ability to produce lipid immiscibilities at relatively low concentrations are discussed. Since it has been described that only some isomers of diacylglycerol elicit biological responses, the distinct types of perturbation of the phospholipid bilayer produced by the different isomers of diacylglycerol tested here may be a significantly important phenomenon to be considered when studying the mechanism of action of these compounds.

Diacylglycerols (DG's)[1] are nonpolar molecules which are currently the focus of attention of many workers. DG's are generated in response to the stimulation of phosphoinositide breakdown by extracellular agents, acting then as a second messenger by activating protein kinase C (Nishizuka, 1984; Downes & Mitchell, 1985) together with calcium and phosphatidylserine. It has been also described that DG's enhance the activity of a variety of phospholipases when incorporated into their substrates (Dawson et al., 1983; Dawson et al., 1984). The generation of DG's has been observed in many different cells, eliciting a great variety of biological activities [see Kikkawa and Nishizuka (1986), Abdel-Latif (1986), and Berridge (1987) for recent reviews].

The crystalline structure of DG's has been studied, indicating that 1,3-DG's and 1,2-DG's have different structures. 1,3-DG molecules pack in a triclinic lattice with T parallel subcells and the chain extended on both sides of the glycerol (Larsson, 1963). 1,2-DG's, however, pack in a stable mono-

†This work was supported by Grants 3401(83)01 from CAICYT (Spain) and PA-86-0211-C02-01 from DGICYT (Spain).

[1] Abbreviations: ΔH, enthalpy change of the phase transition; DG, diacylglycerol; DCG, dicapryloylglycerol; DOG, dioleylglycerol; DPG, dipalmitoylglycerol; DPH, diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; T_c, onset temperature of the phase transition.

0006-2960/88/0427-9030$01.50/0

clinic crystalline form with orthorhombic-perpendicular subcell chain packing (Pascher et al., 1981). It is interesting that ^{13}C NMR studies, using ^{13}C-enriched DG's, show that these molecules lie parallel to phospholipid molecules in the bilayer and that both carbonyls are exposed to the aqueous layer. Thus, interaction at an interface causes major changes from the conformation in the crystal state in the 1,3-DG's, whereas 1,2-DG's at an aqueous interface have conformational states similar to that in the crystal (Small, 1986). In general, DG's undergo polymorphic changes presenting different crystalline structures (Larsson, 1966). The enthalpies of the corresponding transitions have been studied by Timms (1978).

It is known that DG's may produce structural changes in membranes, such as alteration of surface charge (Ohki et al., 1982), bilayer–nonbilayer phase transition (Dawson et al., 1983; Das & Rand, 1984; Cheng & Hui, 1986), or the spreading apart of the polar groups of the phospholipids and production of nonlamellar phases (Das & Rand, 1986). These structural changes may be relevant to the mechanism through which DG's exert their biological activities. Hence, we decided to study in some detail the interaction of a number of different DG's with membranes of DPPC, looking for physical changes in the membrane and phase behavior of the mixtures of DG's and DPPC, by using DSC and fluorescence anisotropy of the probe DPH.

First, we will compare the effects of 1,2-DPG with those of 1,2-DCG and 1,2-DOG. It is interesting to compare them since it has been described that DG's containing unsaturated fatty acids, such as 1,2-DOG, are more adequate for the activation in vitro of protein kinase C than 1,2-DG's containing two long-chain saturated fatty acids, such as 1,2-DPG (Kichimoto et al., 1980). Furthermore, DG's containing short-chain saturated fatty acids, such as 1,2-DCG, are as active as those containing long-chain unsaturated fatty acids for eliciting biological activities (Davis et al., 1985; Cabot & Jaken, 1984; Lapetina, 1985; Conn et al., 1985).

Second, we will compare the effects of 1,2-DG's with those of 1,3-DG's. This may be also relevant from the physiological point of view, because it has been shown in platelets (Nomura et al., 1986) that there is stereospecificity for the activation elicited by DG's, so that the 1,2-*sn* but not the 1,3-*sn* configuration activated protein kinase C directly.

These studies show indeed clear differences in the interaction of each type of DG with DPPC, and the significance of these differences will be discussed.

Experimental Procedures

Dipalmitoylphosphatidylcholine, 1,2-dioleylglycerol, 1,3-dioleylglycerol, and 1,3-dicapryloylglycerol were purchased from Avanti Polar Lipids (Birmingham, AL). 1,2-Dicapryloylglycerol, 1,2-dipalmitoylglycerol, and diphenylhexatriene (DPH) were from Sigma (Poole, Dorset, U.K.). All the other chemicals used in this work were of analytical grade. Water was twice distilled in an all-glass apparatus and deionized in a Milli-Q apparatus from Millipore.

Since diacylglycerols may undergo rapid racemization (Nomura et al., 1986), the purity of each isomer was checked by DSC, monitoring the melting points of the anhydrous DG's and confirming that only one isomer was present in each case.

