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Interactions of Saturated Diacylglycerols with Phosphatidylcholine Bilayers: A ^{2}H NMR Study†

Hilde De Boeck‡ and Raphael Zidovetzki*
Department of Biology, University of California, Riverside, California 92521
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ABSTRACT: The interactions of a series of saturated diacylglycerols (DAGs) with fatty acid side chain lengths of 6–14 carbons with multilamellar phospholipid bilayers consisting either of dipalmitoylphosphatidylcholine (DPPC) or of a mixture of DPPC and bovine liver phosphatidylcholine (BL-PC) extracts were studied by ^{2}H NMR spectrometry. We found that the perturbation induced by the DAGs into the bilayer structure strongly depends on the length of the DAG fatty acid side chain. Shorter chain 1,2-*sn*-dihexanoylglycerol and, to a larger degree, 1,2-*sn*-dioctanoylglycerol (diC_8) induce transverse perturbation of the bilayer structure: the order parameters of the phospholipid side chains are increased by the intercalating DAG molecules in the region adjacent to the phospholipid headgroups and decreased toward the terminal methyls, corresponding to the bilayer interior. The longer chain DAGs ($C \geq 12$) studied in this and previous [De Boeck & Zidovetzki (1989) *Biochemistry 28*, 7439] work induce lateral phase separation of the lipids into DAG-enriched gellike domains and relatively DAG-free regions in the liquid-crystalline phase. Each of the DAGs studied induces a decrease in the area per phospholipid molecule, and a corresponding *increase* in the lateral surface pressure of the bilayers. Since numerous biochemical studies consistently report that diC_8 is the most effective of saturated DAGs in activating protein kinase C, we may conclude that the activation of this enzyme is associated with a transverse perturbation of the lipid bilayer structure and a decreased ordering in the interior of the bilayer membrane, and is less affected by the lateral phase separation of the lipids into regions of different fluidities, as induced by the longer chain DAGs. The DAG-induced lateral phase separation may, however, play a role in activating other enzymes, such as pig pancreatic phospholipase A_2.

Diacylglycerols (DAGs)[1] are endogenous second messengers, produced as a result of cell activation by a variety of stimuli [see Berridge (1987) for a review]. Several exogenously added DAGs will stimulate a variety of biological responses. Exogenous (mostly short-chained) DAGs stimulate protein kinase C (PK-C) in C62B glioma cells (Brooks et al., 1982), pituitary HL-60 cells (Ebeling et al., 1985), and human carcinoma A431 cells (Davis et al., 1985a,b). Tumor-promoting activity of 1,2-*sn*-dioctanoylglycerol (diC_8) and 1,2-*sn*-didecanoylglycerol (diC_{10}) on mouse skin was reported by Verma (1988), Smart et al. (1989), and Hansen et al. (1990). Recently, 1,2-*sn*-dihexanoylglycerol (diC_6) and diC_8 were shown to enhance light-induced stomatal opening in plants (Lee & Assmann, 1991). In the studies where the homologous series of saturated short-chained DAGs was employed, the emerging consensus is that the most potent PK-C activator is diC_8 with a somewhat smaller effect of diC_6 and diC_{10}, while shorter or longer chain DAGs are usually ineffective (Mori et al., 1982; Jetten et al., 1985; Ebeling et al., 1985; Cohn et al., 1985; Go et al., 1987; Sekiguchi et al., 1988). The biological effects of short-chain DAGs were reviewed by Abdel-Latif (1986).

A growing number of studies indicate that exogenously added DAGs can stimulate other intracellular enzymes. Goppelt-Strübe et al. (1987) reported that diC_8 inhibits membrane-bound lysophosphatide acyltransferase, probably

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‡ Deceased.

[1] Abbreviations: BL, bovine liver; DAG, diacylglycerol; diC_6, 1,2-*sn*-dihexanoylglycerol; diC_8, 1,2-*sn*-dioctanoylglycerol; diC_{10}, 1,2-*sn*-didecanoylglycerol; diC_{12}, 1,2-*sn*-didodecanoylglycerol; diC_{14}, 1,2-*sn*-ditetradecanoylglycerol; DPPC, dipalmitoylphosphatidylcholine; DPPC-d_{62}, diperdeuteriopalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; PC, phosphatidylcholine; PK-C, protein kinase C.

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by modification of interactions of this enzyme with the lipid membrane. Intracellular free Ca^{2+} concentration increase in promyelocytic HL-60 cells or in pancreatic islet HIT T16 cells (Restrepo et al., 1989; Thomas et al., 1991) and inhibition of neuron calcium channels by diC_8 (Hockberger, 1989) do not appear to be mediated PK-C. Stimulation of the exocytosis of the sperm acrosome by diC_8 (Roldan & Harrison, 1990) is also a PK-C-independent process, because spermatozoa lack PK-C. Among the other PK-C-independent effects of DAGs is diC_8-induced translocation of cytidyltransferase to microsomes (Kolesnik & Hemer, 1990). This effect is subject to surface dilution (i.e., it is not diC_8 concentration dependent but proportional to the moles of diC_8 added per cell), suggesting that membranes are the probable site of diC_8 action.

The structural features of hydrophobic domains of activating phospholipids affect the activation of protein kinase C (Snoek et al., 1988), making the effect of such hydrophobic cofactors as DAG on the membrane structure a probable parameter in PK-C activation. Indeed, the length and unsaturation of the DAGs' fatty acid chains affect their function as cofactors in activation of PK-C (Kishimoto et al., 1980; Cabot & Jaken, 1984; Lapetina et al., 1985; Bonser et al., 1988). Lester (1990) and Bell and Burns (1991) recently suggested that optimal PK-C activation depends on both the interaction with the active site of DAG and a suitable physicochemical state of the lipid hydrocarbon environment.

