Diacylglycerol and the Promotion of Lamellar-Hexagonal and Lamellar-Isotropic Phase Transitions in Lipids: Implications for Membrane Fusion

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ABSTRACT Changes in steady-state fluorescence anisotropy of 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) are applied to the detection of lamellar-hexagonal transitions in egg phosphatidylethanolamine. Even low (2 mole%) proportions of diacylglycerol decrease the hexagonal transition temperature considerably, as confirmed by differential scanning calorimetry. Diacylglycerol is also found to promote a lamellar to "isotropic" (Q^{224} cubic) transition in mixtures of phosphatidylcholine:phosphatidylethanolamine:cholesterol. This nonreversible transition is also observed by ^{31}P nuclear magnetic resonance and detected as a large increase in TMA-DPH steady-state anisotropy. The same technique reveals as well that lysophosphatidylcholine counteracts the effect of diacylglycerol and stabilizes the lamellar phase in both transitions. Diacylglycerol and lysophosphatidylcholine are known to respectively promote and inhibit membrane fusion in a variety of systems. These data are interpreted in support of the hypothesis of a highly bent structural fusion intermediate ("stalk"). They also show the interest of lipid-phase studies in predicting and rationalizing membrane fusion mechanisms.

INTRODUCTION

The well-established concept of a lipid bilayer as the structural basis of cell membranes is nonetheless compatible with the idea of transient nonbilayer structures playing important roles in various aspects of cell physiology. In particular, lipid structures of the kind represented by the so-called hexagonal II phase and the various cubic phases (Luzzati, 1968) have been invoked to explain various membrane phenomena, e.g., ion transport by membrane proteins (Cheng et al., 1986), intercell contacts (Hein et al., 1992), or membrane fusion (Seddon, 1990; Walter et al., 1994; Chernomordik and Zimmerberg, 1995; Chernomordik et al., 1995a,b). In turn, the involvement of nonlamellar structures in cell physiology or cell pathology processes has given rise to a renewed interest in lamellar to nonlamellar phase transitions in model systems (Hong et al., 1988; Epand et al., 1988; Siegel et al., 1989; van Langen et al., 1989; Seddon, 1990; Chen and Cheng, 1990; Allen et al., 1990; Han and Gross, 1992; Yao et al., 1992; Epand et al., 1992; Luzzati et al., 1993; Ambrosini et al., 1994; Siegel et al., 1994; Koynova and Caffrey, 1994; Goldberg et al., 1994).

Previous studies from this laboratory have described a model system for membrane fusion in which phospholipase C promotes fusion of large unilamellar liposomes via the in situ generation of diacylglycerol (Nieva et al., 1989, 1993, 1995; Burger et al., 1991; Goñi et al., 1994). This system, which has been further explored by other authors for the case of small unilamellar vesicles (Luk et al., 1993), appears to be the only fusion model in which fusion is driven by a catalytic agent. Under our conditions, optimum fusion is observed with liposomes made of PC:PE:CH (2:1:1, molar ratio) (Nieva et al., 1989). Recent nuclear magnetic resonance (NMR) and x-ray diffraction data (Nieva et al., 1995) suggest that, although the above lipid mixture gives rise to stable lamellae, the substitution of 5–10% of phospholipids by diacylglycerol greatly facilitates the formation of H_{II} hexagonal or cubic phases.

In view of these observations, a series of experiments has been carried out in our laboratory exploring the lamellar to nonlamellar transitions of pure phosphatidylethanolamine (PE) and PC:PE:CH mixtures by means of changes in fluorescence anisotropy of DPH-containing probes, mainly 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH).The latter probe has been extensively used to detect lipid phase transitions (van Langen et al., 1989; Cheng, 1990; Ambrosini et al., 1994), and we have demonstrated its application to monitoring the thermotropic transition from the lamellar to the H_{II} hexagonal phase in an unsaturated PE, and the requirement of vesicle aggregation for the transition to take place (Nieva et al., 1990). In the present paper we extend the application of TMA-DPH to the detection of lamellar-isotropic (cubic) phase transitions, and we report on our observations of the ability of diacylglycerol to facilitate lamellar to nonlamellar transitions in pure PE and in PC:PE:CH mixtures, supporting the proposed mechanism for phospholipase C-promoted liposome fusion. Occasionally, ^{31}P-NMR and differential scanning calorimetry (DSC) have been used in support of our fluorescence measurements. Moreover, lysolecithin has been shown to inhibit fusion in our system (Nieva et al., 1993), as well as in others (Chernomordik et al., 1993, 1995a); the fluorescence anisotropy data indicate that this lipid stabilizes the lamellar phase in systems containing PE, thus opposing the effect of diacylglycerols.

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MATERIALS AND METHODS

Egg phosphatidylcholine (PC), egg PE, and a 1,2-diacylglycerol derived from egg PC were grade I from Lipid Products (South Nutfield, England); egg lysolecithin was from Avanti Polar Lipids (Alabaster, AL); cholesterol was from Sigma (St. Louis, MO). Diacylglycerols contained a trace of the 1,3-isomer. All lipids were used without further purification. DPH, PC-DPH, and TMA-DPH were from Molecular Probes (Eugene, OR).

