Effects of Dipalmitoylglycerol and Fatty Acids on Membrane Structure and Protein Kinase C Activity

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ABSTRACT The individual and combined effects of the saturated diacylglycerol (DAG) dipalmitin (DP) and saturated or polyunsaturated unesterified fatty acids (PUFAs) on both the structure of phosphatidylcholine/phosphatidylserine (PC/PS; 4:1 mol/mol) bilayers and on protein kinase C (PKC) activity were studied using ^2H nuclear magnetic resonance (NMR) and enzyme activity assays. In the absence of DP, PUFAs only slightly activated PKC whereas palmitic acid had no effect. In the absence of fatty acids, DP induced lateral phase separation of the bilayer into liquid-crystalline and gel phases. Under these conditions virtually all DP was sequestered into the gel phase and no activation of PKC was observed. The addition of polyunsaturated arachidonic or docosahexaenoic acids to the DP-containing bilayers significantly increased the relative amounts of DP and other lipid components in the liquid-crystalline phase, correlating with a dramatic increase in PKC activity. Furthermore, the effect was greater with PS, resulting in an enrichment of PS in the liquid-crystalline domains. In the presence of DP, palmitic acid did not decrease the amount of gel phase lipid and had no effect on PKC activity. The results explain the observed lack of PKC-activating capacity of long-chain saturated DAGs as due to the sequestration of DAG into gel domains wherein it is complexed with phospholipids and thus not available for the required interaction with the enzyme.

INTRODUCTION

The protein kinase C (PKC) family of serine/threonine kinases plays a central role in the intracellular signaling network (Nakamura and Nishizuka, 1994) and is known to phosphorylate a wide variety of transmembrane and intracellular proteins including phospholipase D (Kiss, 1996), epidermal growth factor receptor (Ido et al., 1987), lamin B (Fields et al., 1990), DNA-regulatory proteins (Buchner, 1995), and the tumor-suppressor protein p53 (Baudier et al., 1992). It is the key transducer of the effects of the phorbol ester and bryostatin groups of tumor promoters and is implicated in a variety of biological processes including apoptosis (Lucas and Sanchez-Margalet, 1995) and learning and memory (Fagnou and Tuchek, 1995) as well as exocytosis, extracellular signal transduction, cell growth, and cell differentiation (Nishizuka, 1986). The mammalian PKC family consists of at least 12 different isoforms (Dekker and Parker, 1994) found in various tissues and is particularly concentrated in the nervous system (Wetsel et al., 1992). Most cells express several PKC isozymes that differ in activation requirements, suggesting that different isozymes are involved in distinct signaling pathways.

Most models of activation of the conventional or classical PKC isoforms, which include PKCs α, β_I, β_{II}, and γ, involve association of the inactive, cytosolic form of the enzyme with lipid membranes where it binds to acidic lipids, primarily phosphatidylserine (PS), in a Ca^{2+}-dependent manner (Newton, 1995). The presence of the lipid second messenger 1,2-*sn*-diacylglycerol (DAG), which is produced as a result of extracellular stimulus-generated activation of phospholipases C or D, promotes membrane binding (Mosior and Newton, 1995) and may induce conformational changes in PKC, causing its activation (Mosior and McLaughlin, 1991; Zidovetzki and Lester, 1992; Nakamura and Nishizuka, 1994).

A number of lipophilic molecules have been shown to modulate DAG-induced PKC activation. *Cis*-unsaturated unesterified fatty acids (FAs) synergistically enhance DAG-dependent PKC activation (Shinomura et al., 1991; Lester, 1990; Schachter et al., 1996) and can even cause low-level activation without DAG (Murakami and Routtenberg, 1985; Murakami et al., 1986; Lester, 1990) whereas saturated FAs and *trans*-unsaturated FAs do not cause this effect. In sonicated lipid vesicles, lysophosphatidylcholine (lyso-PC) extracts potentiate DAG-induced activation of PKC at low lyso-PC concentrations (Sasaki et al., 1993), although in the same study, higher concentrations of lyso-PC inhibited activity. Sando and Chertihin (1996) showed that in sonicated vesicles, unsaturation of the lyso-PC acyl chain is necessary for enhanced activity. Sphingosine, a naturally occurring cationic long-chain amphiphile, was shown to inhibit PKC activity (Hannun et al., 1986), although this effect is highly dependent upon sphingosine charge and increased activity is observed under appropriate conditions (Senisterra and Epand, 1992).

