up to 1 pH unit and to 83 mV. The deviations of the data from the model at high gradients may be due to nonlinear potential barriers in the membrane or to differences in the pK for H_2O dissociation in the membrane at the membrane interfaces.

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Modification by Diacylglycerol of the Structure and Interaction of Various Phospholipid Bilayer Membranes†

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ABSTRACT: The effects of incorporating diacylglycerol (DG) derived from egg phosphatidylcholine (PC) into PC, egg phosphatidylethanolamine (PE), and bovine phosphatidylserine (PS) have been measured. In excess solution DG induces a multilamellar-to-hexagonal (L–H) structural transition in PE and PC that is temperature dependent. At 37 °C it begins at about 3 and 30 mol %, respectively. In PC at lower DG concentrations a modified lamellar phase is formed; at about 70 mol % DG a single primitive cubic phase forms. An L–H transition induced by 20–30 mol % DG in PS is dependent on ionic strength and degree of lipid hydration, with the appearance of crystalline acyl chains at the higher DG levels. Calcium precipitates of DG/PS (1/1) mixtures have melted chains. Structural parameters were derived for the lamellar phases at subtransition levels of DG in PE and PC. The area per polar group is increased, but by contrast with cholesterol, the polar group spreading is not accompanied by an increase in bilayer thickness. DG does not affect the equilibrium separation of PC or PE bilayers. Measured interbilayer forces as they vary with bilayer separation show that DG at 20 mol % does not effect closer apposition of PC bilayers at any separation. Spreading the polar groups may effect the binding of protein kinase C or the activation of phospholipases; the nonlamellar phases may be linked to the biochemical production of DG in cellular processes involving membrane fusion.

Rapid turnover of a membrane phospholipid, phosphatidylinositol, and its phosphorylated forms is known to occur in a large variety of cells in response to various extracellular stimuli (Hawthorne & Pickard, 1979; Michell, 1975; Nishizuka, 1983; Berridge, 1984; Berridge & Irvine, 1984). Diacylglycerol and inositol trisphosphate produced as a result of this turnover act as second messengers and trigger various cellular responses. The phosphoinositides are soluble in the cytosol and are likely responsible for many cellular responses by mobilization of calcium from intracellular stores (Berridge, 1984). The extremely nonpolar molecule diacylglycerol acts at the membrane level (Nishizuka, 1984). Diacylglycerol has been implicated in several cellular processes, for example, calcium-independent activation of cell secretion and shape changes in platelets (Hokin & Hokin, 1953; Rink et al., 1983), vesiculation and shape changes in erythrocytes (Allan & Michell, 1975; Allan et al., 1978), exocytosis in adrenal medulla chromaffin granules (Knight et al., 1982), intracellular pH changes (Moolenaar et al., 1984), fusion of myoblasts

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(Wakelam, 1983, 1985), and exocytosis in sea urchin eggs (Whitaker & Aitchison, 1985). Diacylglycerol specifically activates protein kinase C (Nishizuka, 1983, 1984). It also promotes the activity of a number of phospholipases when incorporated into their substrates (Dawson et al., 1983, 1984).

Many of these cell responses involve large changes in membrane structure. In a preliminary report we have demonstrated that diacylglycerol can bring about major structural transitions, as suggested by others (Ohki et al., 1982; Dawson et al., 1983), in phospholipid bilayer membranes (Das & Rand, 1984). In this paper we describe the effects of incorporating this unusually hydrophobic molecule in various phospholipid/water systems, effects involving three aspects of their properties: (a) the structure of the lipid bilayers, (b) the forces of interaction between bilayers containing diacylglycerol, and (c) the formation of nonbilayer phases. These effects indicate DG's potential to perturb interactions between neighboring component molecules of membranes. We speculate on how such perturbations could activate membrane enzymes or play a role in membrane fusion.

Materials and Methods

Chemicals. 1,2-Diacylglycerol derived from egg phosphatidylcholine (DG) and dipalmitin were obtained from Serdary Research Laboratories (London, Canada). Egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), and egg phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Egg PE was derived by transesterification of egg PC. Bovine brain PS was converted to the sodium salt by using a chloroform/methanol/water two-phase system. The purity of each lipid as tested by thin-layer chromatography (TLC) was >99%. Monensin was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

Thin-Layer Chromatography. Egg PC, egg PE, and bovine brain PS were chromatographed in chloroform/methanol/7 N NH_4OH (65:35:4 v/v). DG was chromatographed in hexane/ether/acetic acid (65:35:2 v/v). The chromatograms, made of silica gel G, were developed with iodine vapor.

Preparation of Samples. Pure lipids were dried from chloroform solutions, first by a rotary evaporator and then in a vacuum desiccator. The lipids were then stored under nitrogen in sealed containers at low temperature. To make a gravimetric sample, 15–20 mg of lipid was weighed into a small glass vial; the required amount of diacylglycerol was added from a stock solution of 20 mg/mL in hexane. A small amount of chloroform was added to facilitate lipid mixing. Whenever ionophore was required for equilibration of sodium ions, it was added at this stage. The lipid mixture was dried under a gentle stream of dry nitrogen and then vacuum desiccated for 1–2 h. The amounts of individual components were determined by weighing at each stage of sample preparation. The lipid mixtures were then hydrated by weighing in different amounts of 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.4, with the aid of a fine-tipped pipet, or by suspending lipid in a large excess of buffer and recovering it by centrifugation. The samples were sealed under nitrogen and equilibrated for 2–5 days at room temperature (19–23 °C). They were then sealed between mica windows 1 mm apart in X-ray sample holders. Lipid degradation (<1%) was checked periodically by TLC as described above.