Lipids and probe were mixed at a 500:1 ratio in organic solvent; then the solvent was evaporated and the mixture was vacuum-dried for at least 2 h in the dark. In mixtures containing PC:PE:CH (2:1:1 mol ratio) and diacylglycerol or lysolecithin, the latter components are substituting for part of the phospholipid, so the composition of a mixture with, e.g., 10% diacylglycerol is PC:PE:CH:diacylglycerol (43:22:25:10). Multilamellar PE vesicles were formed in borate buffer (5 mM borate, 150 mM NaCl, 0.1 mM EDTA, pH 9.5) at room temperature and pelleted by centrifugation in a bench-top centrifuge for the spectroscopic or calorimetric studies. When required, PE liposomes were dialyzed against citrate buffer (10 mM citrate, 150 mM NaCl, 0.1 mM EDTA, pH 5.0) at 15°C, which induces liposome aggregation. Vesicles based on PC:PE:CH mixtures were prepared in HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.0) and pelleted by centrifugation for spectroscopic analysis.

Steady-state fluorescence anisotropy was recorded in a MPF-66 Perkin-Elmer fluorometer. TMA-DPH fluorescence was excited at 360 nm; emission was recorded at 430 nm. Lipid dispersions were examined using a frontal disposition, in a thermostated assembly consisting of two microscope cover slides cut to fit diagonally a standard 1 × 1 cm fluorescence cuvette filled with water. The pelleted or aggregated lipid was located between the slides, and the four sides were tightly sealed with plasticine (Nieva et al., 1990). A 390-nm cutoff filter was used to prevent scattered light from reaching the detector. The expression used to calculate anisotropy (R) is described by Lakowicz (1983). Samples were heated at 10°C/h. For PE-based samples, data from the first run were neglected; otherwise, subsequent runs gave similar results. In the case of PC:PE:CH mixtures with diacylglycerol, the heating effects were irreversible; thus only one heating run was performed.

Differential scanning calorimetry was performed in a Perkin-Elmer DSC-2C calorimeter operating in the low-temperature mode, with solid CO_2/methanol as a coolant, with a heating or cooling rate of 1.25 or 5°C/min. The aggregated lipid (~10 μl) was enclosed in Perkin-Elmer aluminum "volatile" sample pans. At least two runs were performed on each sample, neglecting the data from the first run.

^{31}P-NMR spectra were recorded in a KM360 Varian spectrometer operating at 300 MHz for protons. Spectral parameters were 45° pulses (10 μs), pulse interval 3s, sweep width 16 kHz, full proton decoupling. One thousand FID were routinely accumulated from each sample; the spectra were plotted with a line broadening of 80 Hz. Samples were equilibrated for 10 min at each temperature before data acquisition.

RESULTS

Phase transitions in pure PE

Changes in anisotropy of DPH, PC-DPH, and TMA-DPH dispersed in egg PE at pH 5.0 (i.e., in the form of lipid aggregates) are shown in Fig. 1 *A* as a function of temperature. All three probes detect a transition starting near 10°C, corresponding to a hydrocarbon chain order-disorder conformational transition (Allen et al., 1990; Nieva et al., 1990). In addition, PC-DPH and TMA-DPH, but not DPH, detect a second transition whose onset is located at ~30°C. The latter change corresponds to a lamellar (L_α) to hexagonal (H_{II}) phase transition (Seddon, 1990; Allen et al., 1990; Cheng, 1990; Nieva et al., 1990; Castresana et al., 1992). There is a wide variation in the published onset temperatures of this transition (T_h), because of the natural variability of the lipid fatty acid composition. Chen and Cheng (1990) have shown the advantages of PC-DPH in the study of this system by time-resolved fluorescence anisotropy, but it is clear from Fig. 1 *A* that the largest signal is obtained from TMA-DPH in the steady state, so that this probe was used in all further experiments. As discussed in our previous paper (Nieva et al., 1990), for the L_α-H_{II} transition to occur, lipid vesicles must first be aggregated, e.g., by dialysis against pH 5.0 buffer. This is seen in Fig. 1 *B*, in which the thermotropic effects on TMA-DPH anisotropy are comparatively shown at pH 5.0 and pH 9.5. The L_α-H_{II} transition is not observed at the higher pH because PE is negatively charged under such conditions, and aggregation is hindered by electrostatic forces (Allen et al., 1990).