It has been suggested that physical perturbations in the lipid membranes may contribute to the modulation of PKC activity (Epand, 1985; Das and Rand, 1986; De Boeck and Zidovetzki, 1989; Bolen and Sando, 1992; Zidovetzki and Lester, 1992; Senisterra and Epand, 1993; Slater et al., 1994; Kinnunen, 1996; Stubbs and Slater, 1996). This is supported by findings demonstrating that lipid bilayer structure is an important factor in both protein-lipid interactions and activity of integral membrane proteins and membrane-

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associated enzymes, including the adenine nucleotide translocator (Streicher-Scott et al., 1994), phosphocholine cytidyltransferase (Cornell, 1991; Arnold and Cornell, 1996), insulin receptor (Lewis and Czech, 1987; Leray et al., 1993; McCallum and Epand, 1995), and particularly the phospholipases (Wilschut et al., 1978; Menashe et al., 1981; Jain and Jahagirdar, 1985; Romero et al., 1987; Gheriani-Gruszka et al., 1988; Jain and Berg, 1989; Roberts and Dennis, 1989; Sen et al., 1991; Zidovetzki et al., 1992; Huang et al., 1996).

A variety of lipid physicochemical properties such as the extent of bilayer unsaturation (Bolen and Sando, 1992), the electrical charge of lipophilic additives (Epand, 1987; Epand and Lester, 1990), transverse membrane perturbations (Goldberg et al., 1994), and the tendency to form nonbilayer phases (Epand, 1987; Epand and Lester, 1990; Senisterra and Epand, 1993; Goldberg et al., 1994; see Zidovetzki, 1997, for review) has been shown to modulate PKC activity. Instabilities in the bilayer, variously referred to as lipid stress or frustration, bilayer packing irregularities, curvature stress, fluctuating defect regions, bilayer packing strain or L_ϵ phase (Israelachvili et al., 1980; Gruner, 1985; Romero et al., 1987; Seddon, 1990; Sen et al., 1991; Kinnunen, 1996), have been implicated in modulating protein-lipid interactions and activity of membrane-associated proteins including PKC (Kinnunen, 1996; Stubbs and Slater, 1996). Kinnunen (1996) advanced the idea that increased lipid frustration could be relieved through a lipid-protein interaction wherein the *sn*-2 chain of a lipid extends out from the bilayer, fitting into a hydrophobic cavity of a membrane-associated protein, which both reduces lipid stress and anchors the protein to the lipid surface. Spacing and conformation of the lipid headgroups have also been suggested as important biophysical properties (Bolen and Sando, 1992; Goldberg et al., 1995; Stubbs and Slater, 1996).

In addition to the required 1,2-*sn* configuration of activating DAG, PKC activity has also been shown to be a function of the DAG acyl chain composition. DAGs with unsaturated chains activate PKC whereas long-chain saturated dipalmitin (DP) and distearin do not (Kishimoto et al., 1980; Mori et al., 1982; Go et al., 1987; Goldberg et al., 1994). Short-chain saturated DAGs (dihexanoin, dioctanoin, and didecanoin) have also been found to activate PKC (Lapetina et al., 1985; Goldberg et al, 1994). In our previous study, we correlated the perturbations of the lipid bilayer induced by different DAGs with their PKC activation properties (Goldberg et al., 1994). Specifically, the lack of PKC-activating capacity of the long-chain saturated DAGs was attributed to the complex-like association of these DAGs with phosphatidylcholine (PC) or PS, with concurrent lateral phase separation of these phospholipid-DAG complexes into gel-like domains. In the present study we demonstrate that the addition of FAs to the DP-containing bilayers reduces the DP-phospholipid interaction and increases the partitioning of DP into liquid-crystalline membrane domains, thereby enabling this DAG to activate PKC. The degree of activation correlated with the effects of DP and FAs on physical properties of the lipid membrane. We employed ^{2}H nuclear magnetic resonance (^{2}H-NMR) using a panel of nonperturbing deuterium-substituted lipid probes, which have the same chemical properties as the native molecules. The ^{2}H-labeled lipids used in this study were chosen to elucidate changes at the lipid-water interface and along the length of the hydrocarbon acyl chains for the PC and PS lipid components as well as for DP.

MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC), diperdeuteriopalmitoylphosphatidylcholine (DPPC-d_{62}), dipalmitoylphosphatidylserine (DPPS), diperdeuteriopalmitoylphosphatidylserine (DPPS-d_{62}), diperdeuteriopalmitoylglycerol (DP-d_{62}), and PC extracts from bovine liver (BLPC) were purchased from Avanti Polar Lipids (Alabaster, AL). DP was purchased from Sigma Chemical Co. (St. Louis, MO). Palmitic acid (PA), arachidonic acid (AA), and docosahexaenoic acid (DHA) were purchased from Nu-Check Prep (Elysian, MN). Perdeuteriopalmitic acid (PA-d_{31}) was purchased from Serdary Research Laboratories (London, Ontario, Canada). All lipids showed a single spot when checked for purity by thin-layer chromatography.

Multilamellar lipid dispersions were prepared by first dissolving the lipid mixture in chloroform and evaporating the solvent with a stream of dry nitrogen. Lipid mixtures consisted of a matrix of BLPC with DPPC and DPPS (3:1:1 mol/mol/mol) with other lipids added as mentioned in the text. Concentrations are expressed in mol % DAG or FA relative to phospholipids (e.g., 25 mol % DP and 25 mol % DHA is 60:20:20:25:25 BLPC:DPPC:DPPS:DP:DHA).

Samples were placed under high vacuum (<1 mtorr) for at least 8 h either immediately after solvent evaporation or after dissolving in cyclohexane and freezing for lyophilization. No differences in results were seen between lyophilized and nonlyophilized samples.

Samples for ^{2}H-NMR were then hydrated with 40 mM MOPS (pH 7.4), 1 mM EDTA, and 0.02% sodium azide buffer solution prepared in ^{2}H-depleted H_2O (Sigma). The samples were always fully hydrated and were typically 1:10 (w/w) lipid/buffer. In samples displaying a significant degree of bilayer orientation by the magnetic field, water content was decreased to 1:2 (w/w). Both quadrupole splittings and phase behavior were identical for the samples with low or high hydration. No sample orientation was observed in samples containing a significant fraction of gel phase. In different samples, DPPC, DPPS, DP, or PA were substituted with DPPC-d_{62}, DPPS-d_{62}, DP-d_{62}, or PA-d_{31} for the corresponding nondeuterated lipid. A uniform lipid suspension was obtained by five freeze-thaw cycles (Westman et al., 1982; Mayer et al., 1985).

PKC activity assay samples were prepared with 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 40 μM $CaCl_2$, 10 μM histone 1 (histone IIIs from Sigma), 40 μM ATP (Sigma), 0.6 μCi [γ-^{32}P]ATP (Dupont NEN, Boston, MA), and 10 ng of PKC (Calbiochem, La Jolla, CA) in a total volume of 75 μl. We assured that the PKC activity measurements were done in the region of lipid cofactor saturation, where changes in the effective lipid concentration do not have any effect on the enzymatic activity. It was reported that the lipid cofactor saturation region starts at 1 mM with PC/PS multilamellar vesicles of various acyl chain compositions (Sando and Chertihin, 1996; Sando, personal communication). PKC activity measurements were performed at 4 mM phospholipid, which places our conditions well above this threshold. We furthermore tested the saturation conditions in our system by performing a number of parallel measurements at lipid concentrations ranging from 2 to 4 mM. In this range, the results were independent of the lipid concentration, proving that PKC assays were in the lipid cofactor saturation region.

Protein kinase C activity assays

PKC activity was measured by phosphorylation of the exogenous substrate histone 1 according to Sando and Chertihin (1996), with modifications.

