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Effects of Diacylglycerols on the Structure of Phosphatidylcholine Bilayers: A ^2H and ^{31}P NMR Study†

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Received February 10, 1989; Revised Manuscript Received May 8, 1989

ABSTRACT: The interaction of four diacylglycerols (DAGs) with multilamellar phospholipid bilayers consisting either of dipalmitoylphosphatidylcholine (DPPC) or of a mixture of DPPC and bovine liver phosphatidylcholine (BL-PC) extracts was investigated by a combination of ^{31}P and ^2H NMR spectrometry. We found that saturated and unsaturated long-chain DAGs induce different types of perturbations into the bilayer structure. The saturated DAGs dipalmitin and distearin induce lateral phase separation of the lipids into (i) DAG-enriched gellike domains and (ii) relatively DAG-free regions in the liquid-crystalline phase. In the latter regions, the order parameters along the fatty acyl chains of DPPC are practically identical with the control. This phase separation effect was observed in both model systems studied, and its extent is dependent upon DAG concentration and temperature. Only bilayer phases were present upon addition of dipalmitin or distearin at all concentrations and temperatures studied. The unsaturated DAGs diolein and DAG derived from egg PC (egg-DAG) affect PC bilayers in the following two ways: (i) by increasing the order parameters of the side chains, as observed for both DPPC and BL-PC model systems; (ii) by inducing nonbilayer lipid phases, as observed for BL-PC, but not DPPC. At a concentration of 25 mol % of an unsaturated DAG in mixed PC bilayers, a peak corresponding to isotropic lipid conformation appeared and increased in intensity with increase in temperature, while at 32 mol % hexagonal and bilayer phases coexisted. Previous studies showed that DAGs containing unsaturated chains can activate a variety of enzymes, such as phospholipases and protein kinase C. Our work indicates that this enzyme-activating ability correlates with the induction of nonbilayer lipid phases by diolein or egg-DAG and is not simply due to the lateral phase separation of the lipids into regions of different fluidities, with corresponding defects in the bilayer, as exhibited by dipalmitin or distearin.

One of the mechanisms of transmembrane signal transduction involves increased metabolism of phosphadidylinositol lipids, and a product of this process is diacylglycerol (DAG)[1] (Michell et al., 1981; Berridge et al., 1983). DAG is produced in vivo from phosphatidylinositols by stripping the polar group of these phospholipids and is a very hydrophobic molecule, acting at the membrane level. Numerous studies demonstrated that DAG and related molecules can directly influence cell behavior. Cellular processes affected by DAG are, for example, activation of cell secretion and induction of morphological changes in platelets (Hokin & Hokin, 1953; Rink et al., 1983) and erythrocytes (Allan & Michell, 1975), exocytosis in adrenal medulla chromaffin granules (Knight et al., 1982) and in sea urchin egg plasma membrane (Whitaker & Aitchison, 1985), the fusion of myoblasts (Wakelam, 1985), and changes in intracellular pH (Moolenaar et al., 1984).

Physicochemical studies of the effects of DAGs on the phospholipid bilayer structure consistently report that DAGs induce destabilization of the bilayers. Observed effects include the appearance of ^{31}P NMR signals characteristic of isotropic or hexagonal lipid phases (Dawson et al., 1984), changes in Ca^{2+}-induced lateral phase separation of lipid mixtures (Ohki et al., 1981), and changes in the bilayer to hexagonal phase transition temperature of phosphatidylethanolamine (Epand,

† This work was supported by grants from the International Union against Cancer and NFWO (to H.D.B.) and from the American Cancer Society (BC-590) (to R.Z.). The ^2H NMR work was done at the NSF Southern California Regional NMR Facility (NSF CHE-791634 and CHE 844-137) located at the California Institute of Technology. The ^{31}P NMR was done with a GN 500 spectrometer (NSF DMB 840491 and NIH BRSG 2 507).
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[1] Abbreviations: BL, bovine liver; DAG, diacylglycerol; DO, diolein; DP, dipalmitin; DPPC, dipalmitoylphosphatidylcholine; DPPC-d_{62}, bis(perdeuteriopalmitoyl)phosphatidylcholine; DS, distearin; PC, phosphatidylcholine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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1984). Addition of DAG to various phospholipid bilayers induces major structural changes as monitored by X-ray diffraction (Das & Rand, 1986). These findings were interpreted as destabilization of bilayers, which was proposed to be responsible for such biological effects of DAG as activation of protein kinase C and phospholipases.

Further support for the hypothesis that DAG induces destabilization of bilayers comes from observations that many phospholipases increase their activity when the lipid substrates contain DAG (Dawson et al., 1983). Earlier works demonstrated the importance of structural defects in lipid bilayer structure for phospholipase A_2 activation (Jain & Cordes, 1973a,b). It was further demonstrated that irregularities in bilayer packing which are present at the gel to liquid-crystalline phase transition temperature (T_c) render phosphatidylcholine (PC) substrate liable to phospholipase A_2 (Op den Kamp et al., 1974, 1975). Similar conclusions were reached by a number of subsequent works (Wilshut et al., 1978; Noordam et al., 1982; Jain et al., 1984; Lichtenberg et al., 1986).

Structural instabilities and nonbilayer phases of the lipids induced by DAGs (Rand et al., 1985) can also affect the capacity of proteins to penetrate into the hydrocarbon region of the bilayers. For example, Ca^{2+}- and phospholipid-dependent protein kinase C, an amphiphilic protein, binds to membranes following production of DAG (Nishizuka, 1984). DAG possibly activates the hydrophilic catalytic site of protein kinase C by permitting the hydrophobic domain of protein kinase C to penetrate into the bilayer (Rand et al., 1985). A similar mechanism may operate in phospholipase A_2 activation because the sequence homology found between the two enzymes probably represents structural elements involved in the interaction with phospholipid (Maraganore, 1987).