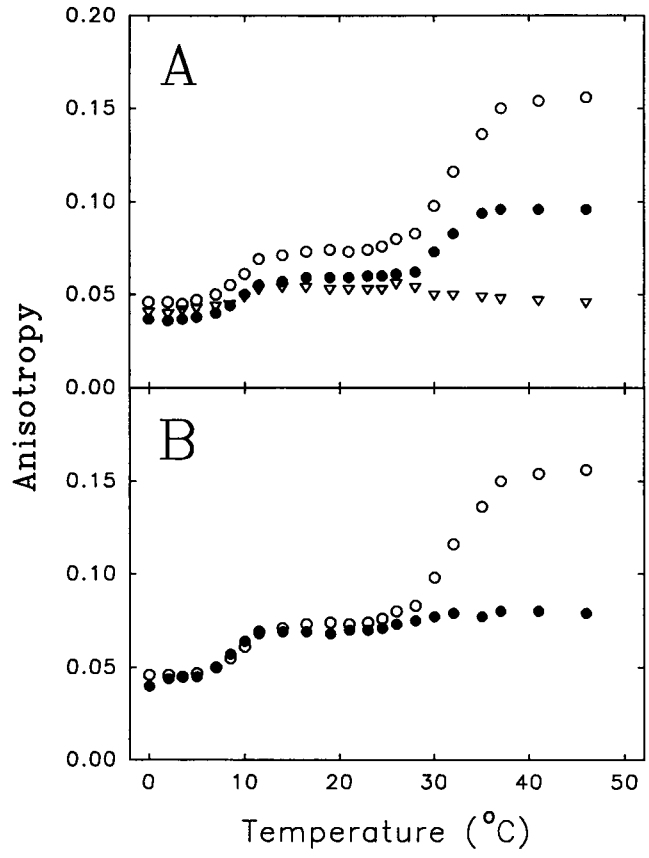

FIGURE 1 Thermotropic phase transitions of egg PE as detected by changes in anisotropy of diphenylhexatriene-containing probes. (*A*) A comparative study of three probes: ▽, DPH; ●, PC-DPH; ○, TMA-DPH. (*B*) Effect of pH on the L_α-H_{II} transition. Probe: TMA-DPH. ○, pH 5.0; ●, pH 9.5.

The frontal disposition technique used in our fluorescence measurements requires the lipid aggregates to be disposed in a sandwich structure between two microscope coverslips. Preliminary observations showed that the anisotropy measurements may vary with the thickness of the lipid layer. For that reason, a systematic study was performed in which the thickness of the lipid sandwich was estimated from its turbidity (absorbance at 550 nm) when located in

the sample holder of a dual-beam spectrophotometer against pure water. Measurements of TMA-DPH anisotropy were performed at 5, 25, and 50°C (temperatures at which L_β, L_α, and H_{II}, respectively, should predominate), for A_{550} values between ~0.15 and 1.1. The results in Fig. 2 show that there is indeed an important variation in the relative anisotropies for preparations with $A_{550} < 0.6$. Furthermore, at very low turbidities the gel to liquid-crystalline transition may go undetected, if it is not marked by an apparent decrease in steady-state anisotropy. The previous data for the L_α-H_{II} transition as detected by TMA-DPH indicate a decline in the order parameter (Cheng, 1990), in agreement with an increased anisotropy, as seen for preparations with $A_{550} > 0.5$. All of our anisotropy studies have been carried out with samples of $A_{550} > 0.8$. The problem of the influence of sample turbidity on TMA-DPH anisotropy has already been described by Storch et al. (1989) for a system involving whole cells.

Effects of diacylglycerol

The effect of diacylglycerol on the thermotropic PE transitions as detected through TMA-DPH anisotropy is shown in Fig. 3. The L_β-L_α transition appears to be largely unaffected, whereas the L_α-H_{II} transition is shifted to lower temperatures as the diacylglycerol concentration is increased. The effect of this lipid is already appreciable at 2% (mol:mol). With 10% diacylglycerol, a single transition is observed starting below 10°C and consisting probably of the simultaneous L_β-L_α and L_α-H_{II} transitions. Chen and Cheng (1990) have made similar observations, including the merging of both transitions, by infrared spectroscopy.

These phenomena can be better understood by looking at the phase transitions by differential scanning calorimetry (Fig. 4).

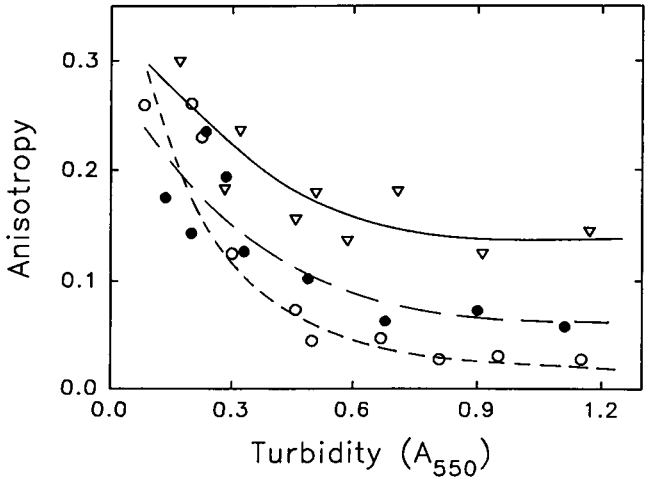

FIGURE 2 Effect of lipid film thickness on the apparent anisotropy of TMA-DPH in egg PE (pH 5.0). Thickness is measured as turbidity (A_{550}) in a spectrophotometer, as detailed under Materials and Methods. Anisotropies are measured at 5°C (○), 25°C (●), and 50°C (▽), temperatures at which L_β, L_α, and H_{II} should, respectively, predominate. The curves shown are only meant to guide the eye and do not have any theoretical foundation.