Samples were temperature-equilibrated with substrate and ATP shortly before performing the assay, which was then initiated by addition of PKC. Reaction time was 5 min at 30°C and the reaction was terminated by spotting 60 μl of the reaction mixture onto Whatman P-81 cation exchange paper (Whatman International, Maidstone, UK). The papers were then washed three times with 250 ml of 50 mM NaCl to remove unreacted ATP. Linear reaction kinetics were maintained under all conditions of the assay. The amount of ^{32}P transferred to histone was determined by liquid scintillation counting. Ca^{2+}-independent activity was less than 13% and phospholipid-independent activity was less than 7%.

NMR measurements

^2H-NMR spectra were acquired at 11.74 T (corresponding to 500.13 MHz ^1H and 76.77 MHz ^2H) on a General Electric GN500 spectrometer. ^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 500 kHz and the refocusing time was 60 μs, with a 90° pulse of 3.5 μs and a recycle time of 200 ms. Spectra were de-Paked by numerical deconvolution according to Sternin et al. (1983) using a computer analysis program modified for the IBM personal computer, kindly supplied by Dr. S. Wassall. De-Paked spectra correspond to the spectra that would be obtained from a planar membrane with its bilayer normal aligned parallel to the applied static magnetic field, enhancing resolution and facilitating analysis of individual spectral peaks. These spectra were compared with the original spectra to ensure that the relevant features were maintained through the de-Paking process. First moments (M_1) of the spectra were determined according to the following equation (Davis, 1983):

$$M_1 = \frac{\int_0^\infty \omega f(\omega) d\omega}{\int_0^\infty f(\omega) d\omega} \tag{1}$$

where ω is the frequency with respect to the central Larmor frequency ω_0.

Calculation of percent gel phase

An empirical method was designed to estimate the percentage of the deuterated lipid probes in gel phase. The fraction of spectral intensity in the region enclosed by the outermost symmetric peaks of the L_α phase component in a two-phase spectrum was used to calculate the amount of L_β phase present as follows. In each two-phase spectrum the area of this region was determined and normalized by the total spectral area to get the fraction (F) of area between the outermost peaks of the L_α spectral component. The same algorithm was used to determine the fraction of spectral area (F_α) between the outermost peaks for samples that were fully liquid crystalline. The fraction (F_β) of the area in a 100% L_β spectrum within the same frequencies used in the 100% L_α spectrum was then calculated. These values then defined a linear scale for the percent L_β calculation, establishing end points for 0 and 100% gel phase accordingly, and percent L_β for a two-phase spectrum was calculated according to the following equation:

$$\%\, L_\beta = 100\% \cdot \frac{F - F_\alpha}{F_\beta - F_\alpha} \tag{2}$$

A separate scale was calculated for each probe (DPPC-d_{62}, DPPS-d_{62}, and DP-d_{62}) to compensate for any change in L_β profiles for the different lipid molecules. This method allows for a more accurate determination of the fraction of lipids in gel phase than do M_1 values, because the latter do not correct for the possible changes in the order parameters of the lipids in L_α phase. This procedure is useful when it may be difficult or impossible to apply other mathematical approaches in determining fractions of gel and liquid-crystalline phases (Nichol et al., 1980; Jarrell et al., 1981).

RESULTS

The ^2H-NMR spectra of DPPC-d_{62} in PC/PS mixtures at 30°C in the presence or absence of DP and/or one of the three FAs studied are shown in Fig. 1. The ^2H-NMR spectrum of fully hydrated lipid bilayers is the superposition of the powder patterns arising from each of the deuterons along the acyl chains of the DPPC-d_{62} molecule. The quadrupole splittings ($\Delta\nu$), distances between pairs of symmetric peaks, are related to the order parameters of the C-D bonds as defined by Eq. 3.

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

where (e^2Qq/h) = 167 kHz is the static quadrupolar coupling constant for a deuteron in a C-D bond (Burnett and Muller, 1971). The order parameter is highest at the carbon segments next to the glycerol backbone, continuously decreasing along the acyl chain toward the terminal methyl group. This gives rise to the peak pattern shown in Fig. 1 *A*, consisting of 11 Pake doublets, with the outermost peak pair corresponding to the plateau of superimposed peaks from carbon segments two through eight (Seelig, 1977; Davis, 1979) and the innermost pair of peaks representing the terminal methyls.

