Siegel DP, Banschbach J & Alford D. et al Physiological levels of diacylglycerols in phospholipid membranes induce membrane fusion and stabilize inverted phases. Biochemistry28: 37...

ARTICLE *in* BIOCHEMISTRY · JUNE 1989

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Physiological Levels of Diacylglycerols in Phospholipid Membranes Induce Membrane Fusion and Stabilize Inverted Phases†

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Received April 29, 1988; Revised Manuscript Received August 31, 1988

ABSTRACT: In the preceding paper (Ellens et al., 1989), it was shown that liposome fusion rates are substantially enhanced under the same conditions which induce isotropic ^{31}P NMR resonances in multilamellar dispersions of the same lipid. Both of these phenomena occur within the same temperature interval, ΔT_I, below the L_α/H_{II} phase transition temperature, T_H. T_H and ΔT_I can be extremely sensitive to the lipid composition. The present work shows that 2 mol % of diacylglycerols like those produced by the phosphatidylinositol cycle in vivo can lower T_H, ΔT_I, and the temperature for fast membrane fusion by 15–20 °C. N-Monomethylated dioleoylphosphatidylethanolamine is used as a model system. These results show that physiological levels of diacylglycerols can substantially increase the susceptibility of phospholipid membranes to fusion. This suggests that, in addition to their role in protein kinase C activation, diacylglycerols could play a more direct role in the fusion event during stimulus–exocytosis coupling in vivo.

Diacylglycerols are second messengers produced in cellular membranes by the PI cycle (Majerus et al., 1984, 1986; Sekar & Hokin, 1986) at levels of about 1 mol % with respect to phospholipid (Preiss et al., 1986). One known function of PI cycle diacylglycerols is to activate protein kinase C. It has been speculated that these diacylglycerols are also involved in exocytosis in that they make the relevant membranes more susceptible to fusion (Pickard & Hawthorne, 1978). We have shown that there is a liposome fusion mechanism associated with lamellar/inverted phase transitions (Ellens et al., 1989), and it is known that diacylglycerols lower lamellar/inverted phase transition temperatures (Dawson et al., 1984; Das & Rand, 1986; Epand, 1986; Coorsen & Rand, 1987). In this work, we studied the effect of 1–3 mol % diacylglycerols on the phase behavior and fusion kinetics of phospholipid systems that can form inverted phases.

Surprisingly, as little as 2 mol % diacylglycerols can lower the equilibrium lamellar/inverted hexagonal (L_α/H_{II})[1] phase transition temperature (T_H) of monomethylated dioleoylphosphatidylethanolamine (DOPE-Me)[1] by as much as 20 °C.

† This investigation was supported by Research Grant NIH-GM-31506 and UCSF Academic Senate Grant (J.B.), a senior postdoctoral grant from the California Branch of the American Cancer Society (H.E.), Grant NIH-HL-23853 (P.L.Y.), and Grant GR/E 33769 from the Science and Engineering Research Council (U.K.) (P.J.Q.).

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[1] Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; CHOL, cholesterol; DEG, dieicosenoin; DG, diacylglycerol; DLG, dilinolenin; DOG, diolein; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, monomethylated dioleoylphosphatidylethanolamine; DPA, dipicolinic acid; DPG, dipalmitin; DPX, *p*-xylylenebis(pyridinium bromide); DSC, differential scanning calorimetry; NTA, nitrilotriacetic acid; OAG, 1-oleoyl-2-acetylglycerol; OArG, 1-oleoyl-2-arachidonoylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; R_0, spontaneous radius of curvature of the monolayers of a lipid–water system, as defined by Gruner (1985); T_H, lamellar/inverted hexagonal (L_α/H_{II}) phase transition temperature; ΔT_I, range of temperatures at which isotropic ^{31}P NMR resonances are observed from fully hydrated bulk multilamellar preparations of a lipid system; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

0006-2960/89/0428-3703$01.50/0

Multilamellar dispersions of DOPE-Me yield isotropic ^{31}P NMR resonances and form inverted cubic phases at temperatures tens of degrees below T_H (Gagné et al., 1985; Gruner et al., 1988). Isotropic resonances are observed in the same temperature range, ΔT_I, as enhanced fusion rates in unilamellar liposome dispersions (Ellens et al., 1986b, 1989). ΔT_I extends from the temperature at which isotropic resonances are first observed to the calorimetric T_H. We have proposed that a common intermembrane structure produces membrane fusion, isotropic ^{31}P NMR resonances, and inverted cubic phases in these systems (Ellens et al., 1986b, 1989; Siegel, 1986b,c; Siegel et al., 1988a). Here it is shown that low levels of diacylglycerols lower the onset of ΔT_I and the temperature for rapid membrane fusion almost in parallel with T_H. These data are consistent with the proposed mechanism and show that diacylglycerols produced in vivo could substantially affect the susceptibility of biomembranes to fusion. From X-ray diffraction and DSC data, we infer that diacylglycerols lower T_H by reducing the spontaneous radius of curvature of the lipid systems (Gruner, 1985).