Physicochemical effects of DAGs on lipid bilayers have been examined by X-ray diffraction (Das & Rand, 1983, 1986), electron microscopy and electron spin resonance (Ohki et al., 1982), differential scanning calorimetry (DSC) (Epand, 1985; Ortiz et al., 1988; Siegel et al., 1989), and NMR (Dawson et al., 1984; Siegel et al., 1989; Hamilton et al., 1991a,b). These studies demonstrate that the presence of DAGs affects structural changes of lipid membranes. Such structural changes may be partially responsible for the induced biologial responses, both through PK-C-dependent and through PK-C-independent pathways.

In the present paper, we extend our previous work (De Boeck & Zidovetzki, 1989) to include shorter chain (6–14 carbons), saturated DAGs and demonstrate the strong dependence of DAG-induced bilayer perturbation on the length of the DAG side chains.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC), diperdeuteriopalmitoylphosphatidylcholine (DPPC-d_{62}), and phosphatidylcholine (PC) extracts from bovine liver (BL) were purchased from Avanti Polar Lipids (Birmingham, AL). 1,2-*sn*-Dihexanoylglycerol (diC_6), 1,2-*sn*-dioctanoylglycerol (diC_8), 1,2-*sn*-didecanoylglycerol (diC_{10}), 1,2-*sn*-didodecanoylglycerol (diC_{12}), and 1,2-*sn*-ditetradecanoylglycerol (diC_{14}) were obtained from Serdary Research Laboratories (London, Ontario).

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)-propane-1,3-diol] (pH 7.4) buffer solution, prepared in ^2H-depleted H_2O (Sigma) for ^2H NMR experiments 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 three freeze–thaw cycles. Single-component DPPC-d_{62} multilayers and mixtures of DPPC-d_{62} and BL-PC extracts at a molar ratio of 1:3 were prepared.

^{31}P and ^2H NMR spectra were acquired at 11.74 T (corresponding to 500.13-MHz ^1H, 202.49-MHz ^{31}P, and 76.78-MHz ^2H frequencies) on a General Electric GN500 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 70° pulse was used instead of the more common 90° pulse, in order to compensate for the limited radio-frequency power. The pulse spacing was 30 μs, and the recycle delay was 1.5 s. Gated broad-band proton decoupling of 10 W was used. ^2H NMR spectra were acquired with a high-power probe (Doty Scientific, Columbia, SC) using the standard quadrupole echo sequence (Davis et al., 1976). The spectral width was 0.5–1 MHz, refocusing time 60 μs, and 90° pulse of 3.2 μs.

Results

The ^2H NMR spectra of DPPC-d_{62} or BL-PC/DPPC-d_{62} mixtures in the absence and in the presence of 25 mol % of the five DAGs are shown in Figures 1 and 2, respectively. A ^2H NMR spectrum of fully hydrated chain-perdeuterated lipids is the superposition of axially averaged powder patterns that correspond to the deuterons in the various CD_2 segments 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:

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

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 ^2H NMR spectral peaks to specific methylene units is based upon ^2H NMR studies with lipids deuterated at specific positions in the side chains, which demonstrated the existence of a 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 eight 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 ^2H 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. Up to 13 splittings of DPPC-d_{62} above the gel to liquid-crystalline phase transition temperature (T_c) were resolved, and their tentative assignment to corresponding CD_2 segmens can be found in Davis (1979).

Visual examination of the spectra in Figures 1 and 2 shows that the basic bilayer structure of the phospholipids is maintained in the presence of 25 mol % DAGs. Addition of 25 mol % diC_{12} or diC_{14} causes the appearance of a broad component, which is characteristic of immobilized lipid molecules (vida infra).

diC_6. The effects of different concentrations of diC_6 on the quadrupole splittings of DPPC-d_{62} are summarized in Figure 3. Although the shape of the ^2H NMR spectrum in the presence of 25 mol % diC_6 is similar to the control (Figure 1A,B), diC_6 at this concentration causes a small but significant decrease in the quadrupole splittings for peaks 4–13. Peak 2, which corresponds to approximately eight CD_2 segments near the glycerol backbone, has $\Delta\nu$ values which are only slightly higher than those for pure DPPC-d_{62}. The possibility that the observed effect is due to wrong peak numbering in this and the following cases was ruled out by a temperature study: at elevated temperatures, all 13 peaks are resolved for