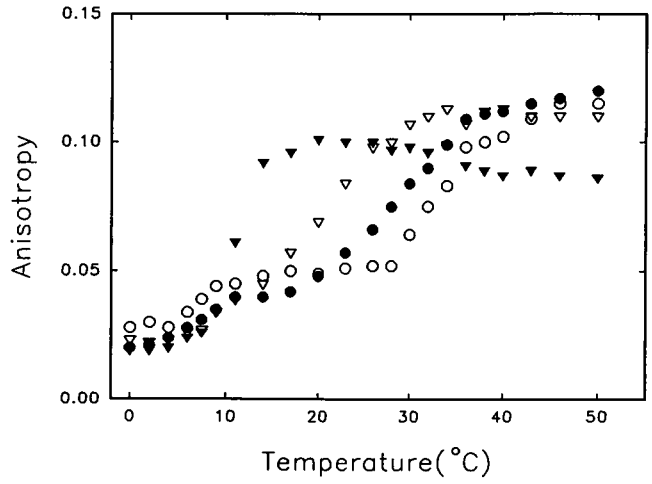

FIGURE 3 Destabilization of the L_α lamellar phase of PE by diacylglycerol. Thermotropic phase transitions are detected through changes in TMA-DPH anisotropy, pH 5.0. Diacylglycerol concentration (mol%): ○, 0%; ●, 2%; ▽, 5%; ▼, 10%.

Again the lipids are aggregated by dialysis versus a pH 5 buffer and encapsulated in gas-tight vials. The thermograms (Fig. 4 *A*) clearly show both transitions; the L_α-H_{II} has a much smaller molar enthalpy change (smaller area under the peak). The midpoint temperature of the L_β-L_α transition (T_m) increases slightly (Fig. 4 *B*), whereas the corresponding value for the hexagonal transition is clearly depressed. Ortiz et al. (1988) showed that diacylglycerol increased the T_m of dipalmitoyl PC, and Epand et al. (1988) detected a decrease in the L_α-H_{II} transition temperature of dielaidoyl PE. Diacylglycerol also makes the L_α-H_{II} transition less cooperative, as indicated by the increased width of the corresponding endotherm (Fig. 4 *A*). The thermogram corresponding to the sample with 10% diacylglycerol is interesting, because it shows unequivocally that both transitions occur and that the early part of the L_α-H_{II} transition occurs simultaneously with the final part of the L_β-L_α melting (see on this point Katsaras et al., 1993).

In a different series of experiments the effect of diacylglycerol on the phase behavior of PC:PE:CH (2:1:1 mol ratio) mixtures was studied. Liposomes with this composition have been found to fuse with each other when diacylglycerol is generated in situ by the action of phospholipase C (Nieva et al., 1989). TMA-DPH anisotropy does not display any abrupt change by heating the PC:PE:CH (2:1:1) mixture from 20 to 80°C (Fig. 5 *A*), in agreement with previous observations on its stability (Nieva et al., 1995). However, in the presence of 5% DG, a transition is detected starting at ~55°C. The nature of this transition is made clear by the ^{31}P-NMR spectra shown in Fig. 5 *B*. In the absence of diacylglycerol, the mixture PC:PE:CH (2:1:1) shows the asymmetric lineshape, with a shoulder at the lower field side, that is characteristic of the lamellar phase (Seelig, 1978). However, the presence of 5% diacylglycerol in the sample produces, above 50°C, the formation of an isotropic phase that becomes predominant at ~70°C and beyond. The