Materials and Methods

Lipids. Monomethylated dioleoylphosphatidylethanolamine (DOPE-Me), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), and 1-oleoyl-2-arachidonoyl- (OArG), 1-oleoyl-2-acetyl- (OAG), 1,2-dioleoyl- (DOG), and 1,2-dilinolenoylglycerol (DLG) were all purchased from Avanti Polar Lipids, Inc. (Birmingham, AL), and were used without further purification. Cholesterol (CHOL) was purchased from Sigma Chemical Co. (St. Louis, MO). Dieicosenoin (mixed 1,2 and 1,3 isomers; DEG) and 1,2-dipalmitin (DPG) were purchased from Sigma Chemical Co. All the diacylglycerols were used without further purification. All lipids and diglycerides were stored as chloroform stock solutions under argon at −40 °C until used, except for OArG and DLG, which were stored at −70 °C.

Sample Preparation. Lipids and diacylglycerols were cosolubilized in HPLC-grade chloroform. For DSC, concentrations of diacylglycerols in the samples accurate to ±2% were obtained via gravimetry on vacuum-dried samples. Chloroform was removed by rotary evaporation (20 min) and subsequent vacuum drying (1 h). Subsequent steps were as described in Ellens et al. (1989).

Buffers. DOPE-Me was dispersed in either (i) 100 mM NaCl, 50 mM sodium acetate (pH 4.5), and 0.1 mM EDTA ("acetate buffer"); (ii) 100 mM NaCl, 10 mM TES (pH 7.4), 0.1 mM EDTA, and 5 mM $MgCl_2$ ("Mg–TES buffer"); or (iii) 100 mM NaCl, 10 mM TES (pH 7.4), and 0.1 mM EDTA ("TES buffer"). For DSC experiments, the Mg–TES and TES buffers contained 20 mM TES, instead of 10 mM.

^{31}P NMR, DSC, X-ray Diffraction, and Membrane Fusion and Leakage Studies. These studies were done as described in Ellens et al. (1989). The *Z*-average diameter of all the liposomes used in fusion or leakage assays was 190 ± 60 nm, measured by dynamic light scattering (Coulter Model N4; Coulter Electronics, Inc., Hialeah, FL). The encapsulated volumes were 5.4 and 3.1 μL/μmol for ANTS- and ANTS/DPX-containing DOPE-Me liposomes and 3.2 and 5.4 μL/μmol for the DOPE-Me/2% OArG liposomes, respectively. This variability in encapsulated volume has been noted before with pure PE liposomes (Ellens et al., 1986a,b; Bentz et al., 1987). Using an area per DOPE-Me headgroup in the bilayer of about 60 Å^2 (Mulukutla & Shipley, 1984; Gruner et al., 1988), it can be shown that the liposomes probably have two or more lamellae.

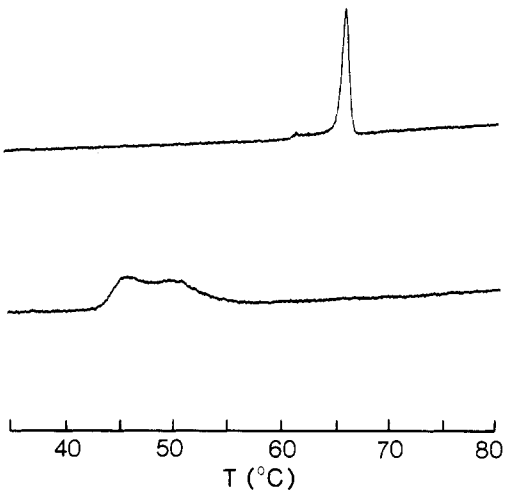

FIGURE 1: Thermograms of DOPE-Me without (top) and with (bottom) 2.0 mol % OArG, obtained in Mg^{2+}–TES buffer at a scan rate of 13 °C/h. The sensitivity used to obtain the bottom trace is twice that used for the top trace. The onset temperature is defined as the temperature at the intersection of the extrapolated base line and the extrapolated slope of the low-temperature shoulder of the endotherm. Note that two peaks are visible in the OArG-containing endotherm: this was typical of the diacylglycerol systems. The peak temperatures reported in Table II are those of the lowest temperature peaks.

Table I: L_α/H_{II} Phase Transition Temperatures As Determined via DSC

lipid system	cations (all buffers contain 100 mM NaCl)	transition temp (°C) onset	peak
POPE	pH 7.4	68 ± 1	69 ± 1
DOPE-Me	pH 4.5	64 ± 2	66 ± 2
	pH 7.4/5 mM Mg^{2+}	65 ± 2	66 ± 2
DOPE-Me + 2 mol % OArg	pH 4.5	41 ± 2	45 ± 3
	pH 7.4/5 mM Mg^{2+}	42 ± 2	45 ± 2
DOPE-Me + 2 mol % DOG	pH 4.5	44 ± 2	47 ± 2