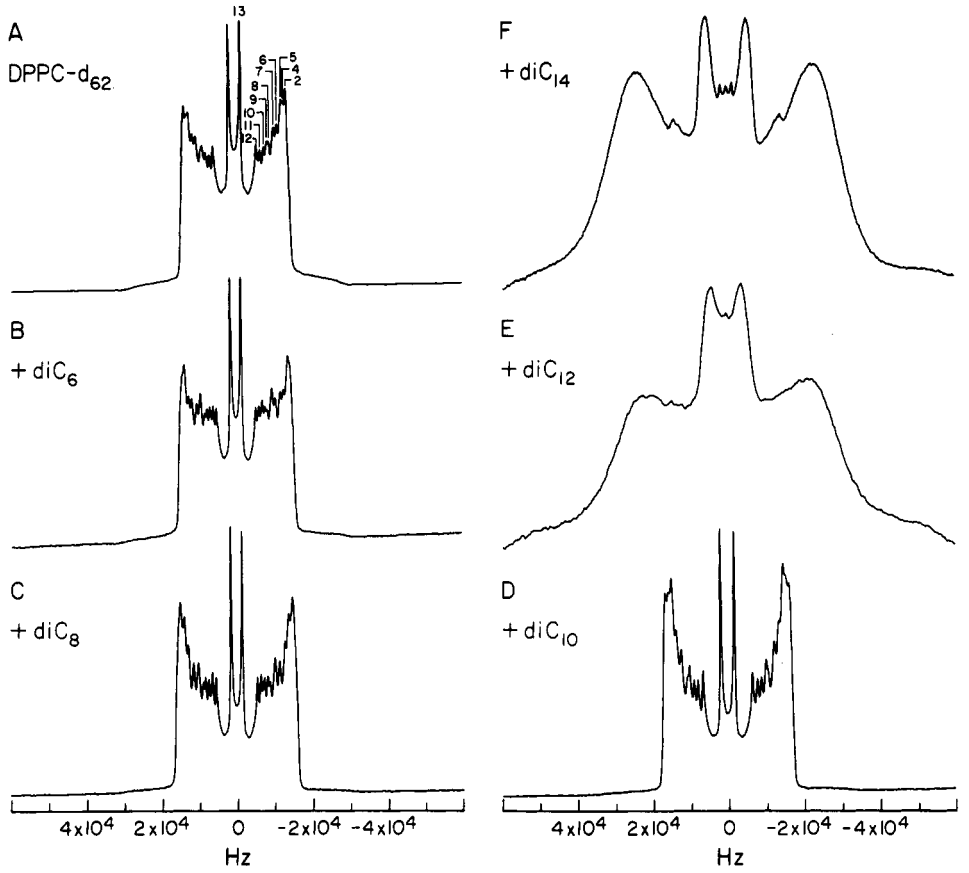

FIGURE 1: ^2H NMR spectra of DPPC-d_{62} in the absence or presence of 25 mol % DAGs at 40 °C. Peaks 1 and 3 are resolved from peak 2 only at higher temperatures.

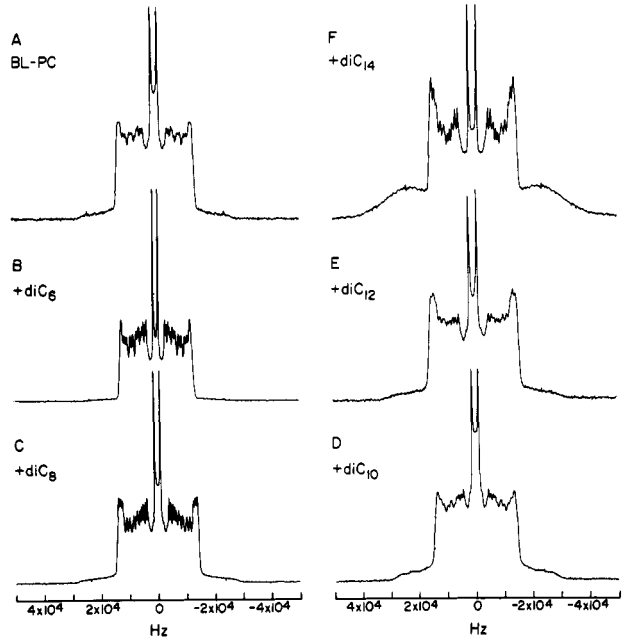

FIGURE 2: ^2H NMR spectra of DPPC-d_{62}/BL-PC mixtures (1:3 molar ratio) in the absence or presence of 25 mol % DAGs at 37 °C.

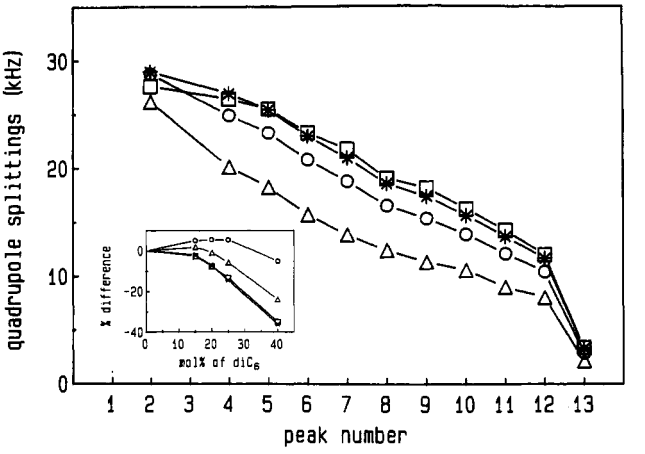

FIGURE 3: Plots of the quadrupole splittings of DPPC-d_{62} vs peak number in the absence and in the presence of different concentrations of diC_6 at 40 °C. The size of the symbols corresponds to the experimental error. (□) DPPC-d_{62}; (*) with 15 mol % diC_6; (○) with 25 mol % diC_6; (△) with 40 mol % diC_6. Inset: Change of quadrupole splittings of DPPC-d_{62} upon addition of diC_6 at 40 °C. (○) Peak 2; (△) peak 4; (□) peak 8; (×) peak 13.

control and diC_6-containing samples, allowing straightforward comparison. Gradual lowering of the temperature to 40 °C allowed us to follow the redistribution of the peaks and assured consistent peak numbering.

The changes of the quadrupole splittings as a function of the molar ratio of diC_6 are shown in Figure 3 (inset) for four peaks, which correspond to different positions on the DPPC-d_{62} side chains. The effect of diC_6 on quadrupole splittings of DPPC-d_{62} has a biphasic character: little change is observed up to 15 mol % of diC_6; further increase in diC_6 concentration causes a significant decrease of order parameters for all positions with the most prominent effect for peaks 8 and 13 which correspond to carbons located near the center of the bilayer.

The effect of diC_6 on quadrupole splittings of DPPC-d_{62} incorporated into BL-PC was similar to the effect of high concentration of diC_6 on pure DPPC-d_{62}: at 25 mol %, all splittings decreased, except for peak 2, which corresponds to the carbon position nearest to the headgroup. The effect was most prominent further from the headgroup region (Figure 4A).