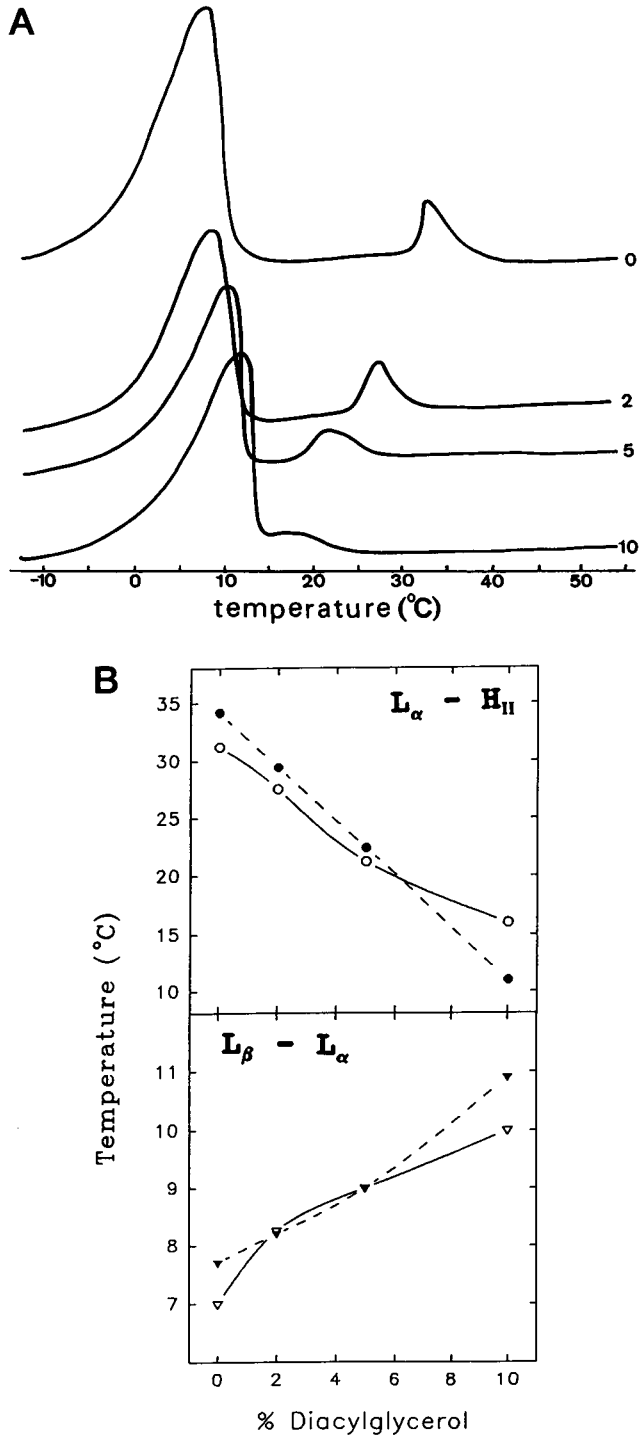

FIGURE 4 Thermotropic phase transitions of egg PE as detected by differential scanning calorimetry, and the effect of diacylglycerol. (*A*) DSC thermograms. The low- and high-temperature endotherms correspond, respectively, to the L_β-L_α and L_α-H_{II} transitions. The diacylglycerol concentration (mol%) is indicated by each curve. The calorimetric pans contained similar amounts of lipid. (*B*) Change in transition temperatures produced by diacylglycerol. Circles: T_m of the L_α-H_{II} transition. Triangles: T_m of the L_α-L_β transition. Open symbols: data from DSC thermograms. Filled symbols: data from TMA-DPH anisotropy. Note the different scales in the top and bottom of *B*.

nature of this isotropic phase cannot be further determined from the ^{31}P-NMR data, but our previous x-ray diffraction studies suggest that it is a cubic phase (Q^{224}) (Nieva et al., 1995). Thus the increase in TMA-DPH anisotropy observed in the sample containing 5% diacylglycerol appears to be due not to a L_α-H_{II} transition but to a lamellar-cubic transition. (The present data do not allow further speculation on, e.g., intermediate steps between the L_α and Q_{224} phases.) One important point on this transition is that it is virtually irreversible; as the system is cooled back down to room temperature, the anisotropy does not decrease (Fig. 5 *A*) and the NMR signal remains isotropic (data not shown). Previous studies of PC:PE:CH mixtures with diacylglycerol by ^{31}P-NMR and x-ray diffraction have shown that the isotropic (cubic) phase is stable for days after the samples are annealed at room temperature, and chemical analysis indicates that the thermal treatments described here do not lead to any detectable degradation of the lipids under study (Nieva et al., 1995; Nieva et al., unpublished observations). Israelachvili's theory of molecular shapes of lipidic membrane components (Israelachvili et al., 1980) predicts that lipid molecules of "conical" shape, e.g., PE or diacylglycerol (lipids that have a negative spontaneous curvature, in the nomenclature of Helfrich, 1973), will favor the formation of inverted hexagonal (H_{II}) structures, whereas lipids of "inverted conical" shape (or positive curvature), e.g., lysophospholipids, will counteract the latter tendency. Because we found that PE was essential for fusion to occur in our PC:PE:CH system in the presence of phospholipase C (Nieva et al., 1989), we tried successfully to confirm the inhibitory role of lysophosphatidylcholine (Nieva et al., 1993). To establish on molecular grounds this inhibitory effect, we have tested the effect of lysolecithin on the two cases of lamellar to nonlamellar transition studied above. For the L_α-H_{II} transition of PE, the data in Fig. 6 indicate that 10% lysophospholipid completely abolishes this transition, as seen both by changes in TMA-DPH anisotropy (Fig. 6 *A*) or by differential scanning calorimetry (Fig. 6 *B*). Note that both techniques show that the L_β-L_α transition remains unaltered.

In a similar way, TMA-DPH fluorescence anisotropy data reveal that lysolecithin abolishes the lamellar to isotropic transition brought about by diacylglycerol in the PC:PE:CH (2:1:1) mixture (Fig. 7). ^{31}P-NMR spectra (not shown) confirm the presence of a lamellar phase between 20 and 80°C for PC:PE:CH:diacylglycerol:lysophospholipid (47:23:25:5:10, mole ratio).

DISCUSSION

The above results deserve some discussion at least from two points of view, the mechanism by which diacylglycerol facilitates the phase transitions, and the implications for cell membrane fusion. In addition, there is a methodological aspect that raises some questions, and we shall discuss this first.