Results

Differential Scanning Calorimetry. Thermograms of DOPE-Me in the presence and absence of 2.0 mol % of OArG are shown in Figure 1. Previous NMR and X-ray diffraction experiments (Ellens et al., 1989) confirmed that in this buffer DOPE-Me undergoes an L_α/H_{II} phase transition at the indicated temperature. The onset temperature is the lowest temperature at which L_α/H_{II} phase coexistence is possible and is taken as the temperature at which the extrapolated base line meets the extrapolated low-temperature shoulder of the endotherm (Figure 1). Reproducibility of thermograms run on the same lot of each lipid was ca. ±1 °C. In most, but not all, thermograms of pure DOPE-Me obtained at a scan rate of 13 °C/h, a very low-amplitude shoulder was visible beginning at temperatures between 55 and 61 °C (Figure 1, top). The presence of this shoulder did not affect the position of the peak at 66 °C. A similar feature is seen in the DSC data of Gruner et al. (1988). While the origin of this feature is not known, it may represent the formation of a well-ordered cubic lattice as discussed in Ellens et al. (1989).

The onset and peak temperatures for the L_α/H_{II} phase transition for several lipid systems used in this study are given in Table I. Epand (1985a) reported a peak temperature for POPE that was 2.4 °C higher than our value, using lipid from the same supplier. We believe that lot-to-lot variations in POPE are probably responsible. Different batches of DOPE-Me occasionally yielded transition temperatures differing from the average value by as much as 3 °C. This is

Table II: Reduction in L_α/H_{II} Transition Temperatures by Diacylglycerols[a]

phospholipid	diacylglycerol	reduction in temp/mol % diacylglyceride (°C)
DOPE-Me	OArG (1-C18:1, 2-C20:4)	10.0 (12)
	DEG (C20:1)	10 (4)
	DOG (C18:1)	9 (4)
	DLG (C18:3)	9 (5)
	DPG (C16:0)	7 (3)
	OAG (1-C18:1, 2-C2:O)	2 (2)
POPE	DEG (C20:1)	8 (2)
	OArG (1-C18:1, 2-C20:4)	8 (5)
	DLG (C18:3)	7 (5)
	DOG (C18:1)	6.3 (8)

[a] The temperatures of the lowest temperature peaks in the endotherms were determined as indicated in Figure 1. The reductions relative to the transition temperatures in the absence of diacylglycerol were calculated and divided by the mole percent diacylglycerol present in each case. The results were averaged to yield the values in this table. Peak transition temperatures for pure DOPE-Me (acetate buffer, pH 4.5) and POPE (pH 7.4, TES buffer) were 65.7 ± 2.0 °C (11 determinations) and 68.8 ± 0.5 °C (6 determinations), respectively. The number of determinations per composition is given in parentheses in the third column. Concentrations ranged from 2 to 3 mol % diacylglycerol, except in the case of OAG, where approximately 3.5 and 5 mol % were employed. The standard deviations were 1.1 °C for OArG in DOPE-Me and the same or less for the other cases.

not surprising in light of Epand's later results (Epand, (1985b) and the present data, which show the great sensitivity of T_H to very low levels of hydrophobic impurities. The DOPE-Me values we cite here are averaged over several lots.

The endotherms for the mixed diacylglycerol/phospholipid systems were usually broad and often contained more than one peak, indicating complex phase behavior in the phase coexistence region. We found that 2 mol % diacylglycerol (DG) was sufficient to decrease the onset temperature and the temperature of the lowest peak of the endotherms by ca. 20 °C. The efficacies of different DGs in reducing the L_α/H_{II} transition temperature are listed in Table II. These were determined via DSC on samples containing from 1 to 3.6 mol % DG, although most samples contained 2.0 mol %. The extents to which these DGs reduce T_H are roughly comparable to the extents observed by others, working with other systems (Epand, 1985b; Das & Rand, 1986).

Epand (1986) previously determined the effects of DLG on the transition in POPE. At levels of 1 mol % or less, DLG was reported to lower the peak transition temperature by roughly 8.5 °C/mol % DLG. This slope is slightly larger than the one reported here (7 °C/mol %; Table II). Epand (1985b) also measured the effects of ca. 0.5 mol % DOG, distearin, and dilinolenin on T_H in POPE. The slopes reported were in the range 5–8.5 °C/mol % DG, which is roughly compatible with the values in Table II. The very small amounts of DGs used by Epand (1985b) make accurate evaluation of the slopes difficult. Here we determined transition temperatures at DG contents of 2–3 mol %. This produced larger differences in transition temperatures and permitted more accurate comparison of the effects of different DGs. In the range of 2–3 mol % DLG and 1–3 mol % OArG, we found that the onset and peak temperatures always yielded slopes within 1.5 °C or less of the values in Table II. Thus, the dependence of the extent of decrease in T_H on DG concentration is probably linear in this range. Das and Rand (1986) determined the effects of DG (obtained by the hydrolysis of egg PC) on the transition of PE (obtained by transesterification of egg PC). They found that DG reduced T_H in PE by roughly 5.5 °C/mol % over the range 0–4 mol %.