In the present work, we used a combination of ^{31}P and ^{2}H NMR to characterize the perturbations induced in the structure of phosphatidylcholine bilayers by four diacylglycerols: diolein and DAG derived from egg-PC (egg-DAG), which were previously shown to be potent activations of various phospholipases (Dawson et al., 1983, 1984), and two diacylglycerols with saturated side chains, dipalmitin (DP) and distearin (DS), which did not exhibit the phospholipase activation effect. Our results show that contrary to the effect predicted by previous studies, only "non-activating" DAGs, DP and DS, but not DO and egg-DAG, induce defect formation in the DPPC bilayers. The effect is more complex in bilayers formed from bovine liver PC, where DO and egg-DAG induce nonbilayer lipid phases at higher temperatures.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC), bis(perdeuteriopalmitoyl)phosphatidylcholine (DPPC-d_{62}), and phosphatidylcholine (PC) extracts from bovine liver (BL) were purchased from Avanti Polar Lipids (Birmingham, AL). Dipalmitin, distearin, diolein, 1,3-diolein, and 1,2-dioleoyl-*sn*-glycerol were from Sigma. Egg-PC-derived diacylglycerol was obtained from Serdary Research Laboratories (London, Canada).

Multilamellar lipid dispersions were prepared by first dissolving phospholipid or the phospholipid/DAG mixture in chloroform. The solvent was then evaporated with a stream of dry nitrogen, and the sample was placed under a vacuum (<1 mtorr) for at least 8 h. The thin film thus formed was hydrated with a 25 mM Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] (pH 7.4) buffer solution, prepared in ^{2}H-depleted H_2O (Sigma) for ^{2}H NMR or in H_2O for ^{31}P NMR experiments. The samples were always fully hydrated and were typically 1:10 (w/v) in lipid to water. A uniform lipid suspension was obtained by placing the sample in a sandbath at 50 °C and periodically vortexing it between three freeze–thaw cycles. Single-component DPPC multilayers and mixtures of DPPC and BL-PC extracts at a molar ratio of 1:3 were prepared.

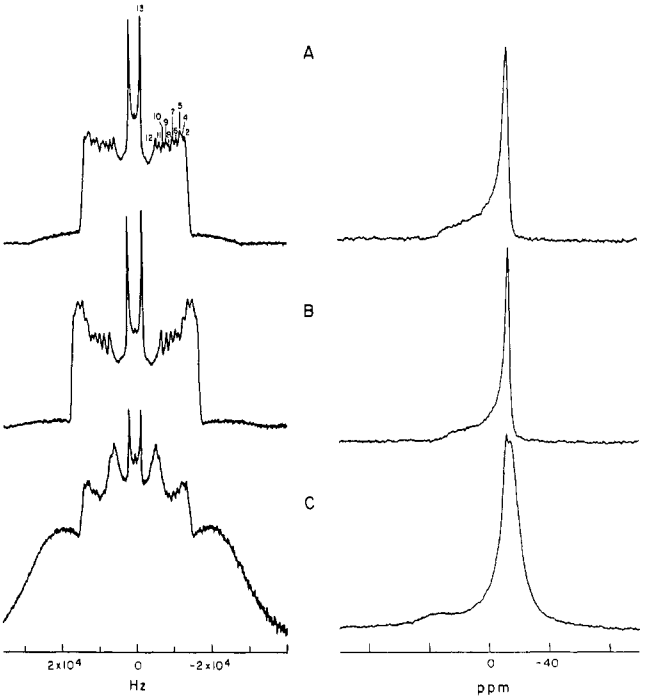

FIGURE 1: (Right) ^{31}P NMR spectra of DPPC/DAG mixtures above T_c of DPPC (T > 42.5 °C). (Left) ^{2}H NMR spectra of DPPC-d_{62}/DAG mixtures at 40 °C (T_c of DPPC-d_{62} is 38.0 °C). (A) No DAG added; (B) with 25 mol % DO; (C) with 25 mol % DP.

^{31}P and ^{2}H NMR spectra were acquired at 11.74 T (corresponding to 500.13-MHz ^{1}H, 202.49-MHz ^{31}P, and 76.78-MHz ^{2}H frequencies) on a Bruker AM500 (^{2}H NMR) or General Electric GN500 (^{31}P NMR) spectrometer. ^{31}P NMR spectra were obtained with a phase-cycled Hahn echo with a $70°-\tau-70°)_n$ pulse sequence (22-μs 70° pulse) and full phase cycling (Rance & Byrd, 1983). The pulse spacing was 30 μs, and the recycle delay was 1.5 s. Gated broad-band proton decoupling of 10 W was used. ^{2}H NMR spectra were acquired with a high-power home-built probe (Müller & Chan, 1983) using the standard quadrupole echo sequence (Davis et al., 1976). The spectral width was 166 kHz, refocusing time 50 μs, and 90° pulse of 4.1 μs.

Results

^{31}P NMR spectra of DPPC multilayers in the absence and presence of diacylglycerols are summarized in Figure 1 (right). The shape of a ^{31}P NMR spectrum of phospholipids generally allows one to distinguish among bilayer, hexagonal, or micellar states of the lipids (Seelig, 1978). The shape of the spectrum in Figure 1A (right), representing DPPC alone, is typical of the bilayer conformation of the lipid molecules (Seelig, 1978). A similar line shape is obtained in the presence of 25 mol % DO (Figure 1B, right), egg-DAG (not shown), DP (Figure 1C, right), or DS (not shown). No indication of nonbilayer conformations of DPPC/DAG mixtures was found up to the highest studied temperature of 60 °C.