The lipid mixtures for the microcalorimetry experiments were prepared by combination in a small glass tube of chloroform solutions containing 2 mg of DPPC (2.72 μmol) and the appropriate amounts of DG's, giving a final volume of 50–200 μL. The solvent was evaporated under an O_2-free N_2 stream, and the last traces of solvent were eliminated by desiccation under vacuum during a period of time longer than 4 h. After the addition of 50 μL of water, multilamellar liposomes were formed by carefully mixing in a bench vibrator and keeping the samples at 50–55 °C, i.e., above the temperature of the phase transition of pure DPPC. Mixing was continued until a homogeneous and uniform suspension was obtained.

A total of 15 μL of the above suspensions was then sealed in small aluminum pans and scanned in a Perkin-Elmer DSC-4 instrument, using a reference pan containing water. The heating rate was 4 °C/min. Peak areas were measured by weighing paper out-outs of the peaks. The instrument was calibrated with indium as standard.

In order to construct the phase diagrams, the main transition region was defined by the onset temperatures on heating and cooling experiments (Phillips et al., 1970). The onset temperatures were determined from the thermograms as previously described (Eliasz et al., 1976), and these were found to be reproducible to better than 0.5 deg. We omitted the pretransitions from the phase diagrams for simplicity.

The samples for fluorescence depolarization measurements were prepared by mixing 2 mg of DPPC, the appropriate amounts of DG's, and DPH to give a 200:1 DPPC/DPH molar ratio, all of them in chloroform solution. After evaporation as in the case for the microcalorimetry samples, 100 μL of water was added, and multilamellar vesicles were formed by shaking. The sample was diluted in water in the fluorescence cuvette until a maximum and constant reading of anisotropy was obtained, this indicating that light scattering was not affecting the measurements. A Shimadzu RF-540 spectrofluorometer instrument, equipped with Polaroid polarizers, was used. Fluorescence was measured by excitation of DPH at 360 nm, while emission was monitored at 430 nm parallel and perpendicular to the plane of excitation. Steady-state fluorescence anisotropy was calculated as described by Barenholz et al. (1976). Heating curves were constructed in all cases.

Lipid phosphorus was assayed by the method of Bartlett (1959). DG's were quantitatively assayed by gas–liquid chromatography, using internal standards, in the cases of DPG and DOG.

In the case of DCG, the estimation was done through the analysis of glycerol. Samples were treated with tetraethylammonium hydroxide which specifically hydrolyzes the acyl esters of glycerol and does not cleave phosphate esters (Brockerhoff, 1963). The procedure described by Chernick (1969) was followed without essential modifications. Free glycerol was then quantitatively assayed with an enzymatic coupled assay kit purchased from Boehringer Mannheim (Barcelona, Spain) according to the instructions given by the manufacturer. The solution assay included 0.24 mM glycylglycine, 3.2 mM $MgCl_2$, 100 mM KCl, 0.27 mM NADH, 1.06 mM ATP, 1.47 mM phosphoenolpyruvate, 1.9×10^3 IU of pyruvate kinase/L, 1.76×10^3 IU of lactate dehydrogenase/L, and 2.7×10^2 IU of glycerol kinase/L. The oxidation of NADH was followed at 340 nm.

The incorporation of DG's into liposomes was measured by centrifugation of the vesicles at 69500*g* during 20 min. Phospholipids and DG's were then analyzed in the pellet. The results obtained indicated that in all cases more than 90% of the initial DG's added to the sample were incorporated into the vesicles.

Results

Differential Scanning Calorimetry of 1,2-DG/DPPC Systems. Figure 1a shows that increasing concentrations of 1,2-DPG in DPPC multibilayers induce a progressive decrease

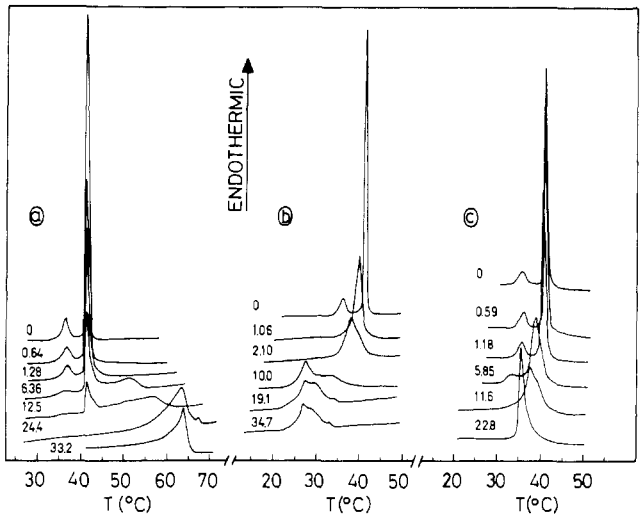

FIGURE 1: (a) DSC thermograms for the mixture DPPC/1,2-DPG. Mole percent of 1,2-DPG is expressed on the curves. Only heating experiments are shown. (b) DSC thermograms for the mixture DPPC/1,2-DCG. Mole percent of 1,2-DCG is expressed on the curves. Only heating experiments are shown. (c) DSC thermograms for the mixture DPPC/1,2-DOG. Mole percent of 1,2-DOG is expressed on the curves. Only heating experiments are shown.