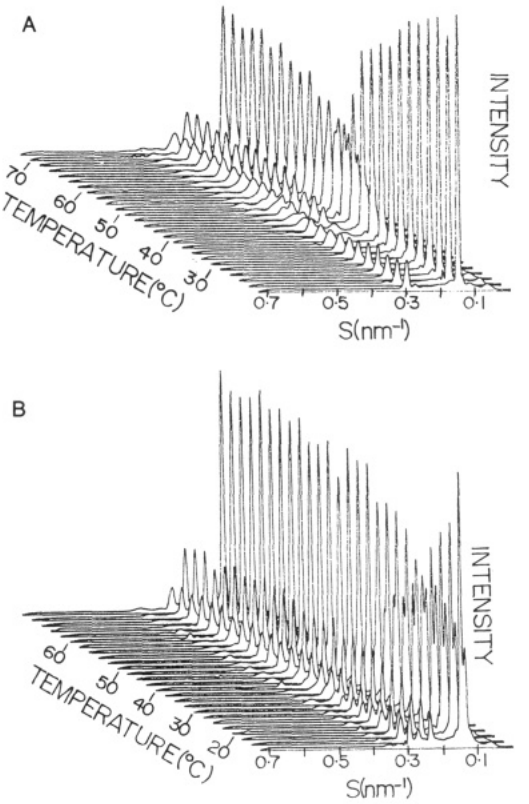

FIGURE 2: Time-resolved X-ray diffraction patterns of phospholipid and phospholipid/diacylglycerol systems heated through the L_α/H_{II} phase transition region. Each trace is a plot of diffracted intensity vs detector position, the scattering angle increasing from right to left. Successive traces, each obtained in 1.25 s, are staggered from right to left (bottom to top) in order of increasing temperature. Every tenth trace (of a total of 255) is displayed. (A) DOPE/DOPC/CHOL (1.1:1.0:1.0 mol/mol) in acetate buffer, heated from 22 to 71 °C. A broad lamellar/hexagonal transition is evident between 36 °C and ca. 49 °C. (B) DOPE/DOPC/CHOL with 3 mol % OArG. Lamellar/hexagonal phase coexistence is evident at the lowest temperature (16 °C). The transition to the hexagonal lattice is complete at ca. 34 °C.

X-ray Diffraction. X-ray diffraction data were obtained to confirm that the transitions in DG/phospholipid samples also correspond to L_α/H_{II} phase transitions [see also Ellens et al. (1989)]. Time-resolved X-ray diffraction data were obtained on representative systems. Figure 2 shows time-resolved data obtained using samples of DOPE/DOPC/CHOL (1.1:1.0:1.0 mol/mol) in the absence and presence of 3 mol % OArG. In the absence of OArG, the lattice is lamellar at low temperatures and commenced a transition to a hexagonal lattice at ca. 39 °C (Figure 2A), with the two lattices coexisting for ca. 10 °C. The lattice constant of the lamellar phase at the onset of the transition is 6.3 ± 0.1 nm, and the lattice constant of the hexagonal phase is 7.8 ± 0.1 nm near the end of the transition (47 °C).

In the presence of 3 mol % OArG (on a total lipid basis), the onset temperature for the transition is less than the lowest temperature at which diffraction data were obtained (16 °C; Figure 2B); 3 mol % OArG thus reduced the onset of the L_α/H_{II} coexistence region by more than 23 °C. Lamellar and inverted hexagonal phases (lattice constants of 6.4 and 8.0 ± 0.1 nm at 17 °C, respectively) coexisted over an interval ca. 17 °C wide. The breadth of this interval is compatible with the broad endotherms observed in such systems (Figure 1B). In both of the systems in Figure 2, the lamellar/hexagonal transition appeared to be a two-state, first-order process, and the lattices of the two phases were clearly incommensurate. In Figure 2A, the peaks in the lamellar phase diffraction

Table III: Lattice Constants of Diacylglycerol-Containing DOPE-Me H_{II} Phases

diacylglycerol	H_{II} tube diameter (nm) (±0.1 nm)	change in H_{II} tube diameter (nm) relative to dodecane-doped DOPE-Me at same temperature[a]
2 mol % OArG	7.6 (50 °C)	−0.4
2 mol % DOG	7.15 (58 °C)	−0.6
2 mol % DPG	7.5 (61 °C)	−0.2

[a] Data from Gruner et al. (1988).

patterns broaden slightly as the temperature approaches the lamellar/hexagonal transition temperature. This effect is particularly noticeable in the second-order diffraction peaks. As noted previously (Ellens et al., 1989), this may indicate disordering of the lamellar lattice accompanying the formation of isotropic or inverted cubic phase domains.

Similar but lower resolution data were obtained for DOPE-Me in acetate buffer in the absence and presence of 2 mol % OArG. The transformation from lamellar to hexagonal lattices commenced at ca. 64 °C in the absence of OArG and ca. 36 °C in its presence, with a coexistence region in the latter case of ca. 10 °C (data not shown). The lattice constants of the lamellar and hexagonal phases of OArG-doped DOPE-Me were 6.2 ± 0.1 nm (16 °C) and 7.6 ± 0.1 nm (50 °C), respectively. This was determined from time-resolved data and data obtained on conventional rotating-anode equipment. This demonstrates that low levels of OArG substantially reduce the L_α/H_{II} transition temperature in this system, as well. Despite the fact that more than one peak is observed in the DG/phospholipid endotherms, there is no suggestion of more than one lamellar or one hexagonal lattice in any of the X-ray data, although the resolution in the DOPE-Me case is low.