The useful parameter which can be obtained from such bilayer-type ^{31}P NMR spectra is the residual chemical shift anisotropy, $\Delta\sigma$, measured as a width between the edges of the spectrum at the half-height of the low-frequency shoulder. Above the liquid-crystalline to gel phase transition temperature (T_c), the values of $\Delta\sigma$ primarily reflect the average orientation

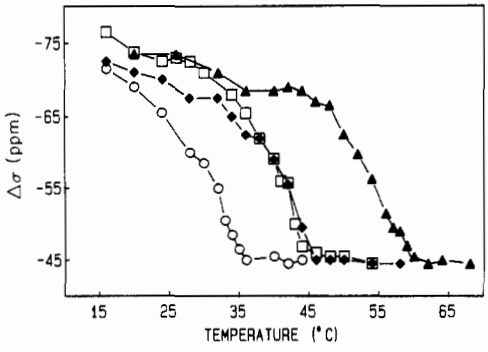

FIGURE 2: Plot of $\Delta\sigma$ vs temperature for aqueous dispersions of DPPC/DAG (25 mol %) mixtures. The experimental errors in $\Delta\sigma$ are ±1 ppm above T_c and ±2 ppm below T_c. (□) DPPC only; (▲) DPPC with DP; (○) DPPC with DO; (♦) DPPC with egg-DAG.

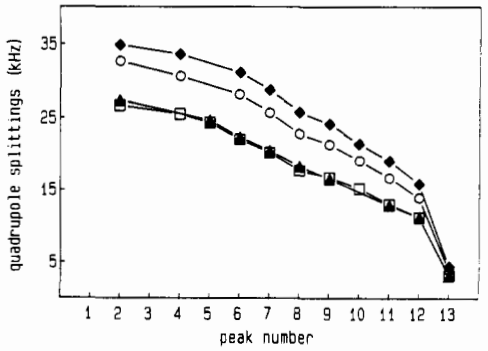

FIGURE 3: Plot of the quadrupole splittings of DPPC-d_{62} vs peak number in the absence and in the presence of DAGs (25 mol %) at 40 °C. The size of the symbols corresponds to the experimental error. (□) DPPC-d_{62}; (▲) DPPC-d_{62} with DP; (○) DPPC-d_{62} with DO; (♦) DPPC-d_{62} with egg-DAG.

of the phosphorus moiety of the phospholipid headgroup relative to the bilayer normal (Banerjee et al., 1985). Furthermore, as $\Delta\sigma$ is also a function of the mobility of the phospholipid headgroups and undergoes a sharp increase in the absolute value at T_c, it can be used to estimate T_c. We confirmed our results on DAG-induced changes in T_c by performing corresponding ^{2}H NMR measurements and observing characteristic broadening of ^{2}H NMR spectra at T_c and differential scanning calorimetry measurements. As previously reported (Browning & Seelig, 1980), the phase transition temperatures obtained by NMR were identical within the experimental error with those determined by differential scanning calorimetry.

DPPC. We studied the effects of DAGs using DPPC or DPPC-d_{62} bilayers, which is a simplified model system with a clearly defined phase transition. The temperature dependences of $\Delta\sigma$ of DPPC with different DAGs added are shown in Figure 2. The absolute value of $\Delta\sigma$ of the control DPPC sample undergoes a sharp increase upon the decrease of temperature at 42.5 °C, corresponding, within the error of our temperature control, to the reported T_c of DPPC of 41.4 °C (Mabrey & Sturtevant, 1976). The presence of 25 mol % DO (Figure 2) *decreases* T_c by 8.5 °C to 34 °C, while the same concentration of egg-DAG does not affect the phase transition temperature (Figure 2). The presence of either 25 mol % DP (Figure 2) or DS (not shown) increases the T_c of DPPC bilayers to 58 °C. The presence of any of the DAGs does not affect $\Delta\sigma$ either below or above of the corresponding T_c, indicating that the average orientation of DPPC headgroups is not affected by DAGs.

The line shape of the ^{31}P NMR spectra of DPPC in the presence of 25 mol % DP (Figure 1C, right) or DS (not shown) between the T_c of pure DPPC (42.5 °C) and the T_c of DPPC/DP or DPPC/DS mixtures (58 °C) can be described as weighted superposition of the spectra of the lipids in the liquid-crystalline and gel states. These results indicate the coexistence of laterally separated lipid domains in the liquid-crystalline and gel phases and were further supported by the ^{2}H NMR results (vide infra).

The ^{2}H NMR spectra of DPPC-d_{62} above the T_c of pure DPPC-d_{62} (38.0 °C) in the presence and absence of DP and DO are shown in Figure 1 (left). A ^{2}H NMR spectrum of fully hydrated chain-perdeuterated lipids is the superposition of axially averaged powder patterns corresponding to the deuterons in the various CD_2 segments along the acyl chains and the terminal CD_3 segment. The observed peak to peak quadrupole splittings, $\Delta\nu^i$, are related to the order parameter S^i_{CD} for each segment according to the equation:

$$\Delta\nu^i = (3/4)(e^2Qq/h)S^i_{CD}$$

where e^2Qq/h = 170 kHz is the quadrupole coupling constant of a deuteron in an aliphatic C–D bond (Burnett & Muller, 1971). The assignment of the ^{2}H NMR spectral peaks to specific methylene units is not unambiguous. ^{2}H NMR studies with lipids deuterated at specific positions in the side chains demonstrated the existence of the characteristic order parameter profile along the chains of the lipid molecules in the liquid-crystalline bilayer phase (Seelig, 1977). For DPPC, this profile has a plateau of relatively higher S_{CD} values corresponding to approximately seven CD_2 segments near the glycerol backbone, which is reflected in the case of chain-perdeuterated DPPC-d_{62} in the overlapping of the corresponding peaks near the edge of a ^{2}H NMR spectrum. Further from the glycerol backbone the side chains become more disordered, and the corresponding $\Delta\nu$ values decrease, producing well-resolved peaks with the smallest quadrupole splitting corresponding to the terminal CD_3 group. Thirteen splittings of DPPC-d_{62} above T_c were resolved, and their tentative assignments to corresponding CD_2 segments can be found in Davis (1979).