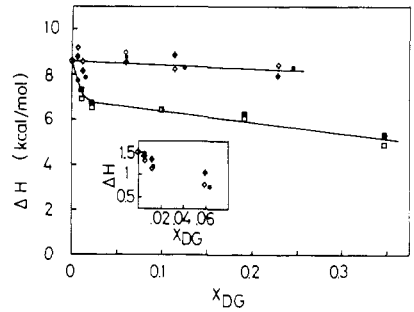

FIGURE 2: Dependence of the ΔH of the main gel to liquid-crystalline phase transition on the molar fraction of (●) 1,2-DPG, (□) 1,2-DCG, (◇) 1,2-DOG, (■) 1,3-DCG, and (◆) 1,3-DOG. The insert shows the ΔH of the pretransition for (●) 1,2-DPG, (◇) 1,2-DOG, and (◆) 1,3-DOG.

in the area under the pretransition peak, this peak totally disappearing at 12.5 mol % 1,2-DPG. The variations of ΔH of the pretransition are shown in the insert of Figure 2. The main transition is also affected by the presence of 1,2-DPG, with a new peak arising at temperatures higher than that of the main endotherm. This new peak is observed at increasing temperatures as the percentage of 1,2-DPG is increased, and it seems to enlarge at the expense of the main transition. Apparently a new phase, rich in 1,2-DPG, separates laterally when the mole percent of this DG reaches a certain value (greater than 6.36 mol %), and this new phase is the prevalent one at 33.2 mol %. However the total ΔH, i.e., the addition of the ΔH values of both peaks, does not change in the range of 1,2-DPG concentrations tested (Figure 2). We have not studied the effect of higher concentrations of 1,2-DPG in the membrane which could lead to the appearance of nonlamellar phases (Das & Rand, 1986) because these high concentrations are not likely to be present in biological membranes.

The interaction of 1,2-DCG with DPPC seems to be different than that of 1,2-DPG with DPPC. Figure 1b shows that 1.06 mol % 1,2-DCG already makes the pretransition disappear, while the main transition is broadened and shifted to lower temperatures. At 2.1 mol % the transition endotherm has been more displaced with respect to pure DPPC, and it appears to have a shoulder on its high-temperature side. At 10 mol % two peaks are clearly distinguished, indicating the

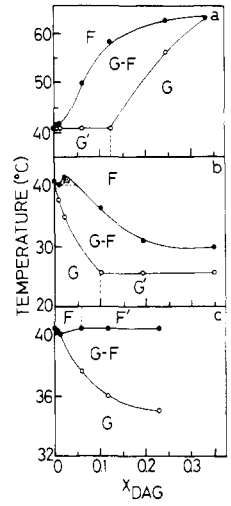

FIGURE 3: Partial phase diagrams obtained from heating and cooling DSC experiments for the mixtures of DPPC with (a) 1,2-DPG, (b) 1,2-DCG, and (c) 1,2-DOG. (●) correspond to the fluidus line and (○) to the solidus line. The points were obtained as described under Experimental Procedures. F indicates a fluid phase and G a gel phase. F′ and G′ indicate that different fluid or gel phases coexist. Dashed lines separate different regions of the diagram.

presence of two phases, one of them rich in DPPC and the other in 1,2-DCG. At 19.1 mol % three peaks are distinguishable, corresponding probably to three phases with different compositions, and at 34.7 mol % the same pattern is seen, the most important peak appearing at the lowest temperature. Figure 2 shows that total ΔH is decreased as the concentration of 1,2-DCG is increased, in contrast with the trend observed for 1,2-DPG (see above).

The effect of 1,2-DOG is presented in Figure 1c. It can be seen that concentrations of 1,2-DOG up to 1.18 mol % affect only slightly the DPPC thermogram. At 5.85 mol % the pretransition is slightly decreased and shifted to lower temperatures, completely disappearing at 11.6 mol %. ΔH values for the pretransition are shown in the insert of Figure 2. On the other hand, the main transition is broadened and shifted to lower temperatures at 5.85 mol %. At 11.6 mol % a composite transition is found again. Figure 2 shows that ΔH does not change with the incorporation of 1,2-DOG, remaining similar to that of pure DPPC, as was found for 1,2-DPG, but different from that of 1,2-DCG.

On the basis of these calorimetric results corresponding to 1,2-DG's partial phase diagrams were elaborated, and they are shown in Figure 3. The pretransitions were omitted for the sake of simplicity.

Totally different partial phase diagrams were found for the three types of mixtures studied here. It can be seen in the mixture DPPC/1,2-DPG (Figure 3a) that a gel separation takes place at concentrations below 12.5 mol % DPG. However, the fluidus line indicates good miscibility in the liquid-crystalline state. A homogeneous phase is observed at 33.2 mol % 1,2-DPG, indicating that a single component is present at a molar ratio DPPC/1,2-DPG of 2:1. 1,2-DCG, however, shows as the main feature that gel-phase immiscibility appears at concentrations higher than 10 mol % (Figure 3b). Apart from that, a fluid-phase separation may occur at low concentrations of DCG with an eutectic point at 1.06 mol %. Very interesting is the case of DPPC/1,2-DOG mixtures (Figure 3c) where fluid-phase immiscibility appears at concentrations higher than 0.59 mol %. On the other hand, the solidus line indicates good miscibility in the gel phase.