In order to determine the mechanism of H_{II} phase stabilization by DG, we studied the effect of DG on the H_{II} phase lattice constant. X-ray diffraction was used to determine the lattice constant in samples of DOPE-Me containing different DGs (Table III). The effects of DGs on these constants were compared with the effects of dodecane, as determined by Gruner et al. (1988). The data of Gruner et al. are applicable to our systems: at 55 °C, using hexadecane-doped DOPE-Me, we obtained the same lattice constant as those authors to within an accuracy of ±0.1 nm. The H_{II} lattice constants (equal to the H_{II} tube diameters) in the DG-doped systems are consistently smaller than in the dodecane-doped systems at the same temperature. This implies that DGs lower T_H by decreasing the spontaneous radius of curvature of the lipid system (Gruner, 1985; see Discussion).

^{31}P NMR. DOPE-Me with and without OArG was examined via ^{31}P NMR in order to (a) independently confirm the phase assignment and (b) determine if the system exhibits the isotropic resonances associated with the occurrence of membrane fusion at temperatures near T_H. Spectra of multilamellar DOPE-Me preparations in Mg^{2+}–TES buffer with and without 2 mol % OArG are shown in Figure 3. In the absence of OArG (top), the NMR powder patterns have the symmetry associated with lamellar phases at temperatures up to 45–50 °C, where a small isotropic component appears. At 65 °C, the spectrum consists of two components: the major component has the symmetry associated with the existence of H_{II} phase lipid (Cullis & de Kruijff, 1978), and there is still a significant isotropic component. These results are consistent with DSC data (Figure 1) indicating a lamellar/H_{II} phase transition at ca. 65 °C. The emergence of the isotropic component at temperatures more than 15 °C below T_H is consistent

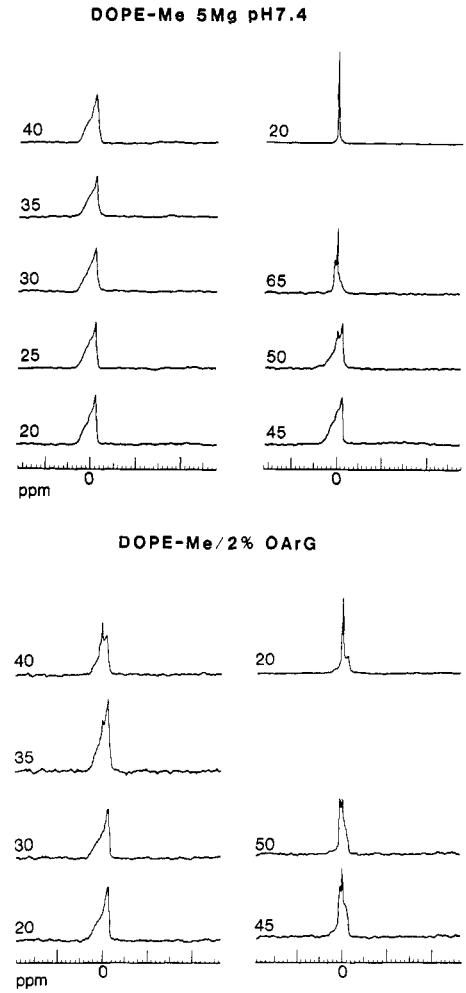

FIGURE 3: ^{31}P NMR spectra of DOPE-Me (top) and DOPE-Me with 2 mol % OArG (bottom) in Mg^{2+}–TES buffer as a function of temperature. The spectra are consistent with lamellar phase samples at low temperature in each case. With increasing temperature, isotropic components arise at 45–50 °C (DOPE-Me) and 35 °C (DOPE-Me/OArG). Components consistent with H_{II} phase appear in the resonances at ca. 65 °C (DOPE-Me) and 40 °C (DOPE-Me/OArG). OArG concentrations in these samples are not as accurately known as in the DSC samples, and temperature control is less accurate; hence, transition temperatures differ by a few degrees.

with previous observations of DOPE-Me (Gagné et al., 1985; Ellens et al., 1989) and indicates the appearance of structures which produce isotropic averaging of headgroup orientations through lipid diffusion on the NMR time scale [ca. 20 μs (Larsen et al., 1987)]. These resonances may mark the first step in the formation of the inverted cubic phases described in DOPE-Me at neutral pH (Gruner et al., 1988). Upon cooling to 20 °C, only the isotropic component is observed. The persistance of this resonance upon cooling is typical [e.g., see Boni and Hui (1983), Gagné et al. (1985), and Ellens et al. (1989)] and reflects the metastability of isotropic and inverted cubic structures (Siegel, 1986c; Gruner et al., 1988).

The spectra of the sample containing 2 mol % OArG (Figure 3, bottom) indicate that an isotropic component appears at ca. 35 °C. H_{II}, lamellar, and isotropic components are all present at 45 °C, and the sample is mostly H_{II} phase at temperatures of 50 °C and above. These data are consistent with the presence of a broad L_α/H_{II} phase transition beginning at ca. 43 °C observed via DSC (Figure 1).