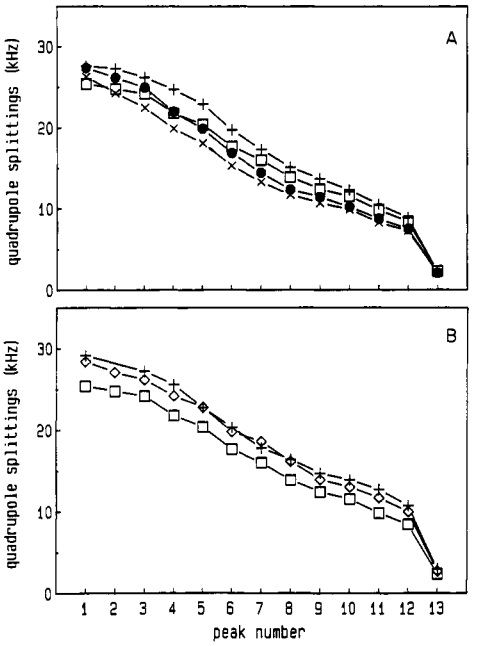

FIGURE 4: Plots of the quadupole splittings of DPPC-d_{62} in DPPC-d_{62}/BL-PC mixtures in the absence and in the presence of 25 mol % DAGs at 37 °C. (A) (□) DPPC-d_{62}/BL-PC only; (×) with diC_6; (●) with diC_8; (+) with diC_{10}. (B) (□) DPPC-d_{62}/BL-PC only; (◊) with diC_{12}; (+) with diC_{14}.

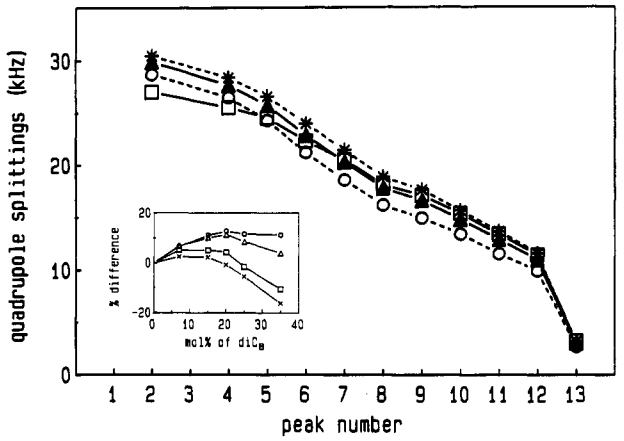

FIGURE 5: Plots of the quadrupole splittings of DPPC-d_{62} vs peak number in the absence and in the presence of different concentrations of diC_8 at 40 °C. (□) DPPC-d_{62}; (*) with 20 mol % diC_8; (▲) with 25 mol % diC_8; (○) with 35 mol % diC_8. Inset: Change of quadrupole splittings of DPPC-d_{62} upon addition of diC_8 at 40 °C. (○) Peak 2; (△) peak 4; (□) peak 8; (×) peak 13.

diC_8. The presence of different concentrations of diC_8 induces relatively small changes in the values, but obvious changes in the *profile* of the quadrupole splittings of DPPC-d_{62} (Figure 5). At concentrations of up to 20 mol % diC_8, the quadrupole splittings are higher than the control values for the chain segments near the headgroup (peaks 2–4), but the increase in $\Delta\nu$ values becomes insignificant further away from the glycerol backbone (peaks 5–13). Due to overlapping symbols for 7, 15, and 20 mol % diC_8, only 20 mol % is shown in Figure 5. The inset in Figure 5 shows that the concentration effect of diC_8 is biphasic. Moreover, the concentration effect is a function of the position on the lipid side chains: at low concentration, diC_8 affects primarily lipid side chains close to the headgroup (Figure 5, inset, peaks 2 and 4). This effect reaches maximum at 20 mol % diC_8. Further increase of the molar fraction of diC_8 affects primarily lipid side chain segments close to the center of the bilayer (peaks 8 and 13) and causes *disordering* of this part of the bilayer. The biphasic

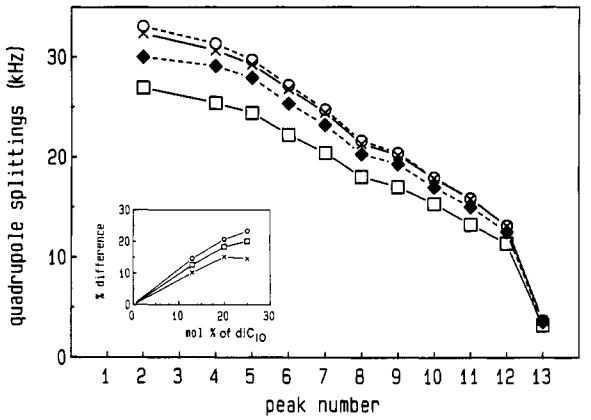

FIGURE 6: Plots of the quadrupole splittings of DPPC-d_{62} vs peak number in the absence and in the presence of different concentrations of diC_{10} at 40 °C. (□) DPPC-d_{62} only; (♦) with 13 mol % diC_{10}; (×) with 20 mol % diC_{10}; (○) with 25 mol % diC_{10}. Inset: Change of quadrupole splittings of DPPC-d_{62} upon addition of diC_{10} at 40 °C. (○) Peak 2; (□) peak 8; (×) peak 13.

character of the effects of diC_6 and diC_8 (Figures 3 and 5, insets) suggests that the presence of a small amount of diC_8 opens up a new positioning site for diC_8 and the presence of diC_8 at this new site disorders the DPPC-d_{62} side chains in the region near the terminal methyls.