Differential Scanning Calorimetry of 1,3-DG/DPPC Systems. Two 1,3-*sn*-DG's were tested: 1,3-DCG and 1,3-DOG.

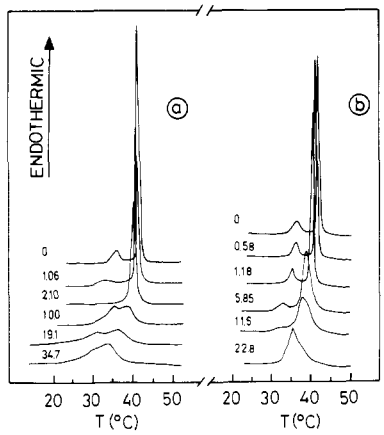

FIGURE 4: (a) DSC thermograms for the mixture DPPC/1,3-DCG. Mole percent of 1,3-DCG is expressed on the curves. Only heating experiments are shown. (b) DSC thermograms for the mixture DPPC/1,3-DOG. Mole percent of 1,3-DOG is expressed on the curves. Only heating experiments are shown.

Figure 4a shows DSC measurements of increasing concentrations of 1,3-DCG incorporated into DPPC multibilayers. The effect is to decrease the onset of the main phase transition, the pretransition being very much broadened at 0.0106 mol % and totally abolished at 0.021 mol %. The effect is apparently similar to that observed for 1,2-DCG (Figure 1b) in the sense that more than one peak is also observed at concentrations higher than 0.1 mol % 1,3-DCG, and the explanation for this fact may be also that there is a separation in phases with different composition. Also, total ΔH for 1,3-DCG decreases similarly as was found for 1,2-DCG-containing samples (Figure 2).

The DSC measurements of 1,3-DOG/DPPC samples (Figure 4b) show a similar pattern to that of 1,2-DOG-containing samples (Figure 1c). It is found again that the pretransition does not disappear until concentrations higher than 5.85 mol % are reached, with the main T_c being shifted to lower temperatures. In samples containing 11.6 mol % 1.3-DOG a broad and asymmetric peak is observed, and also at 22.81 mol % a broad peak is found. This highest concentration is the only one at which a clear difference is observed when the thermograms of 1,3-DOG/DPPC samples are compared with those corresponding to 1,2-DOG/DPPC samples where a narrower peak was observed (Figure 1c). The variation of ΔH of the pretransition is shown in the insert of Figure 2, and this figure also shows that total ΔH does not change in the range of concentrations of 1,3-DOG studied here.

Figure 5 shows the phase diagrams corresponding to the two 1,3-*sn*-DG's stereoisomers, constructed similarly to those of Figure 3. It is remarkable that the diagram corresponding to 1,3-DCG (Figure 5a) shows a pattern different from that of 1,2-DCG (Figure 3b). Whereas good miscibility is observed here for the gel phase, fluid immiscibility seems to happen up to 10 mol % 1,3-DCG, in contrast to the gel immiscibility observed in 1,2-DCG samples. On the other hand, 1,3-DOG/DPPC samples give a phase diagram very similar to that observed for 1,2-DOG/DPPC samples, with fluid-phase immiscibility.

Fluorescence Anisotropy of DPH. In order to investigate the effect of the diacylglycerols on the fluidity of the DPPC bilayer, the steady-state emission anisotropy of DPH included in DPPC multibilayers containing different DG's was studied at different temperatures. It was seen that 1,2-DPG (Figure 6a) induced a shift to higher temperatures with two transitions in the sample containing 6.36 mol % whereas 10 mol % 1,2-DCG induces a shift of the transition to a lower temperature

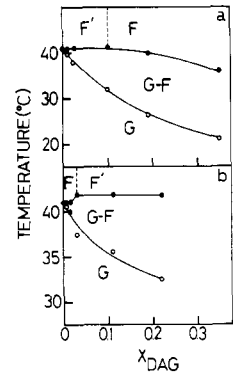

FIGURE 5: Partial phase diagrams obtained from heating and cooling DSC experiments for the mixtures of DPPC with (a) 1,3-DCG and (b) 1,3-DOG. (●) correspond to the fluidus line and (○) to the solidus line. The points were obtained as described under Experimental Procedures. F indicates a fluid phase and G a gel phase. F′ and G′ indicate that different fluid or gel phases coexist. Dashed lines separate different regions of the diagram.