Liposome Fusion and Leakage. Fusion and leakage of DOPE-Me liposomes were studied as a function of temperature. Fusion was monitored by the mixing of aqueous contents, using both the ANTS/DPX assay (Ellens et al., 1985) and

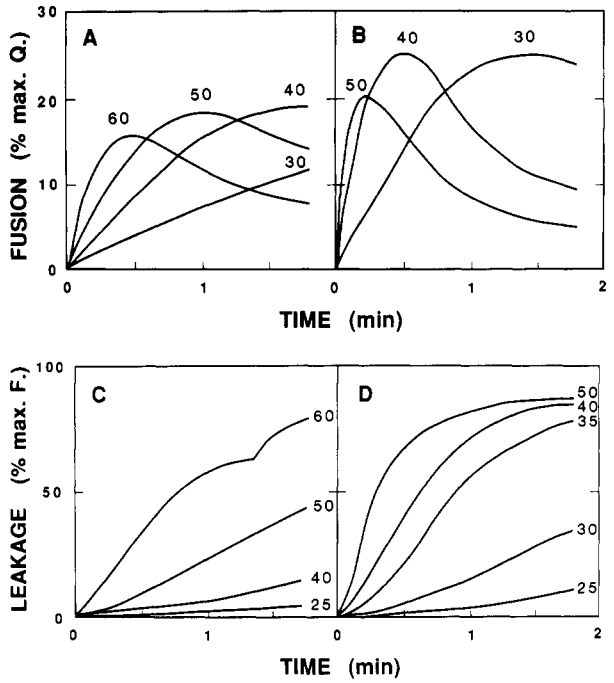

FIGURE 4: Fusion and leakage of DOPE-Me liposomes induced by 5 mM Mg^{2+}/pH 7.4, as measured by the ANTS/DPX assay for contents mixing and retention. The temperature (degrees centrigrade) is noted beside each curve. Fusion data in the absence (panel A) and presence (panel B) of 2 mol % OArG in the liposomes are shown. The ratio of ANTS-containing to DPX-containing liposomes is 1:9. Leakage data in the absence (panel C) and presence (panel D) of 2 mol % OArG are shown. The total lipid concentration was 100 μM in all cases. The calibration of the fluorescence scales is as described in Ellens et al. (1989). Briefly, if all liposomes fused to doublets and there was no leakage, then 90% quenching would be expected, because of the ratio of ANTS- to DPX-containing liposomes in the mixture.

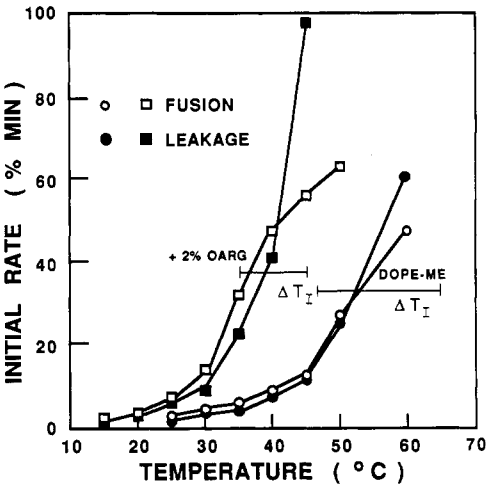

FIGURE 5: Initial rates of fusion and leakage of DOPE-Me liposomes at pH 7.4 in the presence of 5 mM Mg^{2+} as a function of temperature. The initial slopes of plots like the curves shown in Figure 4 are shown, except that fusion data for a 1:1 mixture of ANTS-containing and DPX-containing liposomes are used here (total lipid concentration 100 μM). The curves at the right show the initial rate of fusion (open circles) and leakage (closed circles) for DOPE-Me liposomes. The curves at the left show the initial rates of fusion (open squares) and leakage (closed squares) for liposomes containing 2 mol % OArG. The horizontal bar (ΔT_I) is the temperature range from the first appearance of the isotropic ^{31}P NMR resonances in spectra of bulk, multilamellar samples of these lipid systems to the peak temperature of the transition to the H_{II} phase, as measured by DSC (Table I). All of the rates are in percent per minute, where percent quenching is used for fusion and percent dequenching for leakage.

the Tb/DPA assay (Wilschut & Papahadjopoulos, 1979; Bentz et al., 1985a). The same temperature dependences were obtained, although the absolute rates were slower with the Tb/DPA assay. This quantitative difference between the two assays has been noted previously (Ellens et al., 1985; Düzgünes et al., 1987; Bentz et al., 1988; Düzgünes & Bentz, 1988). We also note that the Tb/DPA assay is difficult to calibrate accurately at temperatures above 50 °C (Ellens et al., 1989). We will present the results of the ANTS/DPX assay.