Determination of Structural Parameters. X-ray diffraction was used to characterize the structures of various phases formed by the lipids and to measure the structural parameters of these phases. The Cu $K\alpha_1$ line (λ = 1.540 Å) from a fixed anode tube was isolated with a bent quartz crystal monochromator. Diffraction patterns were recorded photographically with Guinier X-ray cameras. The temperature of the samples was controlled by using thyristor-controlled thermoelectric elements and was monitored by a thermistor. All samples contained powdered Teflon as an internal standard to measure the repeat spacings of the lamellar phases (∓0.2 Å). Characterizing the phase structure was done by fitting the X-ray reflections to structures of known lattice geometry. Selected X-ray integrated intensities were measured by densitometry. For the lamellar phase, calculation of the thickness of the hydrocarbon layer, d_l, and water layer, d_w, and area occupied by a lipid molecule, A, were determined as described previously (Luzzati, 1968). The molecular area described at all DG contents is that available to one egg PC, PE, or bovine brain PS head group projected onto the bilayer plane.

Determination of Forces between Interacting Bilayers. The technique used to determine the net repulsive pressure, P, between interacting bilayers and bilayer deformation has been described elsewhere (Parsegian et al., 1979; Lis et al., 1982a,b). It is briefly outlined below. To determine how P varies with d_W, lipids were equilibrated with excess solutions of polyethylene glycol (PEG) (M_r 20 000). After equilibration, the exact concentration of PEG was determined by the index of refraction. Its osmotic pressure, P, was then established from a predetermined calibration curve of directly measured osmotic pressures. Lipid together with a small amount of the PEG solution was mounted in the sample holder. The lamellar repeat spacing, d, bilayer thickness, d_l, interbilayer spacing, d_w, and molecular area, A, were determined from an independently established phase diagram of the same lipid. Plots of d_w against log P were constructed for egg PC containing 12.5% DG and egg PE containing 4% DG.

Results

Structural Transitions Induced by Diacylglycerol

DG by itself is a viscous liquid, immiscible with and highly buoyant in water. Pure DG equilibrated with excess water at room temperature shows no sharp reflections and produces only diffuse scattering around 28 Å and at 4.5 Å, a band characteristic of melted hydrocarbon chains. Dipalmitin, dry or in excess water, shows frozen chains and three-dimensional crystalline structures.

Diacylglycerol can induce nonbilayer phases in charged and uncharged phospholipids under the conditions shown in Figures 1–4. In general these transitions depend on DG concentration, water content, and temperature. We describe below the effect of DG on the phase behavior of egg PC, egg PE, and bovine brain PS.

DG in Egg PC. Figure 1 shows the phases formed by egg PC containing various amounts of DG in excess solution and as a function of temperature. In the single lamellar phase region, up to about 20 wt % DG at 20 °C, the total lipid changed from being sedimented to being buoyant in those samples prepared by centrifugation. No pure DG was detected either by centrifugation or by X-ray diffraction at 28 Å. The intensity ratio of the first and second reflection of these lamellar phases decreased systematically with DG content (Table I).

A lamellar-to-hexagonal (L–H) transition is complete at around 30 wt % DG at room temperature. The L–H transition region is complicated by the coexistence of several phases. This demanded detailed investigation of this region, and the results are presented in Figure 2. Two coexisting lamellar phases of different dimensions were observed (68 and 75 Å) before the hexagonal phase appears. The presence of the second

Table I: X-ray Data for Phospholipid/DG Mixtures[a]

lipid	lamellar repeat (Å)	wt % DG	$I_{001}/I_{002}/I_{003}/I_{004}$	hexagonal d_{100} (Å)	wt % DG	$I_1/I_{\sqrt{3}}/I_2$
DG/egg PC	62.5	0	100/164	61.5	31.8	100/120/100
	55*	0	100/34/7/1.6	61.5	41.2	100/90/28
	53*	0	100/40/63/16	61.5	60	100/96/72
	62.5	8.1	100/240			
	63	12.5	100/160			
	55*	12.5	100/72/11/0.16			
	53*	12.5	100/40/90/160			
	64	15	100/136			
	65	18	100/124			
DG/egg PE	52.8	0				
	53	4				

[a] I refers to relative integrated intensities of the indicated X-ray reflections. An asterisk denotes lamellar phases of restricted hydration; all others are in equilibrium with excess solution.

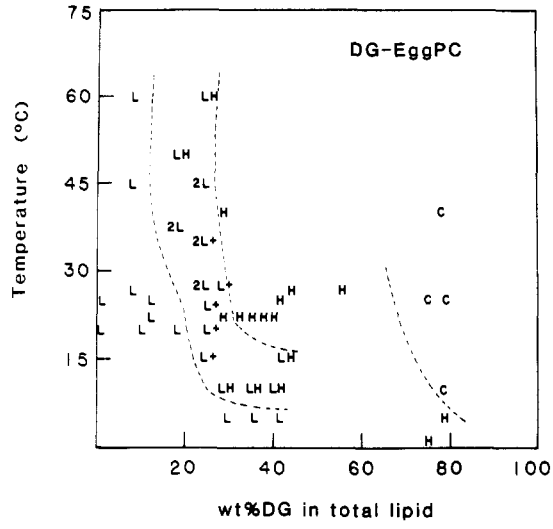

FIGURE 1: Phase behavior of egg phosphatidylcholine containing various amounts of diacylglycerol, in excess 10 mM Tris-HCl, pH 7.4, as a function of temperature (±0.5 °C): L, lamellar; H, hexagonal; C, cubic; +, additional uncharacterized phases. Each symbol represents an individual X-ray experiment.