diC_8 has a qualitively similar effect on the quadrupole splittings of DPPC-d_{62} in BL-PC bilayers: increased values for peaks 2–4, which correspond to about nine carbons next to the headgroup, and decreased $\Delta\nu$ values for peaks 5–13 (Figure 4A); the increase of the quadrupole splittings is, however, smaller than in the case of pure DPPC-d_{62}.

diC_{10}. As can be seen in Figure 1D, the presence of 25 mol % diC_{10} does not disrupt the bilayer structure of DPPC-d_{62} at 40 °C, but there is an increased spectral intensity in the plateau region. To assure consistent peak numbering, lower concentrations of diC_{10} were used, and the fate of the peaks was followed up to 25 mol % diC_{10}. All quadrupole splittings for DPPC-d_{62} increase in the presence of diC_{10} in a concentration-dependent way (Figure 6). From the inset in Figure 6, it is clear that the increase induced by diC_{10} is monophasic, unlike diC_6 or diC_8, up to 25 mol % diC_{10}. An increase of the diC_{10} concentration above 25 mol %, however, induced immobilized lipid phase (not shown). A similar increase of the quadrupole splittings was also observed on the BL-PC/DPPC-d_{62} system (Figure 4A).

Interestingly, ^{31}P NMR spectra of DPPC with 25 mol % diC_6–diC_{10} were identical to those of the control, with no significant change in chemical shift anisotropy ($\Delta\sigma$) values which were −46 ppm with or without the DAGs. Values of $\Delta\sigma$ are very sensitive to the angles formed by the phosphate moiety of the DPPC with the plane of membrane (Banerjee et al., 1985), and could be expected to change upon addition of a DAG with its virtual absence of a headgroup. Our present and previous (De Boeck & Zidovetzki, 1989) data indicate that DAGs do not affect the average orientation of the phosphorus moiety of the PC headgroups.

diC_{12}. The addition of 25 mol % diC_{12} to DPPC-d_{62} at 40 °C induces the presence of an immobilized phase (Figure 1E). This effect was further investigated as a function of temperature. The ^2H NMR spectra of the DPPC-d_{62}/diC_{12} (25 mol %) mixture at different temperatures in Figure 7 illustrate the decrease in intensity associated with the broad component upon increasing temperature and its virtual disappearance above 52 °C. This temperature corresponds to the midpoint of the phase transition, as obtained from DSC measurements. At the one

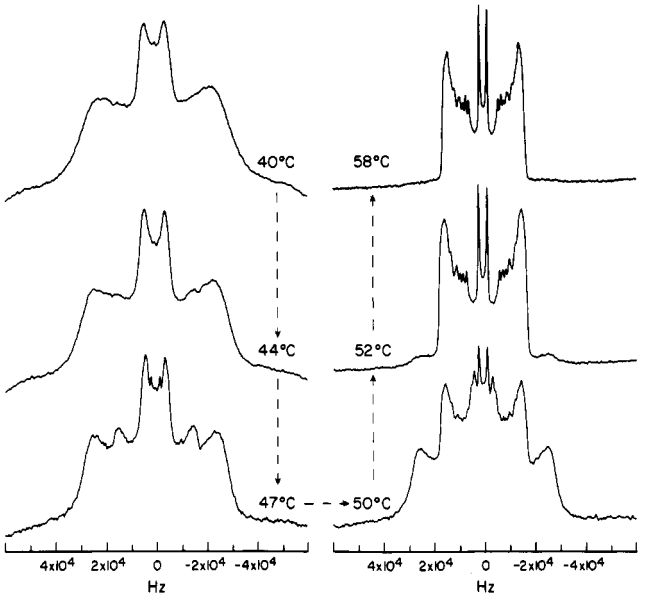

FIGURE 7: ^2H NMR spectra of DPPC-d_{62} with 25 mol % diC_{12} at different temperatures.

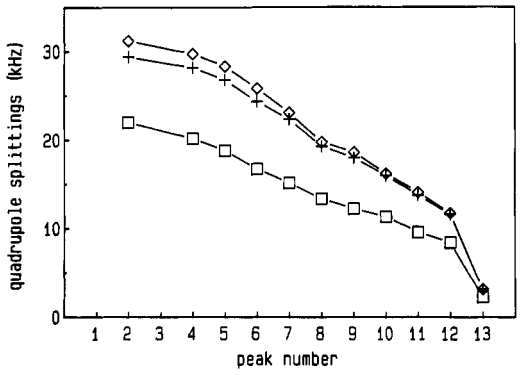

FIGURE 8: Plots of quadrupole splittings of DPPC-d_{62} in the absence and in the presence of 25 mol % diC_{12} or diC_{14} at 58 °C. (□) DPPC-d_{62}; (○) with diC_{12}; (×) with diC_{14}.

phase region, above 52 °C, the presence of 25 mol % diC_{12} has a strong ordering effect on DPPC-d_{62} side chains (Figure 8).

The ^2H NMR spectrum of the BL-PC/DPPC-d_{62} mixture with 25 mol % diC_{12} at 37 °C is shown in Figure 2E. No lateral phase separation is apparent at this temperature, but was observed by us below 30 °C (not shown). Addition of diC_{12} also caused an increase of the order parameters of BL-PC (Figure 4B); however, this increase was much less prominent than in the case of pure DPPC-d_{62}.