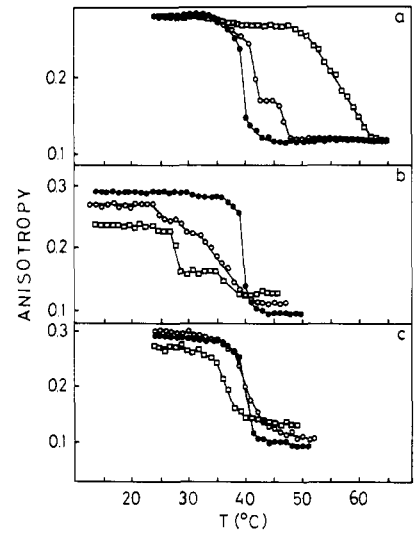

FIGURE 6: Dependence of the anisotropy parameter of DPH incorporated in the bilayer for the mixtures of DPPC with the following: (a) 1,2-DPG, (●) pure DPPC, (○) DPPC containing 6.36 mol % 1,2-DPG, and (□) DPPC containing 24.38 mol % 1,2-DPG; (b) 1,2-DCG, (●) pure DPPC, (○) DPPC containing 10 mol % 1,2-DCG, and (□) DPPC containing 34.37 mol % 1,2-DCG; (c) 1,2-DOG, (●) pure DPPC, (○) DPPC containing 5.85 mol % 1,2-DOG, and (□) DPPC containing 22.81 mol % 1,2-DOG.

(Figure 6b). These results closely agree with those found by DSC. It is noteworthy that the two transitions observed in DSC for samples containing 6.36 mol % 1,2-DPG are also appreciated, and exactly at the same temperatures, by the changes in anisotropy of DPH with temperature. As observed, the anisotropy values are not changed by 1,2-DPG neither above nor below the phase transition interval, whereas 10 mol % 1,2-DCG changes them both above and below this interval but mainly below the phase transition. The changes in anisotropy induced by 1,2-DCG indicate that this molecule decreases the apparent order of the phospholipid acyl chains in the gel phase, and on the contrary, it increases this order in the fluid state. On the other hand, 1,2-DPG does not change the apparent order of the acyl chains either above or below the phase transition.

The effect of 1,2-DOG (Figure 6c) is more similar to that of 1,2-DCG than the effect observed for 1,2-DPG. At temperatures below the phase transition, and at 5.85 mol % 1,2-DOG, the anisotropy parameter of DPH is practically not changed with respect to that of pure DPPC. However, above the phase transition the anisotropy increases. At 22.81 mol

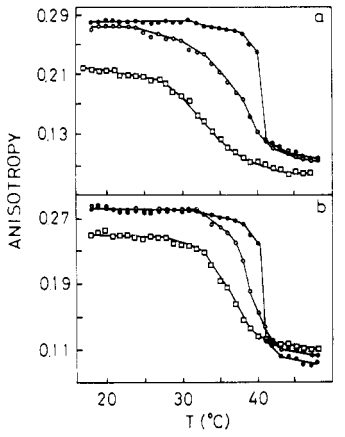

FIGURE 7: Dependence of the anisotropy parameter of DPH incorporated in the bilayer for the mixtures of DPPC with the following: (a) 1,3-DCG, (●) pure DPPC, (○) DPPC containing 10 mol % 1,3-DCG, and (□) DPPC containing 34.37 mol % 1,3-DCG; (b) 1,3-DOG, (●) pure DPPC, (○) DPPC containing 5.85 mol % 1,3-DOG, and (□) DPPC containing 22.81 mol % 1,3-DOG.

% the anisotropy decreases in the gel phase and increases in the fluid phase.

In the case of 1,3-DCG (Figure 7a) we find again a different pattern from that found for 1,2-DCG-containing samples. 1,3-DCG at 10 mol % shifts and broadens the phase transition as observed in DSC but does not appreciably change the anisotropy values outside of the phase transition interval, compared with pure DPPC. Still more striking, at 34.7 mol % 1,3-DCG, the anisotropy is clearly decreased at all the temperatures where measurements were done, with respect to pure DPPC, but more so below the phase transition. The several phases observed in DSC are not well resolved with this technique, as in the case of 1,2-DCG. The results obtained with 1,3-DOG/DPPC samples (Figure 7b) are however fairly similar to those of 1,2-DOG reported in Figure 6c. The 5.85 mol % 1,3-DOG sample shows a shift in the phase transition and no variation in anisotropy below the phase transition, with a slight decrease above of the phase transition. The 22.81 mol % sample shows a decrease in anisotropy below the phase transition and an increase above the phase transition interval, comparing with pure DPPC in all cases.

Discussion

The interaction between DG's and DPPC may affect the structure of the lamellar phase formed by the pure phospholipid. This may occur by changing the van der Waals interactions between the phospholipid hydrocarbon chains and also by affecting the repulsions of the choline head groups (Eliasz et al., 1976). On the other hand, the presence of DG's in phosphatidylcholine bilayers has been shown to spread apart the polar head groups of the phospholipid without changing the bilayer thickness (Das & Rand, 1984). Relatively high concentrations of DG's may induce also the formation of nonbilayer structures. Although these changes in structure will depend on the type of DG's and phosphatidylcholine considered, it seems from the results found by Das and Rand (1984) that most of our samples do not suffer this type of change.

Since DG's may perturb the phospholipid matrix in such a variety of ways, it is not surprising to find complex behavior for the DG/DPPC mixtures that we have examined. Furthermore, these interactions may be expected to be substantially different if we compare the effect of DG's bearing fully saturated fatty acids with that of those having unsaturated ones, or the effect of DG's formed by fatty acids with different chain lengths and even the effect of different stereoisomers.