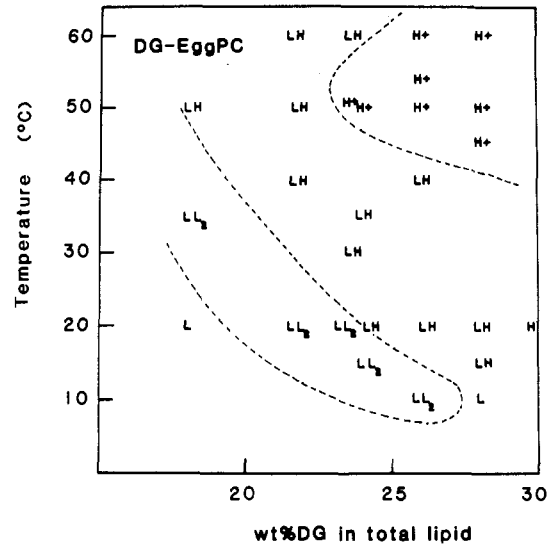

FIGURE 2: Detailed phase distribution of egg phosphatidylcholine containing 15–30 wt % diacylglycerol as a function of temperature. All samples were hydrated with 60 wt % 10 mM Tris-HCl, pH 7.4. L, L_2, two different lamellar phases (see text); H, hexagonal; +, other uncharacterized phase(s). Each symbol represents an individual X-ray experiment.

lamellar phase depended on DG concentration and temperature. At room temperature it was observed at around 22 wt % DG. There was no region of the phase diagram where only the second lamellar phase existed. As DG concentration was increased, the hexagonal phase appeared around 26 and 18 wt % DG at 20 and 50 °C, respectively. In the region of coexistence of L and H phases, as DG content increased, the relative amount of L and H decreased and increased, respectively, as judged by their complement of X-ray intensities.

Over a wide range of DG concentration, about 30–70 wt % DG at 20 °C, a pure hexagonal phase exists whose dimension does not change appreciably but whose relative intensities change (Table I), indicating a modification by DG of the H structure. Increasing temperature produces the pure hexagonal phase at lower DG concentrations.

A hexagonal-to-cubic transition is complete at 75 wt % DG. The single cubic phase has primitive symmetry, established by using the 10 orders of reflections given in Table II. This symmetry is consistent with that observed by Longley and McIntosh (1983) and by Larsson (1983) in a monoolein/water system.

Such effects of DG in PC depend on the hydrocarbon chains of the lipids mixed. Thirty weight percent dipalmitin in dipalmitoylphosphatidylcholine (DPPC) forms a single lamellar phase of repeat distance d = 73 Å. Its hydrocarbon chains are frozen up to at least 65 °C, and no hexagonal phase is observed. Dipalmitin in egg PC produces complex phase separation.

Table II: X-ray Diffraction Data of the Primitive Cubic Phase of Diacylglycerol/Egg Phosphatidylcholine (78.5/21.5 w/w) Hydrated with 70 wt % 10 mM Tris-HCl, pH 7.4, at 25 °C

d (Å)	hkl	unit cell (Å)	rel intensity[a]
78.3	110	110.7	w
47.1	211	114.4	vs
40.1	220	113.4	vs
38.4	221, 300	115.0	s
33.5	222	116.1	m
30.5	321	114.0	m
27.2	411	115.6	w
25.6	420	114.4	m
23.5	422	115.1	m
20.0	441, 522	114.9	vw
		114.4[b]	

[a] Relative intensity of the reflections was estimated visually as vs = very strong, s = strong, m = medium, w = weak, and vw = very weak. [b] Mean value.

DG in Egg PE. Figure 3 shows the phases formed by egg PE containing various amounts of DG in excess solution and as a function of temperature. An L–H transition occurs that is dependent on both DG concentration and temperature. At 37 °C the hexagonal phase begins to appear at 4 wt %, and the transition is complete at 7–8 wt %. Fully hydrated egg PE shows an L–H transition above 65 °C. In contrast to egg PC, no second lamellar phase is observed with egg PE that contains DG. However, over a narrow range of temperature

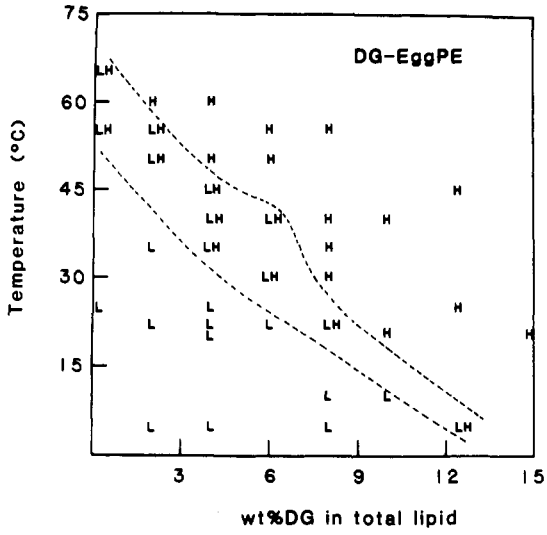

FIGURE 3: Phase behavior of egg phosphatidylethanolamine containing various amounts of diacylglycerol in excess 10 mM Tris-HCl, pH 7.4, as a function of temperature (±0.5 °C): L, lamellar; H, hexagonal. Each symbol represents an individual X-ray experiment.