^{31}P NMR spectra of DPPC-d_{62} mixtures at 60 °C were similar to those of the control, with $\Delta\sigma$ = −45 ppm. At 45 °C, $\Delta\sigma$ of DPPC in the presence of 25 mol % diC_{12} was −55 ppm, intermediate between the values found for the liquid-crystalline and the gel phase of DPPC. Further lowering of the temperature decreased $\Delta\sigma$ to −65 ppm, similar to the value for pure DPPC in the gel phase (Zidovetzki et al., 1989).

diC_{14}. Addition of 25 mol % diC_{14} to DPPC-d_{62} resulted in the coexistence of gel and liquid-crystalline phases at 40 °C (Figure 1F). The fraction of immobilized DPPC-d_{62} molecules decreased upon increasing temperature, reaching zero at 55 °C for 25 mol % diC_{14}. The diC_{14}-caused ordering of DPPC-d_{62} side chains was similar to this induced by diC_{12} (Figure 8). Coexistence of two lipid phases in the presence of 25 mol % diC_{14} was also detected in the BL-PC/DPPC-d_{62} system (Figure 2F). The order parameters of the resolved part

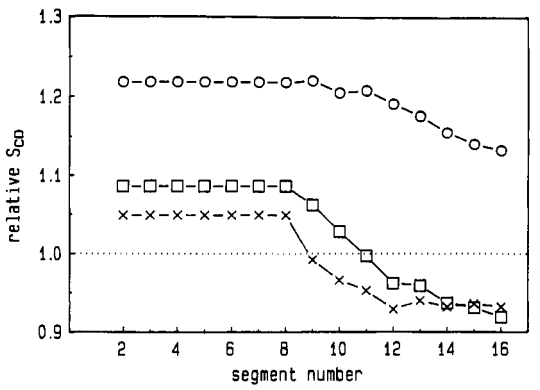

FIGURE 9: Effect of 25 mol % DAGs on the order parameter profile in DPPC-d_{62} multilayers at 40 °C. A relative S_{CD} is the ratio of a segmental order parameter in the presence of DAG to the corresponding value for pure DPPC-d_{62}. (×) DPPC-d_{62} with diC_6; (□) with diC_8; (○) with diC_{10}.

of the spectra were increased in the presence of this DAG (Figure 4B).

None of the DAGs used in this study caused nonbilayer lipid phases in either lipid system as monitored by ^2H or ^{31}P NMR.

Discussion

The present work extends our previous study of DAG–lipid interactions (De Boeck & Zidovetzki, 1989) to include DAGs with fully saturated chains that ranged in length from 6 to 14 carbons. The results demonstrate that the DAG-induced perturbation of phospholipid bilayer structure is strongly dependent on the length of the fatty acid side chains of the DAG molecule.

Effects of 25 mol % diC_{6-10} on the order parameter profiles of DPPC-d_{62} are shown on Figure 9. The peak assignment of the ^2H NMR spectra was done according to Davis (1979). Shorter chain DAGs, diC_6 and diC_8, induce relatively small bilayer perturbation, best described as transverse changes in the order parameter profile of the phospholipid side chains. In the case of diC_6, the order parameters are slightly increased in the "plateau" region (segments 2–8 of the *sn*-1 chain), with segment 9 having the same order parameter as control DPPC-d_{62}, and the lower segments (10–16) being more disordered in the presence of diC_6. A similar picture is obtained in the presence of diC_8, except the ordering effect is stronger and persists down to approximately segment 10, with lower segments (12–16) being more disordered relative to the control. The deeper effect of diC_8 correlates with the longer chain length of this DAG.

The effect on the relative order parameter, similar to the one observed by us upon addition of diC_8, was reported by Boden et al. (1991) upon addition of benzyl alcohol to dimyristoyl-PC bilayers. Using similar arguments, we can conclude that the DAGs intercalate between the bulky PC headgroups and promote a tighter contact between the phospholipid side chains in the region close to the headgroups, thereby increasing their order parameters. The free volume created below the level of DAG penetration is filled up by more disordered phospholipid side chains. The sharp division between ordered and disordered chain segments in the case of diC_6 or diC_8 suggests that the mobility of these DAGs in the direction normal to the bilayer surface is constrained, with minimal penetration into the center of the bilayer. The results are consistent with the recent findings of Hamilton et al. (1991a,b), who showed that DAGs are anchored with their hydroxyl group close to the lipid–water interface. A significant ordering effect was observed in the presence of diC_{10} (Figure 9); the effect gradually decreased from segments 12 to 16.

Increased order of DPPC bilayers by diC_{10} was also observed by Ortiz et al. (1988).

At 40 °C, only short-chain (diC_{6-10}) DAGs are miscible with the liquid-crystalline state of DPPC-d_{62}, as evidenced from the shapes of the ^2H NMR spectra (Figure 1) and the quadrupole splittings of the resolved sharp components of the spectra (Figures 3–6). Addition of 25 mol % of the longer chain DAGs, diC_{12} or diC_{14}, resulted in the formation of a single gellike phase for DPPC-d_{62} at 40 °C (Figure 1), while diC_{16} or diC_{18} at this temperature immobilized only a fraction of DPPC-d_{62} and induced lateral phase separation of the lipids into gellike and liquid-crystalline phase domains (De Boeck & Zidovetzki, 1989). This behavior raises the possibility that more than one mechanism may be involved in the apparent induction of the immobilized phase by longer chain DAGs (C $\geq$ 12). diC_{16} and diC_{18} probably form 1:2 complexes with neighboring DPPC-d_{62} molecules with tight contact between the lipid side chains and resulting predominantly trans conformation of the chains (De Boeck & Zidovetzki, 1989). The gel-state type of the spectra in the presence of diC_{12} may involve partial interdigitation of DPPC-d_{62} side chains induced by the requirement to fill the free volume created below the penetration of diC_{12} [see Boden et al. (1991)]. In the case of diC_{14}, both mechanisms may operate to some extent with partial interdigitation being less extensive than that induced by diC_{12}.