Visual examination of the spectrum with DO confirms that the phospholipids maintain their basic bilayer structure above T_c (Figure 1B, left). Almost identical results were obtained in the presence of 25 mol % egg-DAG, except for the fact that two additional peaks between peak 12 and peak 13 were resolved (not shown). Since 34% of egg-DAG consists of DP, the two additional peaks can be caused by the presence of a small amount of DP-immobilized lipids; prominent peaks at the same position were observed in DPPC/DP mixtures (Figure 5D, vide infra). The addition of 25 mol % saturated DAGs, DP (Figure 1C, left) or DS (not shown), causes the appearance of a broad component in the ^{2}H NMR spectra, and additional intensity at ±6–8 kHz superimposed with the narrower spectrum with the well-resolved peaks. This broad component and the additional intensity are characteristic of the gel phase of the lipid bilayers, and, hence, the spectrum indicates the coexistence of gel and liquid-crystalline phases of the lipids, confirming our ^{31}P NMR observations.

The effects of DAGs on the quadrupole splittings of ^{2}H NMR spectra of DPPC-d_{62} are summarized in Figure 3. Either DO or egg-DAG causes a large uniform increase of the quadrupole splittings and corresponding CD_2 order parameters all along the chains. The DO used throughout this study consists of mixed isomers, with 85% 1,3 and 15% 1,2 isomer. We observed in separate experiments that either 1,2- or 1,3-DO induces an identical effect (not shown). The change of $\Delta\nu$'s of DPPC-d_{62}, induced by DO or egg-DAG, gradually increases with increasing DAG concentration, as shown in Figure 4 for DO. In the case of the saturated DAGs, the

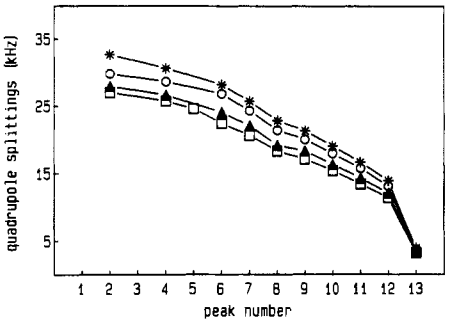

FIGURE 4: Plot of the quadrupole splittings of DPPC-d_{62} vs peak number in the absence and in the presence of different concentrations of DO at 40 °C. (□) DPPC-d_{62}; (▲) DPPC-d_{62} with 7 mol % DO; (○) with 15 mol % DO; (*) with 25 mol % DO.

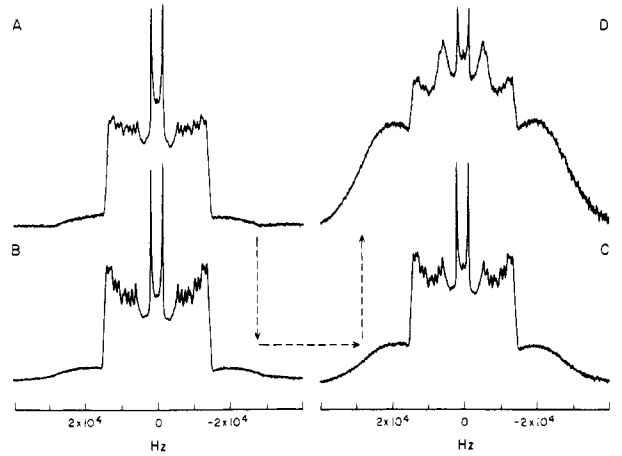

FIGURE 5: ^{2}H NMR spectra of DPPC-d_{62} multilayers in the absence and in the presence of different concentrations of DP at 40 °C. (A) DPPC-d_{62} only; (B) DPPC-d_{62} with 7 mol % DP; (C) with 15 mol % DP; (D) with 25 mol % DP.

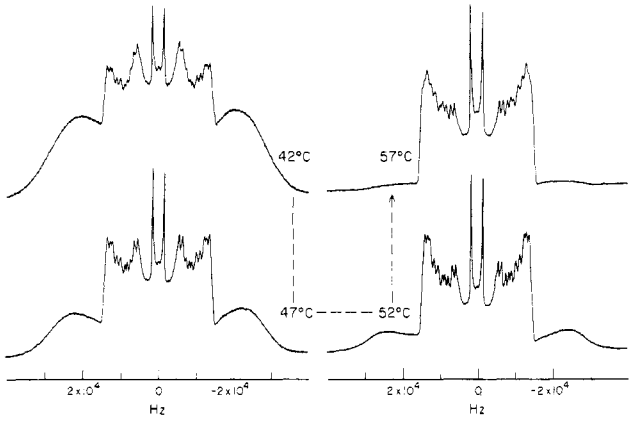

FIGURE 6: ^{2}H NMR spectra of DPPC-d_{62} multilayers with 25 mol % DP at different temperatures.

quadrupole splittings of the resolved peaks of the narrow component of the spectrum in Figure 1C are practically identical with the control, indicating that the laterally separated liquid-crystalline phase of DPPC-d_{62} is unperturbed by the presence of 25 mol % DP (Figure 3) or DS (not shown). We further investigated the coexistence of gel and liquid-crystalline phases of DPPC induced by saturated DAGs as a function of the concentration of DAG (Figure 5) and temperature (Figure 6). The proportion of DPPC-d_{62} in the gel state (broad component) increases with the increased concentration of DP (Figure 5). For each concentration of DP, the resolved quadrupole splittings of the narrow components of the spectra obtained at 40 °C were identical with those of pure DPPC-d_{62} at 40 °C. The ^{2}H NMR spectra of the