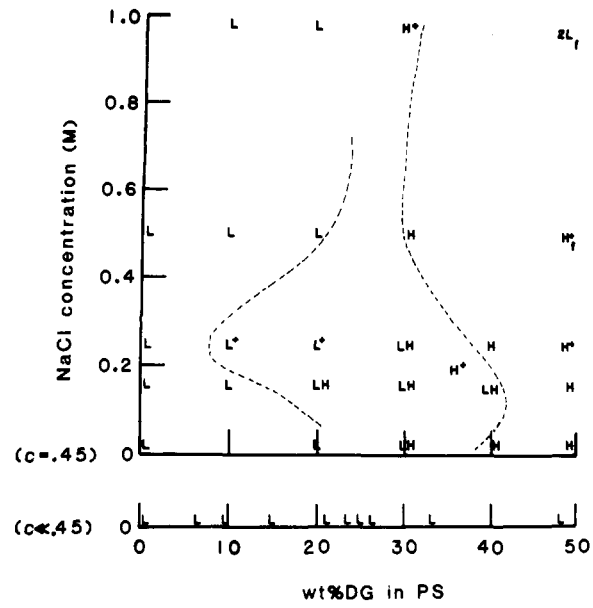

FIGURE 4: Influence of salt, diacylglycerol content, and hydration on phases formed by bovine brain phosphatidylserine hydrated in 10 mM Tris-HCl, pH 7.4: L, lamellar; H, hexagonal; +, other uncharacterized phase(s). Each symbol represents an individual X-ray experiment. The subscript f indicates the presence of crystalline acyl chains. C = weight precent lipid for the cases where no NaCl is present. To avoid indefinite separation of the bilayers in excess 154 (0.9% saline) and 250 mM NaCl, these solutions contained 5 wt % polyethylene glycol (M_r 20000). Charge screening was sufficient to prevent this at higher NaCl concentrations.

and DG concentration, both lamellar and hexagonal phases coexist. This structural sensitivity to DG means that PE bilayers can accommodate only very low levels of DG before undergoing major structural transitions. As a result no changes in X-ray intensities could be detected in the lamellar phase region.

DG in Bovine Brain PS. Figure 4 shows the phases formed by DG/PS mixtures under various conditions. PS bilayers containing various amounts of DG form only lamellar phases when hydrated to greater than 65 wt % water. However, the lamellar phase becomes more disordered as the DG content increases. No L–H transition was observed in these samples up to 40 °C for samples containing more than 65 wt % water. However, at room temperature when the water content of the samples was limited to 55 wt %, the coexistence of a lamellar (d = 85.5 Å) and a hexagonal (d = 65.1 Å) phase was observed at 30 wt % DG. At higher DG content, e.g., around 40 wt %, the transition to a pure hexagonal phase is complete. Thus the L–H transition is sensitive to the degree of hydration of this lipid mixture.

To test the effect of charged head group interaction on the formation of the hexagonal phase, we examined PS containing various amounts of DG in NaCl solutions of different molarities. These results are also shown in Figure 4. To prevent indefinite swelling of the lipid phase with partially screened head groups, i.e., in 154 and 250 mM NaCl, the lipids were hydrated in appropriate solutions containing 5 wt % PEG, which osmotically restricts their uptake of aqueous solution (Loosley-Millman et al., 1982). This was not necessary at higher NaCl concentrations where screening alone prevents indefinite swelling. The main effect of screening the head group charges is to allow the formation of the hexagonal phase. Higher ionic strengths caused a reduction of the region of coexistence of the lamellar and hexagonal phases. The L–H transition begins at 20 wt % DG and is complete at 40 wt % in 154 mM NaCl. High DG and NaCl concentrations result in the coexistence of a lamellar and hexagonal phase or of two lamellar phases and the appearance of frozen acyl chains.

Although NaCl causes the formation of the hexagonal phase in PS bilayers containing DG, presumably by screening the head group charges, calcium ions act in a different manner. In the presence of excess 10 mM $CaCl_2$ solution and calcium ionophore A23187, PS bilayers containing 50 wt % DG form a single lamellar phase of repeat spacing of 53 Å with melted acyl chains. PS alone or PS/PC or PS/PE mixtures in $CaCl_2$ form a dehydrated lamellar phase of repeat spacing of 55 Å with frozen acyl chains, and the neutral lipids segregate from that phase (Portis et al., 1979; Loosley-Millman, 1980; Rand & Parsegian, 1984).

The general pattern that emerges from the data presented here is that DG molecules can be incorporated into phospholipid bilayers but at critical levels, which depend on the lipid and aqueous conditions, induce the transition to nonbilayer structures in various phospholipid systems. As a first step in determining the way DG causes its effects, we have studied how it modifies bilayer structure and bilayer interactions using levels of DG below those that cause the nonbilayer transitions.

Structure and Interactions of DG-Containing Bilayers

We have conducted this study with egg PC and egg PE bilayers containing amounts of DG that are about half of what the bilayers can accommodate at room temperature before nonbilayer structures appear. At higher levels of DG, dehydration leads to the appearance of other phases. The data obtained from such studies are presented below (Figures 5–8).