The results of fusion and leakage assays at several temperatures for DOPE-Me liposomes at pH 7.4 in the presence of 5 mM Mg^{2+} are shown in Figure 4. For the fusion assays (Figure 4A,B), a mixture of 10 μM ANTS-containing liposomes and 90 μM DPX-containing liposomes was used, for a total lipid concentration of 100 μM. For the leakage assays (Figure 4C,D), the total lipid concentration was also 100 μM. The temperature is noted beside each curve. Two points are obvious. First, fusion and leakage are much more rapid at a given temperature when the liposomes contain 2 mol % OArG. Second, the extent of contents mixing starts to decrease at temperatures above T_H (ca. 45 °C in the presence of OArG, Table I) due to the vastly enhanced aggregation-dependent leakage that occurs at and above this temperature. This is consistent with our earlier findings (Bentz et al., 1985b; Ellens et al., 1986a,b, 1989).

It is of interest that the leakage data obtained at 60 °C (Figure 4C) indicate a sudden ejection of the encapsulated contents of the fused liposomes into the medium after about 1.4 min. We have previously referred to this process as "collapse" and have found that it occurs in systems that fuse via the isotropic-state mechanism (Ellens et al., 1989). In this case, the collapse event produced an additional loss of only about 15% of the encapsulated contents, since extensive leakage had already occurred. We also examined the fusion and leakage of DOPE-Me liposomes with and without 2 mol % OArG at pH 4.5 and observed qualitatively the same results (data not shown). The major difference between the two systems is that there is more contents mixing with 5 mM Mg^{2+} at pH 7.4.

The relationship between fusion and leakage rates, ΔT_I, and T_H is shown most clearly in Figure 5, where the initial rates of fusion and leakage are plotted vs temperature. Here we have plotted the data for 1:1 mixtures of ANTS-containing and DPX-containing liposomes at 100 μM total lipid for each type.

The horizontal bar in Figure 5 is the temperature range ΔT_I. T_H for DOPE-Me in the absence and presence of 2 mol % OArG in this buffer is approximately 65 and 45 °C, respectively. It is obvious that the initial rates of both fusion and leakage increase sharply at approximately the temperature at which isotropic ^{31}P NMR resonances are first observed and that leakage proceeds more rapidly than fusion as the temperature increases toward T_H. This is the same temperature-dependent behavior we found for other lipid mixtures for which bulk multilamellar samples exhibited isotropic ^{31}P NMR resonances (Ellens et al., 1986b, 1989). It is also clear that inclusion of 2 mol % OArG reduces the temperature for rapid fusion of these liposomes from ca. 50 to ca. 35 °C.

We also performed the fusion and leakage experiments (using the Tb/DPA assay) with DOPE-Me liposomes containing either 2 mol % OArG or 2 mol % DOG, both at pH 7.4 with 5 mM Mg^{2+}. There was no significant difference between these two DG-containing systems with respect to the fusion or leakage rates or extents from 15 to 60 °C (data not shown). The absolute values for the rates were smaller than those found using the ANTS/DPX assay. These two DGs lower T_H to essentially the same extent (Table II). This

suggests that the beginning of the temperature interval ΔT_I for these systems is also approximately the same.

Discussion

The DSC, X-ray diffraction, and NMR data in this paper demonstrate that 1–3 mol % diacylglycerol (DG) can substantially lower T_H in phospholipid systems. The temperature at which isotropic ^{31}P NMR resonances appear is lowered to nearly the same extent. If these isotropic resonances mark the onset of isotropic or inverted cubic (I_{II}) phase formation (Gruner et al., 1988; Ellens et at., 1989), then this indicates that diacylglycerols reduce this phase transition temperature in parallel with T_H. This is consistent with our theoretical model of the dynamics of these transitions, in which a common intermediate is involved in production of both phases (Siegel, 1986a–c; Ellens et al., 1986b, 1989; Siegel et al., 1988a).

There are several lipid systems which show an enhanced membrane fusion rate in the temperature range ΔT_I (Ellens et al., 1986b, 1989). The present systems are particularly striking in that ΔT_I is changed via addition of such small amounts of a lipid metabolite. The 15 °C drop in the temperature of both the onset of the isotropic resonances and the sharp increase in fusion rate upon addition of 2 mol % OArG (Figure 5) makes it obvious that both processes share a common mechanism.

The data in Figure 5 show that, up to T_H, the initial rates of fusion and leakage are about the same. Whether this indicates that the different pathways leading to leakage and fusion are linked by a common step is not yet known: liposome aggregation is the rate-limiting step in both processes under our experimental conditions.

These DG-doped systems also show aggregation-dependent lysis at temperatures $> T_H$, which has been observed in other similar systems (Bentz et al., 1985b, 1987; Ellens et al., 1986a). The formation of H_{II} phase precursors from liposomal structures results in lysis rather than fusion, although copious lipid mixing occurs.

Mechanism of H_{II} Phase Stabilization by Diacylglycerols. In the formalism of Gruner (1985), hydrophobic molecules like diacylglycerols can stabilize H_{II} phases by either filling hydrophobic interstices between H_{II} tubes or decreasing the spontaneous radius of curvature (R_0) of the lipid/water interfaces in the system. Other factors may affect H_{II} phase stability, but at the present, these two factors provide an adequate qualitative description. R_0 is determined by measuring the H_{II} tube diameter in samples doped with traces of long-chain alkanes in order to remove the interstice-packing stresses (Tate & Gruner, 1987). In DOPE-Me, alkanes at low levels have almost no effect on the tube diameter at a given temperature (Gruner et al., 1988).