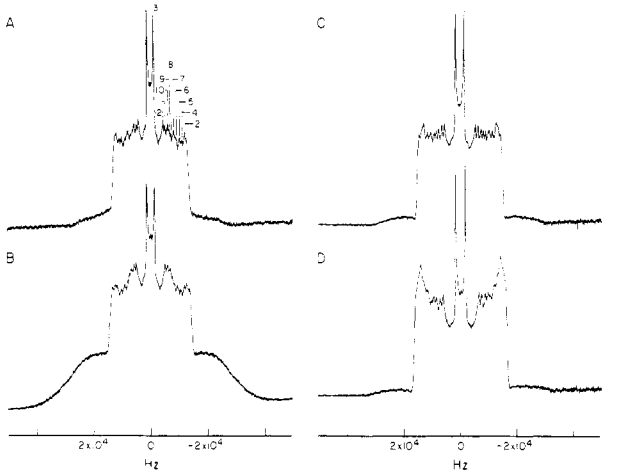

FIGURE 7: ^{2}H NMR spectra of DPPC-d_{62}/BL-PC mixtures (1:3 molar ratio) at 37 °C. (A) No DAG added; (B) with 25 mol % DP; (C) with 25 mol % DO; (D) with 25 mol % egg-DAG.

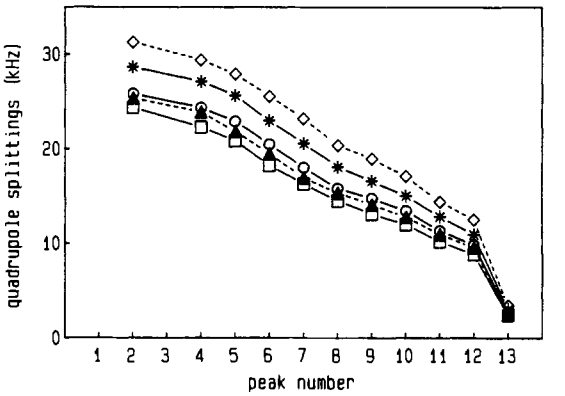

FIGURE 8: Plot of the quadrupole splittings of DPPC-d_{62} in DPPC-d_{62}/BL-PC mixtures (1:3 molar ratio) in the absence and in the presence of 25 mol % DAGs at 37 °C. (□) PC only; (▲) PC with DS; (○) with DP; (*) with DO; (◇) with egg-DAG.

DPPC-d_{62}/DP (25 mol %) mixture at different temperatures illustrate the decrease of the area corresponding to the broad component with increased temperature and its virtual disappearance at 57 °C (Figure 6). At all temperatures studied, the resolved quadrupole splittings of the narrow component of the spectra were similar to each other and to those of pure DPPC-d_{62} at 40 °C. Comparison of the splittings of the ^{2}H NMR spectrum for the DPPC-d_{62}/DP mixture at 57 °C, where only the liquid-crystalline phase is present, with those of pure DPPC-d_{62} at the same temperature shows that there is an increase in the $\Delta\nu$ values in the presence of DP (not shown).

DPPC/BL-PC. In addition to single-component DPPC bilayers, we studied the effect of DAGs on mixtures of DPPC and BL-PC extracts at a molar ratio of 1:3. As in biological membranes, the lipids in this model system have acyl chains of different length and degree of unsaturation. ^{2}H NMR spectra of such PC bilayers with or without DAGs are shown in Figure 7. Again, as in the case of DPPC-d_{62}, the presence of 25 mol % DP (Figure 7B) or DS (not shown) induces coexistence of gel and liquid-crystalline lipid bilayer phases, while DO or egg-DAG does not (Figure 7C,D). The quadrupole splittings obtained from the spectra in Figure 7 are given in Figure 8. The effects of DAGs on the quadrupole splittings of PC bilayers are qualitatively similar to those for pure DPPC-d_{62}, with a difference that small increases of the splittings are now observable upon the addition of the saturated DAGs (Figure 8). This increase is below the significance level

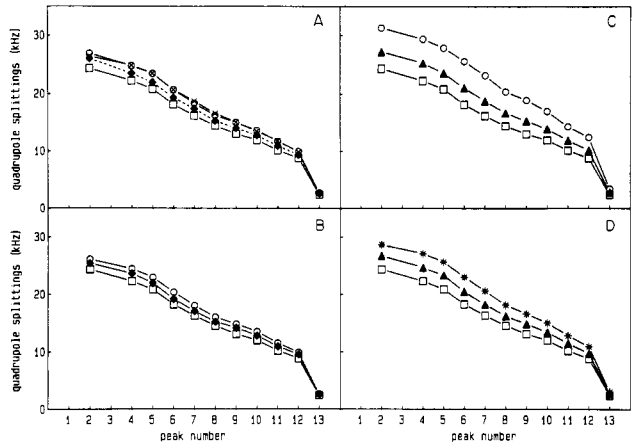

FIGURE 9: Plots of the quadrupole splittings of DPPC-d_{62} in the DPPC-d_{62}/BL-PC mixtures vs peak number in the absence and presence of DAGs at different concentrations at 37 °C. (A) (□) PC only; (♦) PC with 3 mol % DP; (×) with 7 mol % DP; (○) with 25 mol % DP. (B) (□) PC only; (○) PC with 7 mol % DS; (♦) with 25 mol % DS. (C) (□) PC only; (▲) PC with 7 mol % egg-DAG; (○) with 25 mol % egg-DAG. (D) (□) PC only; (▲) PC with 7 mol % DO; (*) with 25 mol % DO.

in the case of DS and slightly above it in the case of DP.

The $\Delta\nu$ values for PC/DAG mixtures at different DAG concentrations are represented in Figure 9. The presence of DS does not significantly affect the quadrupole splittings (Figure 9B), while 7 mol % DP already produces a maximum effect (Figure 9A), indicating that saturated DAGs are not very miscible with PC in the liquid-crystalline state. Both unsaturated DAGs induce an increase of $\Delta\nu$'s with increasing DAG concentration (Figure 9C,D).