Egg PC Bilayers Containing 12.5 wt % DG. Figures 5 and 6 show the behavior of PC bilayers containing DG. The lamellar phase swells with increasing water content above 15 wt % to a repeat spacing of 64 Å in about 46 wt % water. At water concentrations less than 15 wt %, a second phase starts to appear, whereas with pure egg PC bilayers, single lamellar phases exist at these lower water concentrations. The X-ray intensities of these phases measured at constant lamellar repeat spacing differ from those of pure PC (Table I). On this basis and from the behavior of the phase diagram data of Figures 1 and 2 and of the densities of the lipid mixtures, we conclude that all the DG is in the lamellar phase. At maximum water contents the scatter in the lamellar repeat spacing becomes

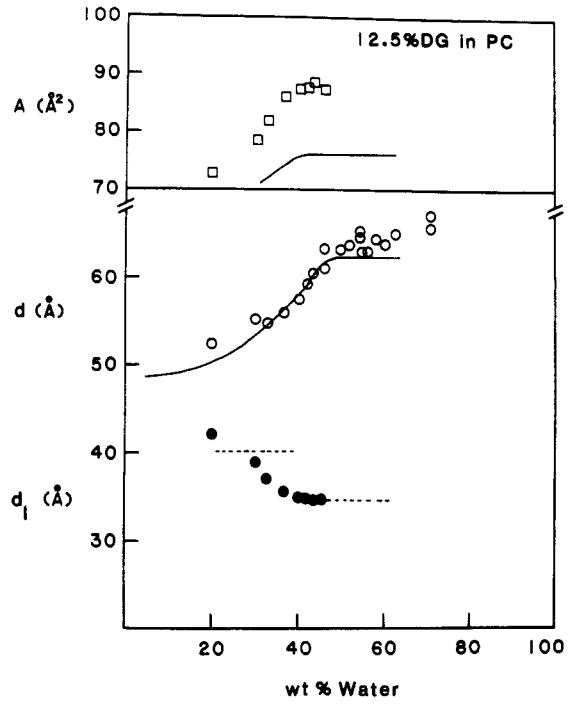

FIGURE 5: Structural dimensions of egg PC bilayers containing 12.5 wt % diacylglycerol, as they vary with weight percent lipid in 10 mM Tris-HCl, pH 7.4, at 25 °C: O, lamellar repeat spacing, d; ●, bilayer thickness, d_l; □, area available to each egg PC head group. The solid lines indicate the same parameters for pure egg PC bilayers. The dotted lines represent the limiting bilayer thickness in excess solution of pure egg PC (lower) and egg PC/cholesterol (1/1) (upper).

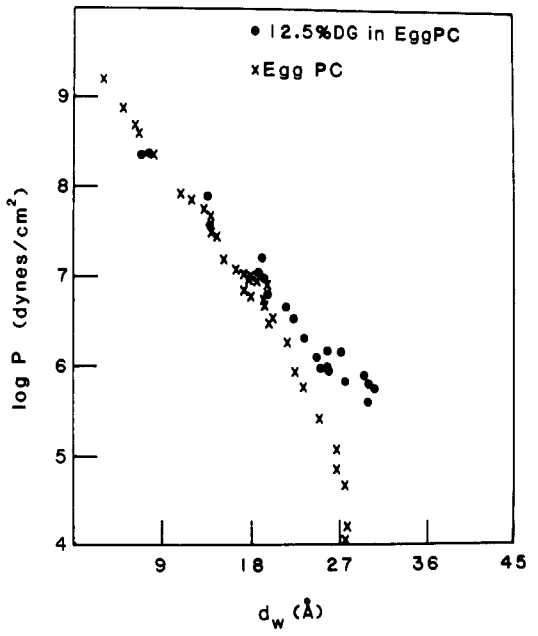

FIGURE 6: Comparison of net interbilayer repulsive pressure, P, as it varies with interbilayer separation, d_w: ×, PC; ●, PC containing 12.5 wt % diacylglycerol.

higher compared to the pure PC lamellar phase. Consequently, the structural parameters can be determined with confidence only up to 46 wt % water. To that level, the bilayer thickness decreases to about 35 Å, and molecular area per egg PC head group increases to about 85 Å². These compare to 35 Å and 75.6 Å² for pure egg PC bilayers, respectively. Thus, DG causes no change in bilayer thickness but does increase the area per PC polar group.

Figure 6 shows the interbilayer pressure P as it varies with bilayer separation for PC bilayers containing 12.5 wt % DG.

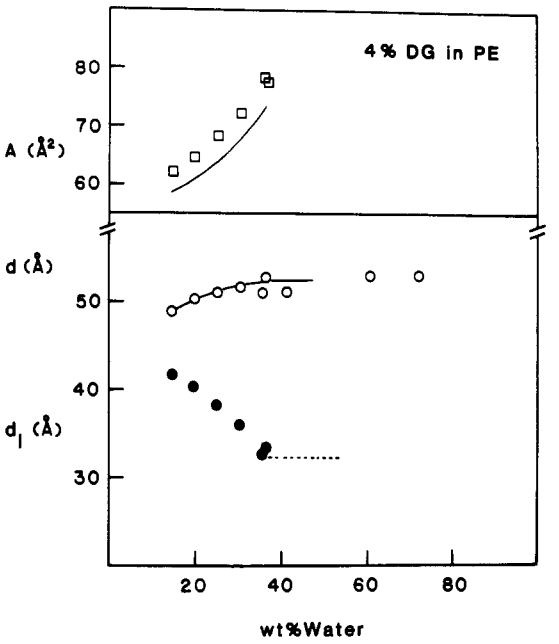

FIGURE 7: Structural dimensions of egg PE bilayers containing 4 wt % diacylglycerol, as they vary with weight percent lipid in 10 mM Tris-HCl, pH 7.4, at 25 °C: O, lamellar repeat spacing, d; ●, bilayer thickness, d_l; □, area available to each PE head group. The solid lines indicate the same parameters for pure egg PE bilayers. The dotted line indicates the limiting value of bilayer thickness in excess solution for pure egg PE.