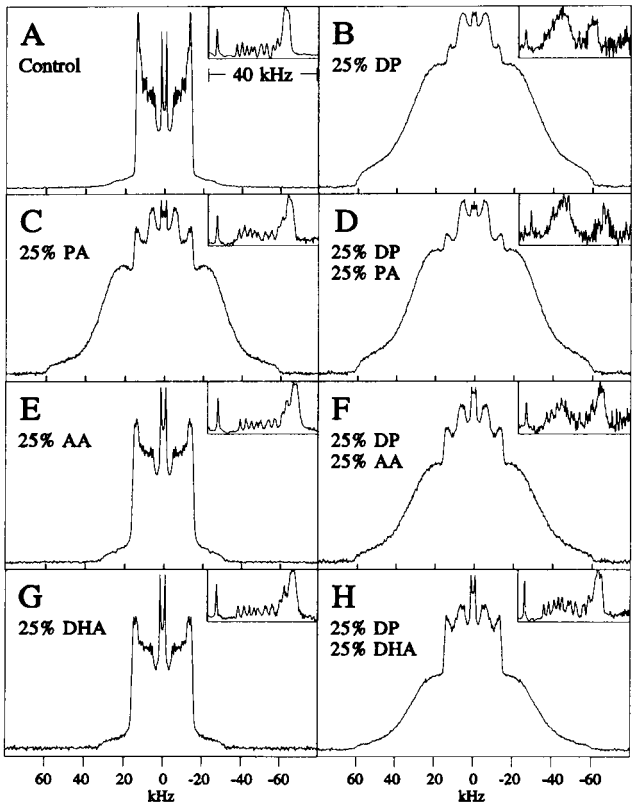

FIGURE 1 ^2H-NMR spectra of DPPC-d_{62} in PC/PS mixtures in the absence (*left*) or presence (*right*) of 25 mol % DP at 30°C and the absence (*A* and *B*) or presence of FAs (*C–H*). Insets are the de-Paked right-hand sides of the spectra.

The spectra in Fig. 1 of samples without DP or PA (Fig. 1, *A*, *E*, and *G*) exhibit the typical profile for a randomly oriented dispersion of multilamellar vesicles in the liquid-crystalline phase. The insets are the de-Paked spectra of the right-hand region of the peaks, which show a general increase in $\Delta\nu$ in the presence of each of the FAs. The presence of 25 mol % DP (Fig. 1 *B*) or 25 mol % PA (Fig. 1 *C*) resulted in the addition of a broad component to the spectrum exhibiting the characteristic features of a gel-phase bilayer. The superposition of the gel-phase and liquid-crystalline components indicates that both DP and PA induced a lateral phase separation of the PC/PS bilayer into liquid-crystalline and gel-like domains. Numerical analysis of these spectra determined that 95 and 70% of the spectral intensity was due to the presence of gel phase domains in the DP and PA samples, respectively. The combined effects of DP with PA appears similar to that of DP alone in that nearly all (~98%) of the DPPC-d_{62} was in L_β phase (Fig. 1 *D*). Both polyunsaturated fatty acids (PUFAs) AA (Fig. 1 *E*) and DHA (Fig. 1 *G*) caused a widening of the liquid-crystalline peaks, corresponding to an increase in lipid order. Addition of AA or DHA to DP-containing bilayers significantly reduced the fraction of lipids in the gel phase (also see below). The spectra of the DPPS component in these PC/PS mixtures were determined using DPPS-d_{62}-labeled samples (data not shown). In general, the results were similar to those for DPPC-d_{62}, indicating that PC and PS did not separate under these conditions. However, it was observed that in samples containing DP, the ratios of L_α to L_β in DPPS-d_{62} spectra were 8–18% higher than in DPPC-d_{62} spectra, suggesting a slight preference for DP-PC over DP-PS interactions.