^{31}P NMR measurements of PC/DAG mixtures further illustrated the difference between the effects of saturated and unsaturated DAGs. Only bilayer-type ^{31}P NMR spectra were observed with PC/DP or PC/DS mixtures up to 60 °C (not shown). The addition of 25 mol % DO to PC bilayers resulted in the appearance of an additional peak, corresponding to isotropic lipid conformation (small vesicles or micelles), at about 45 °C (Figure 10). The intensity of this peak increased with the increase in temperature, and at 60 °C, most of the lipids were in an isotropic conformation (Figure 10). An identical picture was obtained for PC/egg-DAG mixtures (not shown). Subsequent cooling of the samples to room temperature did not restore the bilayer conformation of the lipids, which was only achieved by repeated freeze–thaw cycles. We did not observe the formation of a hexagonal phase in the presence of unsaturated DAGs at up to 25 mol %. The coexistence of hexagonal and lamellar lipid phases was reported, however, by X-ray diffraction in a similar system by Das and Rand (1986). Their phase diagram for egg-PC/egg-DAG mixtures shows such phase coexistence at 35 °C at a DAG content slightly higher than the maximum used in our study. We therefore increased the egg-DAG concentration to 27 wt % (corresponding to 32 mol %) for a more direct comparison with the results of Das and Rand (1986). Indeed, such a relatively small increase in egg-DAG concentration causes formation of the hexagonal phase coexistent with the bilayer phase, already at 35 °C (Figure 11).

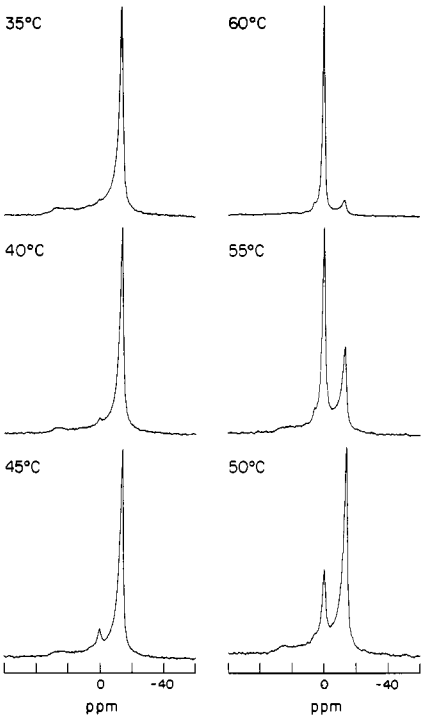

FIGURE 10: ^{31}P NMR spectra of multilayers consisting of DPPC/BL-PC (1:3 molar ratio) and containing 25 mol % DO at different temperatures.

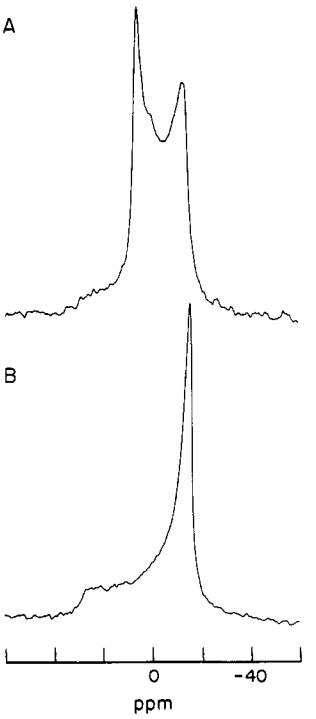

FIGURE 11: ^{31}P NMR spectra of DPPC/BL-PC (1:3 molar ratio) multilayers with egg-DAG at 35 °C. (A) With 32 mol % egg-DAG; (B) with 25 mol % egg-DAG.

Discussion

The present work was initiated to provide comparative information on perturbations induced in PC bilayer membranes by four DAGs, two of which (DO and egg-DAG) activate various phospholipases in in vitro systems and two others (DP and DS) that do not. Our results demonstrate that saturated and unsaturated DAGs differ in respect to the perturbation they induce into the PC bilayer. We found that the addition of DP or DS causes lateral phase separation of PC bilayers into more gellike DAG-enriched domains and a relatively unperturbed region in the liquid-crystalline phase. In the case of DPPC-d_{62}, at 40 °C, this fluid region is probably virtually DAG-free, as concluded from the ^{2}H NMR quadrupole splittings of this phase in the presence of different concentrations of DP or DS being the same as for control DPPC-d_{62}. In the presence of 25 mol % DP, a significant amount of the

lipids remains in the liquid-crystalline state at 40 °C (Figure 5D), which gives the upper limit of less than three immobilized DPPC-d_{62} molecules per one DP molecule at this temperature. The fraction of immobilized DPPC molecules decreases upon increased temperature, reaching zero at 57 °C for 25 mol % DP (Figure 6), at which temperature the quadrupole splittings for the DPPC-d_{62}/DP mixture are significantly higher than for DPPC-d_{62} alone (not shown), indicating that above 57 °C DP is miscible with DPPC-d_{62} and increases the ordering of the side chains of the latter.

Unlike the situation observed with DPPC alone, saturated DAGs probably are present in small amounts in the fluid domains of mixed-chain PC bilayers, because small effects on the quadrupole splittings are noticeable in this case. Apparently, the presence of various fatty acid chains in BL-PC allows a small amount of DP and an even smaller amount of DS to be present in the liquid-crystalline phase of the phospholipids. The DP-induced increase in $\Delta\nu$ is already at its maximum level at 7 mol % of this DAG (Figure 9A), yielding the upper limit for its miscibility with PC in the fluid state at 37 °C.