We will discuss first the case of 1,2-DPG which has the same fatty acid chains as DPPC. The corresponding partial phase diagram (Figure 3a) shows gel immiscibility, and in general, the pattern has a certain similitude to that observed before for the mixture palmitic acid/DPPC (Schullery et al., 1981; Koynova, 1987). The possible explanation of the thermograms and phase diagrams would be that the reticular structure formed by the DPPC molecules below the main T_c transition temperature will have a very limited capability of accommodating 1,2-DPG molecules, so that at 6.6 mol % 1,2-DPG two peaks are already clearly seen (Figure 1a), due to lateral segregation of two phases, one of them rich in DPPC and the other one richer in 1,2-DPG. As more 1,2-DPG is incorporated, the phase rich in 1,2-DPG will have a quantitative predominance. This phase will be gradually richer in 1,2-DPG, and the separation of the bulky polar groups of DPPC will stabilize the membrane, thus producing the observed increase in T_c.

A homogeneous phase is reached at a 2:1 DPPC/1,2-DPG molar ratio. It is interesting that it was observed for the system palmitic acid/DPPC (Schullery et al., 1981; Koynova, 1987) that a homogeneous phase was formed at a 2:1 palmitic acid/DPPC molar ratio. On the other hand, above of the main T_c transition temperature a good mixing of both lipids seems to occur.

The perturbation induced by 1,2-DPG seems to be restricted to the lipid/water interface, and the fluorescent probe experiments show (Figure 6a) that it does not perturb the order of the fatty acyl chains neither above nor below the phase transition interval.

The effect of 1,2-DCG is rather different from that of 1,2-DPG. The relatively short fatty acid chains of 1,2-DCG will position this molecule anchored to the lipid/water interface and close packed in a hexagonal lattice with the palmitoyl chains, so that it will decrease the van der Waals interactions between the terminal methyl and methylene groups of the phospholipid hydrocarbon chains. This is expected to considerably lower the lipid main transition temperature. This effect will counter the dispersion of the head groups produced by the DG, which is expected to increase T_c and which is dominant in samples containing 1,2-DPG. In fact, the transition temperatures of 1,2-DCG-containing samples decrease with respect to that of pure DPPC (Figure 2). Free octanoic acid was also found to decrease the main T_c of DPPC (Eliasz et al., 1976). Nevertheless, the interactions between 1,2-DCG and DPPC are complex as revealed by the thermograms (Figure 1b) and by the partial phase diagram (Figure 3b). At low concentrations (0.1 mol% 1,2-DCG) there is a good miscibility in the gel phase, but the fluidus line shows the presence of a eutectic point at about 0.5 mol %. At concentrations of DCG higher than 10 mol % there seems to be a solid-phase immiscibility.

The effect of 1,2-DCG as seen by the fluorescence anisotropy of DPH is also remarkable, since it produces a decrease in the order of the DPPC bilayer at temperatures below T_c but an increase in this order at temperatures above T_c. The molecular basis for these complex effects are not clear, but we suggest that below T_c the disruption of the van der Waals interactions of the methyl and methylene groups of the phospholipid hydrocarbon chains will be the predominant effect giving a fluidification of the membrane whereas at temperatures above T_c the spacing of the polar head groups of the DPPC molecules by the interposition of the 1,2-DCG molecules decreasing the van der Waals repulsions will be the predominant effect, thus increasing the membrane order.

It is very interesting that 1,3-DCG does not behave like 1,2-DCG. This may be appreciated in the corresponding phase diagram (Figure 5a) where neither the solidus line shows immiscibilities in the gel phase nor the fluidus line indicates immiscibilities in the fluid phase. On the other hand, the experiments measuring the fluorescence anisotropy of DPH show also clear differences with respect to those where samples containing 1,2-DCG were used. First, in the samples containing 1,2-DCG there are different well-resolved peaks, but in the 1,3-DCG-containing samples only wide transitions are seen, although they are shifted with respect to pure DPPC. Second, the fluorescence anisotropy of DPH in the sample containing 19.1 mol % does not change very much with respect to the pure phospholipid, neither above nor below the phase transition interval, and in the sample containing 34.7 mol % 1,3-DCG the anisotropy decreases with respect to pure DPPC in the entire range of temperatures. It is then clear that both stereoisomers interact in a different way with DPPC. Although other experiments should be done to clarify, in molecular terms, the reason for these different types of interactions, it is very interesting to keep in mind that 1,2-DCG has been shown to be capable of stimulating protein kinase C activity in platelets, but 1,3-DCG has been shown to be inactive (Nomura et al., 1986).

With respect to 1,2-DOG/DPPC samples, the patterns found in the DSC thermograms are different from those of the other DG's already considered. Free cis-unsaturated fatty acids have also been shown to behave differently from saturated ones when incorporated into membranes to fully saturated phosphatidylcholines (Ortiz & Gómez-Fernández, 1987). This is to be expected since, apart from affecting the polar head group repulsion of the phosphocholine head groups, the double bonds in the cis form are bulky and thus they will interfere with the interchain interactions of phosphatides. 1,2-DCG certainly causes the phase transition to decrease, but at very low concentrations an eutectic point is appreciated in the partial phase diagram (Figure 3b), showing also a fluid immiscibility at concentrations higher than 6.6 mol %.