The longer chain (C $\geq$ 14) DAGs induce lateral phase separation in BL-PC/DPPC-d_{62} lipid systems [Figure 2F and De Boeck and Zidovetzki (1989)], which is a more realistic model of natural membranes with a diversity of fatty acid side chains. Such DAG-induced lateral phase separation was also observed by us in BL-PC/phosphatidylserine mixtures and in the lipids extracted from normal human erythrocytes (Zidovetzki et al., unpublished observations). The longer chain DAGs are, however, partially miscible with BL-PC/DPPC-d_{62} as indicated by the DAG-induced increase of the quadrupole splittings in this system [Figure 4B and De Boeck and Zidovetzki (1989)].

In order to further analyze the effects of the DAGs, we presented the quadrupole splittings of the plateau region in terms of the surface area per phospholipid molecule (Figure 10). According to De Young and Dill (1988), the area per phospholipid in the bilayer, A, can be presented as the ratio of the area per phospholipid in the crystal, A_0 = 40.8 Å^2, to the surface density, σ, which is derived from ^2H NMR data using the equation:

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

The discussion of assumptions involved in this treatment can be found in De Young and Dill (1988) and in our previous publication (De Boeck & Zidovetzki, 1989).

It is remarkable that, in all cases where a significant effect of a DAG on the area per DPPC-d_{62} or PC molecule was observed, it was a *decrease* in this molecular area. Such a decrease reflects an *increase* in the lateral "surface" pressure in the presence of DAGs. This surface pressure is defined on the level of the top of the lipid chains, just below the glycerol backbone, and not on the level of the headgroups. In the latter case, the discontinuity will exist due to the "empty" spaces (filled with water) between PC headgroups formed by the inserted DAG molecules with their virtual absence of headgroups. In such a case, a concept of surface pressure is not applicable.

A minimum was observed in the plot of area per DPPC-d_{62} vs DAG chain length for diC_{12} in the two-phase region, at 50 °C (Figure 10A). The minimum remained at 58 °C, where

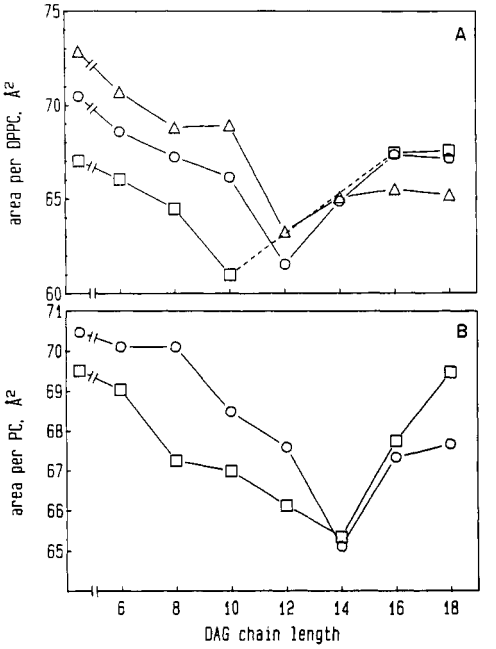

FIGURE 10: Dependence of the area per phospholipid molecule on the chain lengths of added 25 mol % DAGs at different temperatures. The leftmost set of points, before the gap, corresponds to the phospholipids in the absence of DAGs. Data for diC_{16} and diC_{18} are taken from De Boeck and Zidovetzki (1989). (A) DPPC-d_{62} with DAGs: (□) at 40 °C; the dashed line reflects the absence of resolved peaks in the spectra with diC_{12} or diC_{14} at this temperature (see Figure 1); (○) at 50 °C; (△) at 58 °C. (B) DPPC-d_{62}/BL-PC mixture with DAGs: (□) 37 °C; (○) 45 °C.

all DAG/DPPC-d_{62} mixtures were forming one phase. Qualitatively similar effects were observed on the BL-PC/DPPC-d_{62} lipid system, with DAG-induced area decreases being in all cases smaller than with DPPC-d_{62} alone, and the minimum in the plot of area per PC molecule vs DAG chain length being now located at diC_{14} (Figure 10B). The location of the area minimum at diC_{14} instead of diC_{12} for DPPC-d_{62} (Figure 10) probably reflects the longer average chain length of BL-PC.

Interestingly, while the quadrupole splittings of DPPC-d_{62} in the plateau region are monotonously decreasing with increased temperature, reflecting temperature-induced disordering of the chains, in the case of added 25 mol % diC_{14} the quadrupole splittings are in the beginning *increasing* with the increase of temperature, indicating increasing miscibility of diC_{14} with DPPC-d_{62} in the liquid-crystalline state in this two-phase region. The quadrupole splittings of the DPPC-d_{62}/diC_{14} mixture reach a maximum at about 55 °C and then monotonously decrease with temperature. The maximum quadrupole splittings correspond to the minimum value for the area per DPPC-d_{62} molecule (Figure 11). The temperature of this minimum corresponds to the temperature of the transition from the two-phase to the one-phase region, as observed by NMR and DSC (not shown). A similar, but less pronounced, minimum was observed with the DPPC-d_{62}/diC_{12} mixture (Figure 11). Qualitatively similar behavior was reported by Jacobs and White (1984) with hexane/dimyristoyl-PC mixtures.