In contrast to the saturated DAGs, the presence of 25 mol % DO or egg-DAG causes a dramatic *uniform* increase of the quadrupole splittings and corresponding order parameters in both DPPC-d_{62} and BL-PC bilayers. At the same temperature, the effect of egg-DAG on the DPPC-d_{62} quadrupole splittings is larger than that of DO (Figure 3). The difference between DO and egg-DAG, however, becomes negligible if the DO-induced decrease of T_c is taken into account by plotting the quadrupole splittings at reduced temperatures [defined as $\theta = T/(T - T_c)$ where T is the experimental temperature] (not shown).

Because most previous studies suggest that unsaturated DAGs destabilize bilayers, and because the term destabilization is often associated with decreased order, our results were unexpected. The increased order parameters of the lipid side chains by intercalating molecules were, however, described before (Wassall et al., 1986; Zidovetzki et al., 1988) and in this case are probably caused by restriction on the fluctuations of the DPPC side chains imposed by DO or egg-DAG. Such a restriction of fluctuations can be due to the smaller polar headgroup of a DAG molecule which would allow closer contact between lipid side chains. Similar effects were observed by Ohki et al. (1982) using ESR, who showed on a somewhat different system that replacement of yeast phosphatidylinositol by yeast DAG in PC/phosphatidylserine bilayers increases the order parameters of the incorporated spin probe.

Another rather surprising result was the lack of effect of unsaturated DAGs on $\Delta\sigma$ of DPPC above T_c (Figure 2). High sensitivity of $\Delta\sigma$ to even small changes in orientation of phospholipid headgroups was noted previously (Banerjee et al., 1985), and the absence of a large headgroup on a DAG molecule intercalated into DPPC might be expected to alter the environment of the DPPC headgroup with a likely change of orientation of the latter. Our data, nevertheless, indicate that this does not take place.

Our results with unsaturated DAGs and BL-PC bilayers confirm the results of previous studies on similar systems showing that the presence of unsaturated DAGs may induce nonbilayer conformations of the lipids. For example, Cheng and Hui (1986) reported that DO induced a more profound destabilization than DP, as judged from the appearance of an isotropic resonance in ^{31}P NMR spectra. The formation of the H_{II} phase of the lipids in coexistence with the lamellar bilayer phase was reported by Das and Rand (1986) using X-ray diffraction in a system similar to ours but with slightly larger DAG content (25 wt % or 32 mol %) and was also observed by us upon increase of the DAG content (Figure 11). This direct comparison of NMR and X-ray diffraction data shows that in our system, as in most cases described, ^{31}P NMR line shape does correspond to a particular phospholipid conformation. Exceptions from this generalization have been predicted theoretically and by computer simulations (Thayer & Kohler, 1981; Banerjee et al., 1985) and have been reported experimentally (Hui et al., 1981; Noggle et al., 1982). Similar results as ours were observed on a somewhat different system (yeast PC) by Dawson et al. (1984) where an isotropic ^{31}P NMR peak was observed at 15–25 mol % DO at 25–37 °C, and the signal typical of the H_{II} phase appeared upon increase of the DO content to 33 mol %.

Thus, we found that unsaturated DAGs affect PC bilayers in two ways, namely, (i) by increasing the order parameters of the side chains, as observed for both DPPC and BL-PC model systems, and (ii) by inducing nonbilayer lipid phases, as observed with BL-PC but not with DPPC. This raised the question as to which of these effects is responsible for phospholipase activation. Our results indicate that addition of DO or egg-DAG to BL-PC activates pig pancreatic phospholipase A_2, in agreement with earlier works on rat intestinal mucosa phospholipase A_2 (Dawson et al., 1984), but these DAGs totally inhibit this enzyme with a DPPC substrate (Zidovetzki et al., unpublished results). This observation suggests that activation of phospholipase A_2 is associated with the induction of nonbilayer lipid phases. It is worth noting that DO and egg-DAG both induce nonbilayer lipid conformation *and increase* the order of the lipid side chains, indicating that the term "destabilization" should be used with caution.

Additional aspects can be considered in the analysis of our data. These include physicochemical parameters such as bilayer thickness and surface density, which can be calculated from the values of the quadrupole splittings and corresponding order parameters obtained by ^{2}H NMR. We calculated the bilayer thickness by using the assignment of the splittings to specific CD_2 segments of Davis (1979) and the equation which connects the order parameters with the thickness of hydrocarbon regions (Seelig & Seelig, 1974):

$$\langle L \rangle = 1.25\{n - 0.5\textstyle\sum_i [1 - (S_{mol})_i]/1.125\}$$

where n is the number of segments and the molecular order parameter $S_{mol} = 2|S_{CD_2}|$ and $S_{mol} = 6|S_{CD_3}|$ for the terminal CD_3 group (Stockton et al., 1976). It was shown by Salmon et al. (1987) that the assumptions used in this equation are not likely to produce errors of more than ~1 Å. We obtained that DO and egg-DAG increase the bilayer thickness by 1.6 and 2.0 Å, respectively. Such relatively small changes may go unnoticed by X-ray diffraction measurements (Das & Rand, 1986), where additional corrections and assumptions have to be made about the bilayer repeat distance and thickness of the polar headgroup region with associated water and ion molecules. A poor agreement among several X-ray results for DPPC was indeed mentioned by Nagle and Wiener (1988).