The fluorescence anisotropy of DPH is however not very different from that of 1,2-DCG, indicating that at least in the sample with 22.81 mol % 1,2-DOG, and at temperatures below the phase transition, the order of the acyl chains decreases in its presence with respect to pure DPPC, but the order is increased above of the phase transition. 1,3-DOG behaves similarly to 1,2-DOG, at least from a qualitative point of view, in both DSC and fluorescence experiments.

An important aspect to be commented on is that whereas 1,2-DPG produces an increase in the transition temperature compared with pure DPPC, the isomers containing either short or cis-unsaturated fatty acids tend to cause this transition temperature to decrease. Something similar has been previously observed for free fatty acids (Eliasz et al., 1976; Klausner et al., 1980; Ortiz & Gómez-Fernández, 1987), and after solution theory, this can be attributed to their different tendencies of partitioning between fluid and solid-like domains: those increasing T_c partition in solid-like domains, and those decreasing T_c partition in fluid domains (Klausner et al., 1980). This may be important in biological membranes, since the existence of lipid domains in biological membranes has been claimed by some workers, giving place to lateral (Wolf & Voglmayer, 1984; Metcalfe et al., 1986) and transverse (Schroeder, 1978; Cogan & Schachter, 1981; Seigneuret et al., 1984) gradients in fluidity. Apart from free cis-unsaturated fatty acids (Ortiz & Gómez-Fernández, 1987), other lipid molecules like cholesterol (De Kruijff et al., 1974) or α-tocopherol (Ortiz et al., 1987) have been found previously to preferentially partition into fluid domains. The reason for this preference must be, in all the cases, that they do not adequately fit in the hexagonal array of the hydrocarbon chains of the phospholipids in the gel state. This is interesting since it has been shown (Go et al., 1987) that DG's containing either cis-unsaturated chains or short chains (10 or less carbons) are active whereas those which are fully saturated are inactive, so that those partitioning in fluid domains are active and, however, those partitioning in solid-like domains are inactive. This may be relevant to the specificity appreciated for some DG's in the activation of protein kinase C, but other factors must be implicated since, for example, 1,3-DCG, which preferentially partitions into fluid domains and shows fluid-phase immiscibilities, is not active as discussed above.

Another interesting observation made in our experiments is that some DG's may produce fluid-phase separations. The DG's giving this effect are 1,2-DOG, 1,2-DCG, and 1,3-DOG. These fluid immiscibilities are not very oftenly described in the literature, and the first case in which one of them was observed was the binary mixture dipalmitoylphosphatidylethanolamine and dieladoylphosphatidylethanolamine (Wu & McConnell, 1975). This type of immiscibility was predicted on the basis of theoretical calculations for mixtures of DPPC and anesthetics (De Verteuil et al., 1981), where a relatively strong interaction between the anesthetics was supposed, so that clusters were formed. Free cis-unsaturated fatty acids have also been shown (Ortiz & Gómez-Fernández, 1987) to produce these fluid immiscibilities. It may be supposed that these DG's may cluster and this may produce the observed fluid-phase immiscibilities.

The importance of fluid immiscibilities and of the partition of certain DG's in fluid domains can be fully appreciated if we consider the concentration of DG's in a biological membrane. This has not been studied in detail in many cases. Nevertheless, it has been reported, for example, that in fibroblasts supplied with [^{3}H]glycerol about 5% of the radioactivity present in lipids was localized in diacylglycerols in the steady state, and this proportion was increased to up to 11% in *ras*-transformed cells (Wolfman & Macara, 1987). These proportions are relatively low, but they could be substantially higher in certain areas of the membrane if DG's are capable of clustering and partitioning into particular domains.

The ability of certain DG's to modify the properties of specific parts of the membrane, such as lamellar to hexagonal phase transitions, membrane curvature, membrane fusion, or other structural changes which may allow the formation of favorably areas in the membrane to interact with protein kinase C, can explain why some DG's are more active than others. Nevertheless, specific interaction between some DG's and the protein are probably an important mechanism to explain the specificity observed for the activation of protein kinase C by these compounds.

Finally, the different way in which DG's affect membrane order (membrane fluidity), as has been shown by our experiments using the probe DPH, may be also important in determining the specific effects of each DG in the membrane.

The use of other physical techniques may help to complete the picture of how DG's may perturb phospholipid membranes and how these perturbations mediate their biological effects.

Registry No. 1,2-DPG, 761-35-3; 1,2-DCG, 1069-87-0; 1,2-DOG, 2442-61-7; 1,3-DOG, 2465-32-9; 1,3-DCG, 1429-66-9; DPPC, 2644-64-6.

REFERENCES

Abdel-Latif, A. A. (1986) *Pharmacol. Rev. 38*, 227–272.