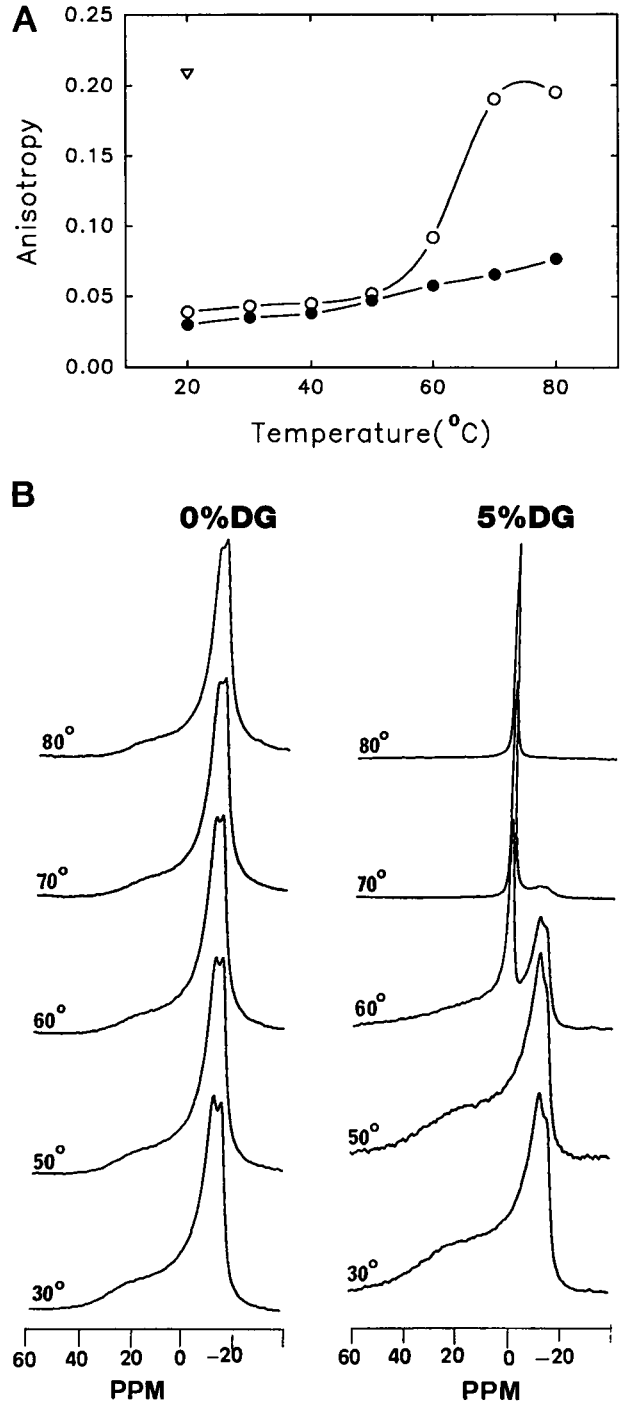

FIGURE 5 Phase transitions in the mixture PC:PE:CH (2:2:1 mol ratio). (*A*) Anisotropy of TMA-DPH, pH 7.0. ●, 0%; ○, 5% diacylglycerol. ▽, Sample containing 5% diacylglycerol, heated to 80°C and cooled back to 20°C. (*B*) ^{31}P-NMR spectra, with and without 5% diacylglycerol, recorded at various temperatures.

Changes in fluorescence anisotropy

The experimental basis of this paper is the observation of an increase in TMA-DPH fluorescence anisotropy upon the lamellar to nonlamellar thermotropic transition in two lipid systems. One of them, unsaturated PE of natural origin, has been studied in detail, and the L_α-H_{II} transition has been characterized by x-ray diffraction (Luzzati, 1968; Seddon, 1990; Castresana et al., 1992), ^{31}P-NMR (Cullis and de Kruijff, 1978), infrared spectroscopy (Mantsch et al., 1981), and differential scanning calorimetry (Gawrisch et al., 1992), among other techniques. The second system, a complex mixture of PC, PE, CH, and diacylglycerol, has not been characterized until recently (Nieva et al., 1995); it appears to undergo a lamellar to isotropic transition, where the isotropic component is a Q^{224} cubic phase. Lamellar-hexagonal transitions have been monitored with a variety of fluorescence spectroscopy techniques. In particular, the case of PE and TMA-DPH has been studied in detail by Cheng and co-workers (van Langen et al., 1989; Cheng, 1990), using angle-resolved and time-resolved fluorescence. According to their data, the local order parameter of TMA-DPH in the H_{II} phase is about 25% higher than that in the L_α phase, and the rotational diffusion constant of the probe decreases with the L_α-H_{II} transition. These data are compatible with the observed increase in steady-state anisotropy (Fig. 1). No such detailed studies have been carried out, to