The surface density, σ, of the bilayer phospholipids can be determined from the maximum quadrupole splitting in the ^{2}H NMR spectrum, which corresponds to the plateau value of the order parameter (De Young & Dill, 1988), assuming that there is a direct relationship between the surface density and the chain organization, characterized by the order parameter profile. This assumption was supported by experimental (Mely et al., 1975) and theoretical (Dill & Flory, 1980; Dill, 1984) studies. The surface density σ is defined as the ratio of the

area per phospholipid molecule in the crystal, A_0 = 40.8 Å^2, to the area per phospholipid in the bilayer, A. Using the equation (De Young & Dill, 1988):

$$\sigma = (16/9)[\Delta\nu_Q(\text{plateau})/170\ \text{kHz}] + (1/3)$$

we found the area of unperturbed DPPC molecules to be 66 Å^2, which agrees well with the reported values of 68 Å^2 (De Young & Dill, 1988) or 64 Å^2 (Seelig & Seelig, 1974) at slightly higher temperatures. In the presence of DO or egg-DAG, the areas per DPPC molecule decrease to 60 and 58 Å^2, respectively. This is opposite to results of Das and Rand (1986) where an *increase* in the area was observed upon addition of egg-DAG to egg-PC bilayers. The reason for this discrepancy is not immediately clear. The calculation of area per PC molecule from X-ray diffraction data requires correction for the presence of DAG molecules, while no corrections are involved in the area calculation from the quadrupole splittings.

Considering the biological relevance of the reported effects of DAGs, it must be noted that DAG concentrations used by us are higher than generally reported in in vivo studies, where the DAG content in an activated cell can reach 2 mol % (Takiwa et al., 1987). It was, however, pointed out by Kramer et al. (1987) that global amounts of DAG generated on the cytoplasmic membrane surface could be 2–6 mol %, which brings it within the range of concentrations where DAGs induce perturbations in the bilayer structure that are observable by our methods. The DAG/bilayer interactions described here are probably also taking place at lower DAG concentrations. Epand (1985) showed, for example, that addition of only 1 mol % unsaturated DAG lowers the bilayer to hexagonal phase transition temperature of phosphatidylethanolamine by 8.5 °C.

Kiss and Luo (1986) suggested that the difference between the effects of DO and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on protein kinase C activation may be due to the difference between their interactions with membranes, where activated protein kinase C translocates. These authors further noted that the protein kinase C interacting parts of TPA and DO are similar, while membrane-interacting portions of those molecules are not. However, TPA, unlike DO, produces only minimal perturbation of order parameters of DPPC-d_{62} (Zidovetzki and De Boeck, unpublished observations), which correlates with the finding that DAGs, but not phorbol esters, stimulate PC degradation by phospholipase A_2 (Kolesnick & Paley, 1987).

In conclusion, we showed that saturated and unsaturated DAGs, which are known to produce different biological effects, exert different types of perturbation of the phospholipid bilayer. With unsaturated DAGs, the perturbing effect is also dependent on the type of phospholipid matrix, with the degree of unsaturation and chain length as important parameters.

ACKNOWLEDGMENTS

We thank Dr. R. Lee for his assistance in performing ^{31}P NMR experiments.

Registry No. DPPC, 2644-64-6; dipalmitin, 26657-95-4; distearin, 1323-83-7; diolein, 25637-84-7; 1,3-diolein, 2465-32-9; 1,2-dioleoyl-*sn*-glycerol, 24529-88-2.

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Spin-Label ESR Studies of Lipid–Protein Interactions in Thylakoid Membranes

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Received January 25, 1989; Revised Manuscript Received May 19, 1989

ABSTRACT: Lipid–protein interactions in thylakoid membranes, and in the subthylakoid membrane fractions containing either photosystem 1 or photosystem 2, have been studied by using spin-labeled analogues of the thylakoid membrane lipid components, monogalactosyldiacylglycerol, phosphatidylglycerol, and phosphatidylcholine. The electron spin resonance spectra of the spin-labeled lipids all consist of two components, one corresponding to the fluid lipid environment in the membranes and the other to the motionally restricted membrane lipids interacting directly with the integral membrane proteins. Spectral subtraction has been used to quantitate the fraction of the membrane lipids in contact with the membrane proteins and to determine the selectivity between the different lipid classes for the lipid–protein interaction. The fractions of motionally restricted lipid in the thylakoid membrane are 0.36, 0.39, and 0.53, for the spin-labeled monogalactosyldiacylglycerol, phosphatidylcholine, and phosphatidylglycerol, respectively. Spin-labeled monogalactosyldiacylglycerol exhibits very little preferential interaction over phosphatidylcholine, which suggests that part of the role of monogalactosyldiacylglycerol in thylakoid membranes is structural, as is the case for phosphatidylcholine in mammalian membranes. Spin-labeled phosphatidylglycerol shows a preferential interaction over the corresponding monogalactosyldiacylglycerol and phosphatidylcholine analogues, in contrast to the common behavior of this lipid in mammalian systems. This pattern of lipid selectivity is preserved in both the photosystem 1 and photosystem 2 enriched subthylakoid membrane fractions.

The photosynthetic apparatus of green plants comprises a series of integral protein complexes embedded in the thylakoid membrane. The conversion of light energy into a chemically useful form takes place at two reaction centers, photosystem 1 (PS1)[1] and photosystem 2 (PS2), which consist of complexes of different integral proteins. In the thylakoids from the mesophyll cells of higher plants, the two reaction centers and associated protein complexes are separately located in the appressed (PS2) and nonappressed (PS1) membrane regions. The appressed membranes are arranged in stacks and are interconnected by the nonappressed membranes, which contain regions of high membrane curvature. Within the thylakoid, the membrane lipids presumably have an important role to play in stabilizing this structural arrangement and, via the lipid–protein interactions, in integrating the protein complexes and possibly in maintaining their spatial distribution. The very active metabolic state of thylakoid membranes is reflected by their relatively high protein content [65–75% (w/w) protein and pigments], the remainder being acyl lipid.

In addition to the specialized protein composition, the thylakoid membrane has a characteristic lipid composition that

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[1] Abbreviations: 12-MGDGSL, 1-oleoyl-2-[12-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-galactose; 14-PGSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol; *n*-PCSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphocholine; PS1, photosystem 1; PS2, photosystem 2; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; ESR, electron spin resonance.

0006-2960/89/0428-7446$01.50/0