H_{II} tube diameters of several DOPE-Me/DG systems are given in Table III, where they are compared with the DOPE-Me tube diameters determined in the presence of low levels of dodecane by Gruner et al. (1988). In all cases, the DG system tube diameters are 0.2–0.6 nm smaller than in the DOPE-Me/alkane systems. This implies that DGs stabilize H_{II} phases by reducing R_0 relative to the pure DOPE-Me or DOPE-Me/alkane systems, and not by filling hydrophobic interstices between H_{II} tubes. Furthermore, recent NMR experiments confirm that DGs do not pack into these interstices (Siegel et al., 1988b). This might be expected, considering the relative hydrophilicity of the glycerol backbone of DG as compared to alkanes.[2] The fact that OAG, with only one long acyl chain, can reduce T_H implies that the nature of the headgroup is the attribute of the DGs most responsible for H_{II} phase stabilization. This is also compatible with DG action through a reduction in R_0, and not through interstice packing. The headgroups of diacylglycerols are less hydrophilic than those of phospholipids. Addition of diacylglycerols should make the lipid/water interfaces more hydrophobic. Yeagle and Sen (1986) pointed out that this may also result in a decrease in R_0 and thus in H_{II} phase stabilization (Yeagle & Sen, 1986).

The total number of cis double bonds in a DG does not seem to determine the extent to which it stabilizes the H_{II} phase (compare DLG with DOG in Table I). DGs with longer acyl chains are more effective in reducing T_H (Table II; compare DEG with DOG, DOG and DLG with DPG). DEG, which has acyl chains substantially longer than those of the host DOPE-Me, is particularly effective in stabilizing the H_{II} phase. This may be because the longer eicosenoic acid chains can extend into the interstices between H_{II} tubes with less entropy reduction than the host chains. A similar effect has been noted for traces of long-chain PCs in PE (Tate & Gruner, 1987).

DOG reduces the T_H of POPE less than it does for DOPE-Me (Table I). This may be because DOG reduces the R_0 of POPE to a smaller extent. The difference in H_{II} tube diameter in 2 mol % DOG/POPE and 3 volume % hexadecane/POPE is only 0.2 nm (data not shown), as compared to a 0.6-nm difference for DOG/DOPE-Me and hexadecane/DOPE-Me (Table I).

Biological Relevance. In vivo, the PI cycle produces transient levels of DGs in biomembranes during stimulus–response coupling (Majerus et al., 1984, 1986; Sekar & Hokin, 1986; Preiss et al., 1986). A principle response in many tissues is exocytosis. The levels of DGs in various unstimulated cells range between 0.003 and 0.01 mol/mol of phospholipid and can increase more than 2-fold upon stimulation (Preiss et al., 1986). Such amounts are sufficient to depress the temperature of the onset of the L_α/H_{II} phase transition by 10 °C or more in phospholipid systems. Many biomembrane lipid compositions actually exist close to this phase boundary under physiological conditions [see Siegel (1986b) and Bentz and Ellens (1988) and references cited therein]. Transient production of such levels of diacylglycerols may bring these lipids into the temperature interval ΔT_I during stimulation. The present and previous work (Ellens et al., 1986b, 1989; Siegel et al., 1988a) has demonstrated that phospholipid membrane fusion is accelerated in this temperature range. While fusion in vivo involves proteins as well as lipids, the fusion properties of biomembranes are probably sensitive to the properties of the lipid components, including changes in their phase behavior. Thus, these results suggest that in addition to their role in protein kinase C activation, DGs may play a more direct role in the fusion event during stimulus–exocytosis coupling.

Acknowledgments

D.P.S. is grateful to Dr. Tom Emge, Dr. Joel Oliver, and W. Broering for obtaining static X-ray diffraction patterns. D.P.S., L.J.L., and P.J.Q. are grateful to Dr. Colin Nave for

[2] Interestingly, the H_{II} tube diameter of a DOPE-Me system containing both hexadecane and 2 mol % DOG is only ca. 0.2 nm smaller than the diameter in hexadecane-doped DOPE-Me at the same temperature (Siegel et al., 1988b). By comparison, the diameter in DOG-doped DOPE-Me is 0.6 nm smaller than in dodecane-doped DOPE-Me (Table III). This implies either that there is some (possibly chain length dependent) synergistic effect of alkanes and DOG on R_0 or that DOG affects H_{II} phase stability in other ways, as well.

expert assistance at the SERC Daresbury Laboratory.

Registry No. DOPE-Me, 87803-74-5; POPE, 10015-88-0; DOPE, 2462-63-7; DOPC, 10015-85-7; OArG, 119616-92-1; OAG, 84746-00-9; DOG, 2442-61-7; DLG, 35098-84-1; CHOL, 57-88-5; DPG, 761-35-3; dieicosenoin, 99491-35-7.

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