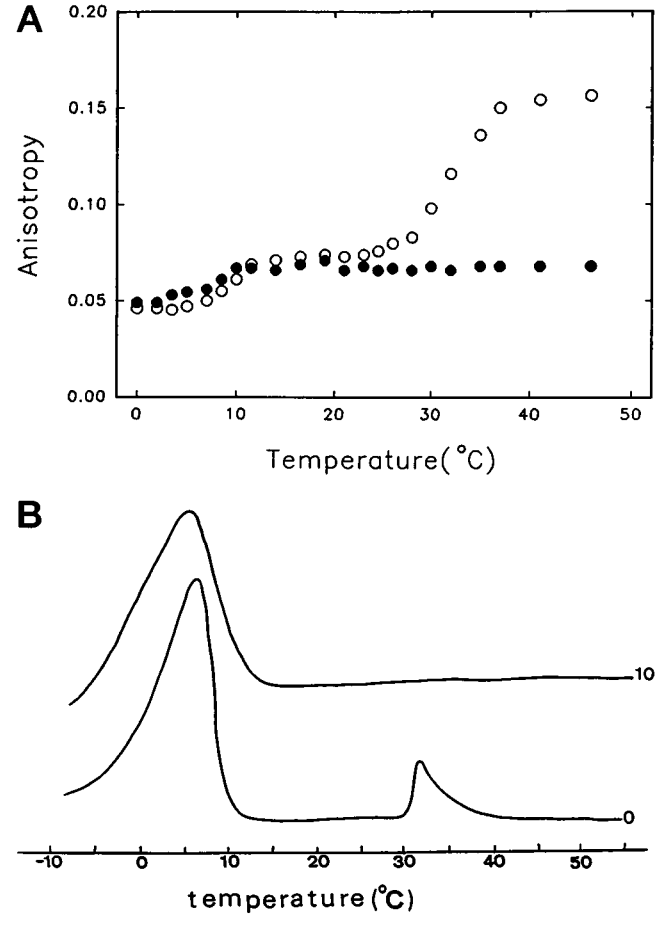

FIGURE 6 Stabilizing effect of lysophosphatidylcholine on the L_α-H_{II} transition of egg PE. (*A*) Anisotropies of TMA-DPH, pH 5.0. ○, Control; ●, +10% lysophospholipid. (*B*) DSC thermograms: 0 and 10% lysophospholipid, as indicated by each curve. The calorimetric pans contained approximately similar amounts of lipid.

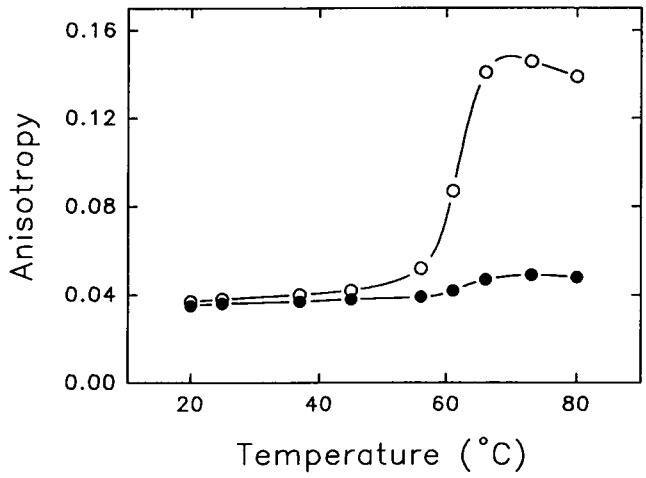

FIGURE 7 Stabilizing effect of lysophosphatidylcholine on the L_α-Q^{224} transition of the mixture PC:PE:CH:diacylglycerol (47:23:25:5). ○, Control; ●, +10% lysophospholipid.

the authors' knowledge, with fluorescent probes in cubic phases; thus the observed increase in TMA-DPH anisotropy that accompanies the lamellar-isotropic transition of PC:PE:CH:diacylglycerol (Fig. 5 *A*) remains to be explained on firm spectroscopic grounds. It should be noted that cubic phases are noted by their high viscosity, much higher than that of the lamellar or hexagonal phases (Luzzati et al., 1993); this property would be compatible with an increased anisotropy as seen in Fig. 5 *A*. The change in anisotropy (ΔR) between fluid lamellar and cubic phases (Fig. 5 *A*) is much larger than the one observed between fluid lamellar and hexagonal phases in Fig. 1 *A* (~0.16 and ~0.08, respectively). The possibility of detecting a lamellar-cubic transition with the relatively simple fluorescence polarization equipment is also important methodologically speaking, particularly when the small enthalpy change associated with such a transition makes difficult its detection by differential scanning calorimetry (Siegel and Banschbach, 1990).

The properties of diacylglycerol

Relatively small proportions of diacylglycerol (2–5 mol%) have marked effects in facilitating lamellar to hexagonal or lamellar to cubic transitions. The formation of nonbilayer lipid phases by diacylglycerol/phospholipid mixtures has been described previously (Goldberg et al., 1994, and references therein). Diacylglycerol may act in at least two ways: by facilitating intermembrane contacts and/or increasing the lipid surface curvature. Intermembrane contacts are required in transitions from lamellar to hexagonal or cubic phases because they imply changes from an essentially one-dimensional to two- or three-dimensional structures. The requirement of vesicle aggregation for a L_α-H_{II} transition to occur was pointed out previously (Allen et al., 1990; Nieva et al., 1990), and it is probably valid as well for lamellar to cubic transitions. In our studies on phospholipase C-promoted vesicle aggregation and fusion (Nieva et al., 1993), we found that diacylglycerol has the effect of increasing vesicle-vesicle aggregation. This can be understood by the relative dehydration brought about by the presence of diacylglycerol in the bilayer surface—thus the partial removal of the major barrier to close contact of phospholipid bilayers (Rand, 1981). L_α-H_{II} transitions are accompanied by dehydration (Cheng, 1990; Castresana et al., 1992; Katsaras et al., 1993; López-García et al., 1994), thus the facilitating role of diacylglycerol is easy to understand. (See also Kozlov et al. (1994) for a discussion of competing bending and hydration energies in a complex case of hexagonal-lamellar-hexagonal phase transitions.) However, Das and Rand (1986) found that removal of up to 18% of PC polar headgroups did not result in closer apposition of the corresponding bilayers, so that the role of diacylglycerol in helping intermembrane contacts, particularly when it is symmetrically distributed in the bilayer, as in the present studies, is debatable.