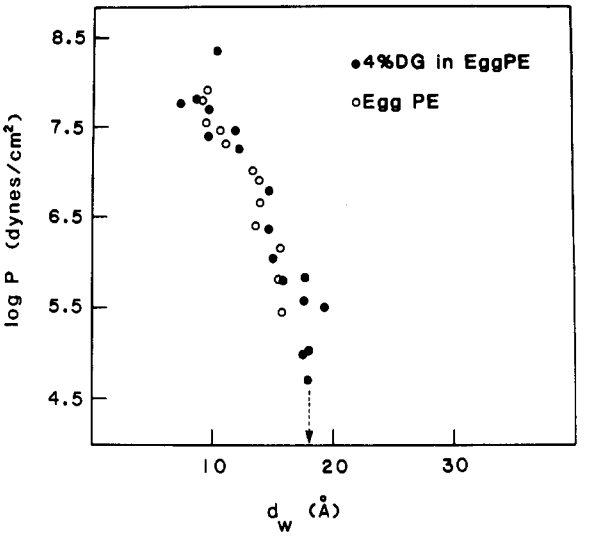

FIGURE 8: Comparison of net interbilayer repulsive pressure, P, as it varies with interbilayer separation, d_w: O, pure egg PE; ●, egg PE containing 4 wt % diacylglycerol.

Also plotted for comparison are similar data for pure egg PC. The force curve of the egg PC bilayers containing 12.5 wt % DG is marginally different from that of pure egg PC bilayers. In spite of the observed scatter at near-maximum hydration, the DG-containing bilayers appear to be, if anything, further apart. Thus, in this case the incorporation of DG does not facilitate any closer approach of two interacting bilayers over their whole observable range of separations.

Egg PE Bilayers with 4 wt % DG. Figures 7 and 8 show comparable data for egg PE containing 4 wt % DG. Figure 7 shows the dimensions and structural parameters of the lamellar phase as they vary with water content. With increasing water the lamellar repeat spacing increases to an equilibrium value of about 53 Å, the bilayer separation increases, the thickness of the bilayer decreases, and the molecular area per

egg PE head group increases. However, as a result of the low levels of DG in these bilayers, no difference in these parameters nor in the X-ray intensities could be detected in comparison to that of pure egg PE bilayers (unpublished data) whose parameters are shown by the solid lines.

Figure 8 shows the net repulsive force between bilayers made from egg PE containing 4 wt % DG as it varies with bilayer separation. It does not differ significantly in comparison to that of pure egg PE bilayers (Figure 8). Assuming these bilayers contain DG, they separate to the same equilibrium spacing under any given external force.

Discussion

These results raise a number of questions about the effects of DG on phospholipid bilayers and the relevance of these to its structural and biochemical activity in native membranes. We consider, first, the effect of DG on bilayer structure, second, DG's effect on the forces of interaction between bilayers, and third, the nonbilayer transitions induced by DG.

Bilayer Structure and Membrane Enzyme Activation. The major detectable effect of DG within bilayers is to spread apart the phospholipids without changing the bilayer thickness. This is clearly seen in the PC system; the small level of DG that can be accommodated by PE bilayers precludes detecting any such an effect. Within bilayers DG itself must occupy an area between 66 and 96 Å^2, depending on whether it spans the whole bilayer thickness or just its hydrocarbon core. In either case, its spreading of the polar groups necessarily exposes hydrocarbon to the aqueous surface, although it is not easy to assess the degree to which the glycerol backbone can act as a polar moiety. The maximum area the PE or PC polar groups can occupy in a bilayer is about 79 and 90 Å^2, corresponding to DG levels of 5 and 20 mol %, respectively. Additional DG causes a nonbilayer transition to occur. A more systematic measure of these structural effects, free of the large region of coexistence of lamellar and hexagonal phases, will require a study using pure PC or PE species.

These structural effects can be contrasted with those produced by cholesterol in phospholipid bilayers. Spreading by interdigitation of cholesterol between PC molecules is significantly offset by the familiar "condensing" effect whereby the rigid steroid nucleus inhibits the motion of the hydrocarbon chains and the bilayer increases in thickness (Lecuyer & Dervichian, 1969; Franks, 1976; Stockton & Smith, 1976). Cholesterol is incorporated up to equimolar concentration in bilayers, at which point the area per phospholipid polar group in egg PC reaches 96 Å^2, an area achieved by about 20 mol % DG. Thereafter, cholesterol has no interaction with the phospholipids. DG, on the other hand, is incorporated into phospholipid structures to very high mole ratios, evoking the major nonbilayer structural transitions described under Results. We conclude that DG and cholesterol have very different effects in the packing of the hydrocarbon chains and of the polar groups within phospholipid bilayers. Further elaboration of these differences will require a detailed study of DG-containing systems, applying the many techniques used to study the effects of cholesterol.