Barenholz, Y., Moore, N. F., & Wagner, R. R. (1976) *Biochemistry 15*, 3563–3570.
Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–471.
Berridge, M. J. (1987) *Annu. Rev. Biochem. 56*, 159–193.
Brockerhoff, H. (1963) *J. Lipid Res. 4*, 96–102.
Cabot, M. C., & Jaken, S. (1984) *Biochem. Biophys. Res. Commun. 125*, 163–169.
Cheng, K. H., & Hui, S. W. (1986) *Arch. Biochem. Biophys. 244*, 382–386.
Chernick, S. S. (1969) *Methods Enzymol. 14*, 627–630.
Cogan, U., & Schachter, D. (1981) *Biochemistry 20*, 6396–6403.
Conn, M., Ganong, B. E., Ebeling, J., Staley, D., Niedel, J. E., & Bell, R. M. (1985) *Biochem. Biophys. Res. Commun. 126*, 532–539.
Das, S., & Rand, R. P. (1984) *Biochem. Biophys. Res. Commun. 124*, 491–496.
Das, S., & Rand, R. P. (1986) *Biochemistry 25*, 2882–2889.
Davis, R. J., Ganong, B. R., Bell, R. M., & Czech, M. P. (1985) *J. Biol. Chem. 260*, 1562–1566.
Dawson, R. M. C., Hennington, N. L., & Irvine, R. F. (1983) *Biochem. Biophys. Res. Commun. 117*, 196–201.
Dawson, R. M. C., Irvine, R. F., Bray, J., & Quinn, P. J. (1984) *Biochem. Biophys. Res. Commun. 125*, 836–842.
De Kruijff, B., Van Dijck, P. W. M., Demel, R. A., Schuijff, A., Brants, F., & Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 356*, 1–7.
De Verteuil, F., Pink, D. A., Vadas, E. B., & Zuckermann, A. (1981) *Biochim. Biophys. Acta 640*, 207–222.
Downes, C. P., & Mitchell, R. H. (1985) in *Molecular Mechanisms of Transmembrane Signalling* (Cohen, P., & Houslay, M., Eds.) pp 3–56, Elsevier, New York.
Eliasz, A. W., Chapman, D., & Ewing, D. F. (1976) *Biochim. Biophys. Acta 448*, 220–230.
Go, M., Sekiguchi, K., Nomura, H., Kikkawa, U., & Nishizuka, Y. (1987) *Biochem. Biophys. Res. Commun. 144*, 598–605.
Kikkawa, U., & Nishizuka, Y. (1986) *Annu. Rev. Cell Biol. 2*, 149–178.
Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., & Nishizuka, Y. (1980) *J. Biol. Chem. 255*, 2273–2276.
Klausner, R. D., Kleinfeld, A. M., Hoover, R. L., & Karnovsky, M. J. (1980) *J. Biol. Chem. 255*, 1286–1295.
Koynova, R. D., Boyanov, A. I., & Tenchov, B. G. (1987) *Biochim. Biophys. Acta 903*, 186–196.
Lapetina, E. G., Reep, B., Ganong, B. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 1358–1364.
Larsson, K. (1963) *Acta Crystallogr. 16*, 741–748.
Larsson, K. (1966) *Acta Chem. Scand. 20*, 2255–2260.
Metcalfe, T. N., Wang, J. L., & Schindler, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 95–99.
Nishizuka, Y. (1984) *Nature (London) 308*, 693–698.
Nomura, H., Ase, K., Sekiguchi, K., Kikkawa, U., Nishizuka, Y., Nakano, Y., & Saton, T. (1986) *Biochem. Biophys. Res. Commun. 140*, 1143–1151.
Ohki, K., Sekiya, T., Yamauchi, T., & Nozawa, Y. (1982) *Biochim. Biophys. Acta 693*, 341–350.
Ortiz, A., & Gõmez-Fernândez, J. C. (1987) *Chem. Phys. Lipids 45*, 75–91.
Ortiz, A., Aranda, F. J., & Gõmez-Fernândez, J. C. (1987) *Biochim. Biophys. Acta 898*, 214–222.
Pascher, I., Sundell, S., & Hauser, H. (1981) *J. Mol. Biol. 153*, 791–806.
Phillips, M. C., Ladbrooke, B. D., & Chapman, D. (1970) *Biochim. Biophys. Acta 196*, 35–44.
Schroeder, F. (1978) *Nature (London) 276*, 528–530.
Schullery, S. E., Seder, T. A., Weinstein, D. A., & Bryant, D. A. (1981) *Biochemistry 20*, 6818–6824.
Seigneuret, M., Zachowski, A., Hermann, A., & Devaux, P. F. (1984) *Biochemistry 23*, 4271–4275.
Small, D. M. (1986) in *The Physical Chemistry of Lipids. From Alkanes to Phospholipids*, Chapter 10, Plenum, New York.
Timms, R. E. (1978) *Chem. Phys. Lipids 21*, 113–129.
Wolf, D. E., & Voglmayer, J. K. (1984) *J. Cell Biol. 98*, 1678–1684.
Wolfman, A., & Macara, I. G. (1987) *Nature (London) 325*, 359–361.
Wu, S. H., & McConnell, H. M. (1975) *Biochemistry 14*, 847–854.