More clear is the participation of this hydrophobic lipid in helping the formation of structures requiring high-curvature monolayers ("negative curvature"; Helfrich, 1973), as in the case of H_{II} or Q^{224} phases. Diacylglycerol increases the relative volume of the hydrocarbon, because unsubstituted glycerol headgroups partition preferentially in the hydrocarbon regions (Gulik et al., 1988); thus it tends to increase the monolayer negative curvature, as discussed previously (Luzzati, 1968; Israelachvili et al., 1980; Das and Rand, 1986; Chernomordik and Zimmerberg, 1995).

Implications for cell membrane fusion

The role of lipids in biological membrane fusion has recently been the object of two important reviews (Chernomordik et al., 1995b; Chernomordik and Zimmerberg, 1995). These authors have proposed that a common mechanism of lipid bilayer rearrangement underlies the various physiological phenomena of membrane fusion. The results in this paper may be considered in the light of those considerations. Essentially, the rearrangement of two lipid bilayers into a single one must involve the bending of the membranes. Most recent data in the literature suggest the existence of a strongly bent intermediate, the "stalk" (Chernomordik et al., 1987; Siegel, 1993), a transient structure formed by the contacting monolayers of two membranes.

The stalk has a net negative curvature (the curvature of a monolayer in the H_{II} phase is defined to be negative; Helfrich, 1973). Thus, including in the monolayer lipids that support formation of the H_{II} phase, will make the monolayer curvature more negative and promote stalk formation. The opposite effect will be found with lipids with a spontaneous positive curvature, i.e., micelle-forming lipids. The observations in this paper on the facilitating and inhibiting effects, respectively, of diacylglycerol and lysolecithin on lamellar to nonlamellar phase transitions explain very well,

through the stalk hypothesis, their respective effects on phospholipase C-promoted membrane fusion (Nieva et al., 1993, 1995). In more general terms, the stalk may be the "structural intermediate" that we proposed earlier (Nieva et al., 1993) and for which we suggest a structure related to a bicontinuous cubic phase (Nieva et al., 1995). Relevant to this point are the observations of Landh (1995) of cubic membranes as subcellular space organizers and the suggestion by Oberhauser et al. (1992) on the role of lipids in the exocytotic fusion pore.

The original stalk model was modified by Siegel (1993) to include the contribution of hydrophobic void spaces within the intermediate. Such void spaces could considerably increase the stalk energy. According to this idea the presence of small amounts of "impurities" in the form of neutral lipids could fill in the voids, thus decreasing energy and promoting stalk formation. This may be an additional mechanism of action for the facilitating role of diacylglycerol in lamellar to nonlamellar transitions and in liposome fusion, apart from those suggested above. It should be noted in this respect that diacylglycerol decreases the optimum temperature for liposome fusion in the presence of phospholipase C (Nieva et al., 1993). Furthermore, Walter et al. (1994) interpreted within this theoretical framework their observation that diacylglycerol increases divalent cation-induced lipid mixing between phosphatidylserine liposomes.

In conclusion, the above results provide clear examples of a lipid (diacylglycerol) that facilitates lamellar to nonlamellar phase transitions in two different lipid systems and promotes membrane fusion in different model systems and, conversely, of a lipid (lysolecithin) that inhibits such phase transitions and suppresses various fusion processes. These observations provide strong support for the hypothesis of the involvement of transient nonlamellar intermediates in the fusion process, and in particular for the so-called stalk hypothesis (Chernomordik et al., 1987; Siegel, 1993). The structure of the Q^{224} cubic phase, consisting of rods rather than micelles (Luzzati et al., 1993), that is formed under our conditions does not favor the putative "inverted micellar intermediates" as the structural fusion intermediates, in agreement with the observations by Siegel et al. (1994). See also on this point Nieva et al. (1995). However, a note of caution should be added at this stage, to dispel sketchy ideas on the dynamics of lipidic structures. The thermodynamic concept of phase should not be confused with the biological concept of structure. And the experimental conditions in which lipid phase transitions are studied differ markedly from those in which membrane fusion takes place, even in vitro. In practical terms, phases and phase structures are but idealizations of real biological structures. The structural fusion intermediate (be it a stalk or otherwise) is not or does not consist of a hexagonal or a cubic phase; it is instead a (hopefully) real though transient structure, in which the relative disposition of a population of lipid molecules resembles the ideal geometry of a hexagonal or a cubic phase. Of course this is not meant to dismiss the biological relevance of lipid phase studies; on the contrary, as shown in this paper, they constitute prime tools of diagnostic and predictive value for biophysical studies of membrane fusion.

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