We have depicted in Figure 9 how DG may affect the activation of two kinds of membrane enzymes. DG potentiates the attack by phospholipases of various kinds of phospholipid bilayers (Dawson et al., 1983, 1984). Again, by contrast, cholesterol does not provide that potentiation. Activity increases with DG content and becomes optimal at levels coincident with the L–H transition boundary. Activity then falls rapidly at higher levels, indicating that the substrate in the hexagonal form is not accessible. This optimum concentration

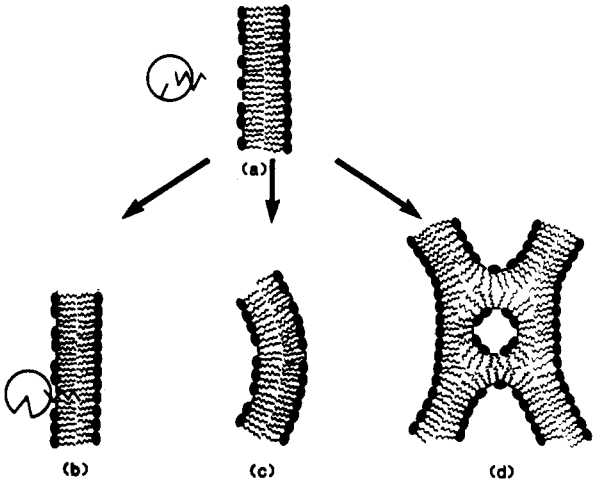

FIGURE 9: Schematic diagram illustrating three possible morphological consequences of the production of diacylglycerol in bilayer membranes. DG is naturally produced on one side of a membrane (a) but in these model studies is uniformly distributed. (b) DG's spreading apart of the phospholipid polar groups enhances access to the membrane hydrocarbon core by proteins that become bound and activated such as protein kinase C (Nishizuka, 1984) or that hydrolyze phospholipids (Dawson et al., 1983, 1984). (c) Rapid transbilayer movement of DG (Allan & Michell, 1978) represents a net mass transfer and transbilayer disparity of area causing membrane bending and vesiculation, first suggested by Allan et al. (1978). (d) The ability of DG to form nonbilayer structures suggests that the formation of inverted micelles in two such apposed membranes represents the first destabilizing step in the lamellar–hexagonal transition and perhaps of membrane fusion. There are about 100 molecules in such an inverted micelle.

coincides with the maximum area occupied by the polar groups of the PC molecules as they are spread apart by DG, with the L–H transition region, with the appearance of the second but ill-defined lamellar phase of the PC transition region, and with the development of an isotropic component of the ^{31}P NMR signal (Dawson et al., 1984). How these structural observations can be translated into enhanced access of the protein for the phospholipid substrate is still unclear. But the destabilizing combination of an enhanced area per polar group without the compensating inhibition of hydrocarbon chain motion that cholesterol imposes appears to provide the necessary structural modification.

Protein kinase C requires PS and calcium, has a preference for the neutral lipid PE, and is activated by diacylglycerol (Nishizuka, 1984). When activated, it becomes membrane bound by a hydophobic region separate from its catalytic sites (Ashendell et al., 1983; Kraft & Anderson, 1983). We suggest that the spread of polar groups allows increased interaction of the bilayer hydrocarbon core and the hydrophobic region of the enzyme molecule (Figure 9a). The preference for PE may reflect the latter's structural sensitivity to DG levels.

The avidity of PS for calcium is such that it causes the separation of PS/PC or PS/PE lipid mixtures into a Ca–PS lamellar phase with frozen chains and a residual lamellar phase (Portis et al., 1979; Loosley-Millman, 1980; Rand & Parsegian, 1984). It is noteworthy that Ca interaction with DG/PS (1/1), on the other hand, produces a single lamellar phase, and one with melted rather than frozen chains. This suggests that DG modulates Ca binding in a significantly different way than the other neutral lipids. That may play a role in the activation of the protein kinase C/lipid complex.

Bilayer Interactions and Membrane Fusion. The major barrier to close contact of phospholipid bilayers, and likely of any hydrophilic surface, is a hydration repulsion that derives from the energy required to remove intervening water (Rand,

1981). This force dominates interactions at all separations less than about 20–30 Å. Figure 6 shows that removal of about 18% of PC polar head groups results in no closer apposition of the bilayers either when water is in excess or when the bilayers are forced closer together osmotically. These results tend to rule out suggestions (Parsegian, 1977; Das & Rand, 1984) that stripping some of the polar head groups and associated water from PC or PE phospholipid bilayers would allow those bilayers to achieve closer apposition and facilitate fusion. If the physical–chemical effects produced by DG have anything to do with the fusion process in membranes, it is more likely to be related either to its asymmetric production, transbilayer diffusion, and consequent induction of membrane vesiculation (Allan et al., 1976, 1978; Ganong & Bell, 1984) or to its ability to cause the structural destabilization necessary for membrane fusion. We have depicted these effects in Figure 9b,c.

Nonbilayer Structures and Membrane Fusion. DG effects the L–H transition by adding hydrocarbon volume to the phospholipid in a way that requires the lipid to form high-curvature monolayers. As the head groups are spread apart to the critical levels of 79 and 90 Å^2 for PE and PC, respectively, we assume that the total hydrocarbon volume has exceeded what the polar groups can cover on a bilayer. Repacking the molecules into inverted cylindrical micelles of the hexagonal phase allows that volume to be sequestered from the water by the available polar groups; i.e., the area per polar group on the water–hydrocarbon interface is significantly reduced by that restructuring (Luzzati, 1962). This interpretation is consistent with the many other causes of the L–H transition involving changes in the polar group to hydrocarbon volume ratio (Luzzati, 1968).