Figure 7 illustrates that ^2H NMR can be usefully applied to monitor phase transitions and phase boundaries, especially in the two-phase region, a method which was recently called "nuclear magnetic calorimetry" by Vist and Davis (1990). In some cases, this method can provide information not obtainable by DSC because DSC can detect only the transitions with a relatively high degree of cooperativity, while ^2H NMR does

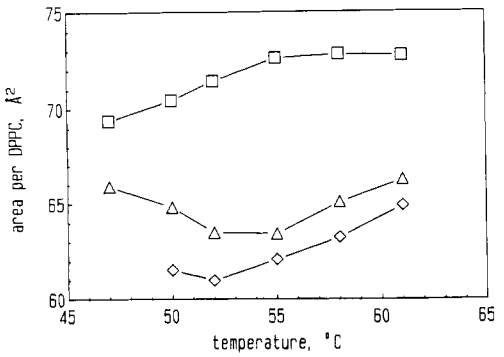

FIGURE 11: Dependence of the area per DPPC-d_{62} molecule on temperature in the absence and in the presence of 25 mol % diC_{12} or diC_{14}. (□) DPPC-d_{62}; (◇) with diC_{12}; (△) with diC_{14}.

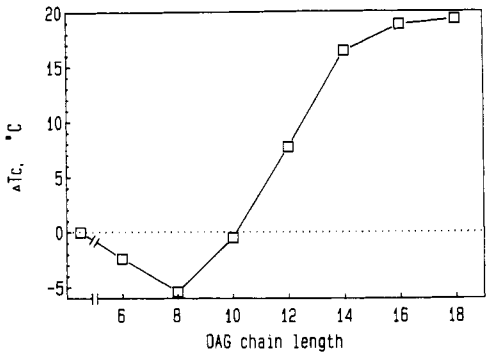

FIGURE 12: Effect of 25 mol % DAGs on the T_c of DPPC-d_{62}. The leftmost point corresponds to pure DPPC-d_{62} (T_c = 38 °C).

not have this limitation. Thus, ^2H NMR spectra show the presence of two-phase coexistence in the BL-PC/DPPC-d_{62} system upon the addition of diC_{14} (Figure 2F) or longer chain DAGs (De Boeck & Zidovetzki, 1989), while no phase transitions are detectable in these mixtures by DSC. The ^2H NMR data, supported by DSC results, showed that DAGs can either decrease or increase the T_c of DPPC, depending on the DAG chain length. Thus, shorter chain DAGs, diC_6 and diC_8, decreased the T_c of DPPC, while longer chain DAGs ($C \geq 12$) increased it, with diC_{10} having only a small effect (Figure 12). Similar behavior occurs upon addition of fatty acids (Lee, 1976a; Eliasz et al., 1976; Usher et al., 1978) or alcohols (Hui & Barton, 1973; Eliasz et al., 1976; Lee, 1976b) to phospholipids. Such a "cut-off"-type dependence of the T_c of DPPC on the chain length of alkanes, alcohols, and fatty acids was recently discussed by Lohner (1991) and is remarkably similar to the effects of DAGs in the present work. The cuttoff effect of higher alcohols was in at least one case correlated with other biochemical effects when Jain and Jahagirdar (1985) showed that the potency of *n*-alcohols to inhibit phospholipase A_2 decreases beyond *n*-decanol.

The information obtained in the course of this work can be used to understand the reported dependence of the activation of PK-C on the chain length of exogenously added DAGs. The emerging consensus in the literature is that the most effective PK-C activator is diC_8 with diC_{10} and diC_6 showing less efficacy and longer chain DAGs being much less, if at all, active. Thus, systematic correlation of DAG chain length with DAG-induced activation of PK-C by Go et al. (1987) showed maximum activation by diC_8 and minimal activation by diC_{18}. In another study, Sekiguchi et al. (1988) measured activation of different PK-C isozymes by a series of unsaturated and saturated DAGs. PK-C isozymes I and II were efficiently activated by diC_8–diC_{12}, with diC_8 being the best. Smaller, but still significant, activation was observed upon addition of diC_{16} or diC_{18}. Thus, the latter work, all disaturated DAGs were shown to activate PK-C isozymes I and II, albeit to a different degree.

It can be thus concluded that lateral phase separation, which is induced by diC_{12}–diC_{18}, is not a relevant membrane perturbation for PK-C activation. This tentative conclusion is supported by the study of Snoek et al. (1988), who did not detect lateral phase separation in the PK-C-activating lipid bilayers. The situation may, however, be different with other DAG-activated processes. Thus, in the PK-C-independent DAG-induced increase of glucose uptake in adipocytes, the most potent DAG was diC_{14} (corresponding to the *minumum* of the area per BL-PC in this work, Figure 10B), followed by diC_{12} or diC_{16}. Only a very small stimulation was observed as an effect of diC_6 or diC_8 (Strålfors, 1988). This emphasizes that these processes, stimulated by DAGs, may require somewhat different types of DAG-induced membrane perturbations—in the latter case, probably formation of laterally separated lipid domains, which is induced by diC_{12-16}, but not by diC_6 or diC_8. We observed a similar correlation of DAG chain length with its capacity to activate pig pancreatic phospholipase A_2 (Zidovetzki et al., unpublished observations).

It was proposed by Exton (1990) that different molecular species of DAG may exert differential effects on the various forms of PK-C. Indeed, diC_8 and diC_{10} are most effective in activation of PK-C type I enzyme (γ subspecies) and less effective with types II and III enzymes (Sekiguchi et al., 1988), showing that differential activation by a DAG can be achieved even in the case of these relatively well-conserved isozymes. The present study, which demonstrated a strong dependence of DAG-induced membrane perturbations on the fatty acid part of a DAG molecule, may provide a mechanism for such differential effects. The strong dependence of DAG-induced activation of phospholipases A_2 from different sources on the DAG fatty acid chain composition was recently demonstrated in our laboratory (Zidovetzki et al., unpublished observations).

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Registry No. diC_6, 30403-47-5; diC_8, 60514-48-9; diC_{10}, 60514-49-0; diC_{12}, 60562-15-4; diC_{14}, 60562-16-5; DPPC, 63-89-8.

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