It has also been established that the L–H transition can be effected by a quite different mechanism (Kirk et al., 1984; Kirk & Gruner, 1985). Some lipids of intermediate polar group to hydrocarbon ratio would form a hexagonal phase of large dimension, i.e., very low monolayer curvature, were it not for the fact that the hydrocarbon chains are not long enough to stretch into the hydrocarbon interstices. Pure hydrocarbons, such as tetradecane, allow these lipids to form the large-dimension hexagonal phase, presumably by filling the interstices between the aqueous cylinders. Since DG effects the L–H transition in PC and tetradecane does not, DG is acting at least to increase the intrinsic curvature of the lipid. Because it does this, we cannot establish by this technique whether it also partitions into the hydrocarbon regions as do the alkanes.

We view conditions that lead to the formation of the hexagonal phase as those that must introduce structural instabilities and distortion of the planar molecular packing in membranes. The critical step, both of the L–H transition and of fusion, necessarily involves major structural rearrangements. The L–H transition has been invoked by many in models of the mechanism by which membranes fuse (Verkleij et al., 1979a,b; Hui et al., 1981; Rand et al., 1981). Critical in that mechanism is the destabilization of the membrane and formation of inverted spherical micelles, as shown in Figure 9d (Siegel, 1984). Interestingly, the contribution of each monolayer to such a micelle involves only about 50 molecules. A single DG molecule among them would represent 2 mol %, a value approximating that which effects the L–H transition in egg PE. This illustrates the potential of low levels of DG to cause the kind of membrane destabilization involved in such models of fusion.

Important in the relevance of these physical–chemical studies to native membranes is the question of DG levels. The global concentrations of DG in these model systems are not related in a simple way to the level in native membranes. First, local levels of DG depend on the rates of its enzymatic production, usually from phosphatidylinositol, of its enzymatic conversion, and of its lateral diffusion. Second, local "concentrations" near single DG molecules in a membrane depend on how many neighbors one includes. More relevant is the question of how many neighboring phospholipid molecules are perturbed by a single DG molecule. A lower limit on that can be set by the levels of DG that effect the complete lamellar-to-hexagonal transition. Thus, for PC and PE at 37 °C, one DG molecule can effect the complete repacking of about 15 and 98 neighboring molecules, respectively. DG produced from phosphatidylinositol has longer and more unsaturated chains than those used in this study and would be expected to produce these perturbations at lower levels. The effects seen in these DG–phospholipid model membranes we expect mimic those that may occur around single or transient groups of DG molecules in native membranes. Such effects will be additionally complicated by the asymmetric distribution of phospholipid species and by DG's asymmetric production and its ability to undergo rapid transbilayer diffusion. Alternatively, DG may be producing its effect biochemically, in which case we are no closer to the crucial step of membrane structural rearrangement required by the fusion process. However, because of its potential to effect such structural changes in bilayers, it is attractive to think that DG production represents one link between the biochemical control of the fusion process and the structural changes in the membrane that are required to effect fusion.

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Thermoinactivation and Aggregation of $\alpha\beta$ Units in Soluble and Membrane-Bound (Na,K)-ATPase†

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ABSTRACT: Stability and conformational transitions of soluble and fully active $\alpha\beta$ units of (Na,K)-ATPase in *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$) are examined. Sedimentation equilibrium centrifugation gave a molecular weight of 143 000 for the $\alpha\beta$ unit eluting from TSK 3000 SW gel chromatography columns. Fluorescence analysis and phosphorylation experiments show that E_1–E_2 transitions between both dephospho and phospho forms of soluble (Na,K)-ATPase are similar to those previously observed in the membrane-bound state. The two conformations can also be identified by their different susceptibilities to irreversible temperature-dependent inactivation. E_1 forms of both soluble and membrane-bound (Na,K)-ATPase are more thermolabile than E_2 forms. Gel chromatography on TSK 3000 SW and 4000 SW columns shows that thermal inactivation of soluble (Na,K)-ATPase at 40 °C is accompanied by aggregation of $\alpha\beta$ units to $(\alpha\beta)_2$ units and higher oligomers. The aggregates are stable in $C_{12}E_8$ but dissolve in sodium dodecyl sulfate. Similar aggregation accompanies inactivation of membrane-bound (Na,K)-ATPase at 55–60 °C. These data suggest that inactivation both in the soluble and in the membrane-bound state involves exposure of hydrophobic residues to solvent. The instability of the soluble E_1 form may be related to inadequate length of the dodecyl alkyl chain of $C_{12}E_8$ for stabilization of hydrophobic protein domains that normally associate with alkyl chains of phospholipids in the membrane. Interaction between $\alpha\beta$ units does not seem to be required for the E_1–E_2 conformational change, but irreversible aggregation appears to be a consequence of denaturation of (Na,K)-ATPase in both soluble and membranous states.

Soluble and fully active $\alpha\beta$ units can be prepared from purified membrane-bound (Na,K)-ATPase from outer renal medulla in *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$)[1] (Brotherus et al., 1981, 1983; Craig, 1982). Each soluble $\alpha\beta$ unit binds one molecule of ATP without evidence for cooperative interaction (Jensen & Ottolenghi, 1983), and the soluble enzyme reconstitutes to fully active Na,K pumps in

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[1] Abbreviations: $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; E_1 and E_2, major conformational states of (Na,K)-ATPase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography.

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