An analysis of the effects of FAs on the relative order parameters of liquid-crystalline DPPC-d_{62} in PC/PS mixtures is shown in Fig. 2. Fig. 2 *A* shows that at 30°C, each FA increased order throughout the acyl chain in a biphasic manner with the greatest increase located between segments 10 through 13. Both PUFAs altered order parameters similarly, whereas palmitic acid was less effective in ordering the portion of the acyl chains nearest the headgroups. As distinct order parameters are observable only for lipids in the liquid-crystalline phase, this was probably due to palmitic acid partitioning more efficiently into gel-phase domains, causing a reduction in the local concentration in the liquid-crystalline domains. ^{2}H-NMR experiments using PA-d_{31} as a probe confirmed that PA was distributed in both gel and liquid-crystalline domains (data not shown). This effect of PA is shown more clearly in Fig. 2 *B* in which order parameters were measured at 60°C, when only L_α phase was present. In this case PA was more efficient at increasing lipid order parameters than either AA or DHA. Addition of 25 mol % DP at 60°C caused a dramatic increase in acyl chain order, which was additive with the effects of PA; however, both PUFAs reduced, to a small degree, the ordering influence of DP. The effects of DP and FAs on the order parameters of DPPS-d_{62} samples were similar to DPPC-d_{62} samples, indicating that in liquid-crystalline domains, DP and FAs interact similarly with both DPPC and DPPS. At 30°C, the relatively small fraction of liquid-crystalline spectral intensity in DP-containing samples prevented the determination of order parameters.

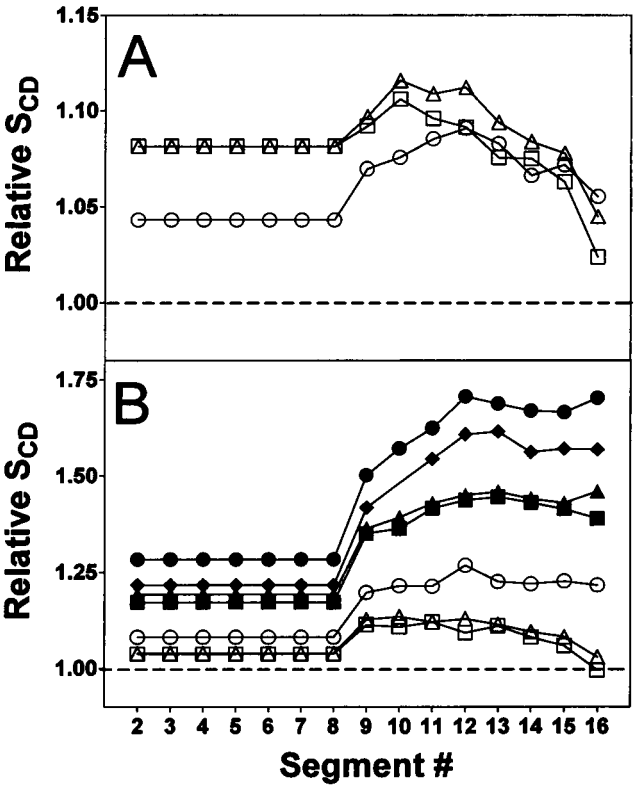

FIGURE 2 Effect of addition of 25 mol % FA and/or 25 mol % DP on the order parameter profile of DPPC-d_{62} in PC/PS mixtures at 30°C (*A*) and 60°C (*B*). The relative S_{CD} is the ratio of segmental order parameters obtained in the presence of FA and/or DP to the corresponding values obtained in the absence of both FA and DP. Open symbols are without and closed symbols with 25 mol % DP: ○ and ●, 25 mol % PA; △ and ▲, 25 mol % AA; □ and ■, 25 mol % DHA; ◆, DP only.

The ability of PUFAs to attenuate the gel-inducing effects of DP was further investigated using DHA. A series of spectra from samples containing 15 mol % DP and 0–25 mol % of DHA illustrates the dependence of the fraction of lipids in the gel phase on the presence of PUFAs (Fig. 3). Increasing the concentration of DHA decreased the fraction

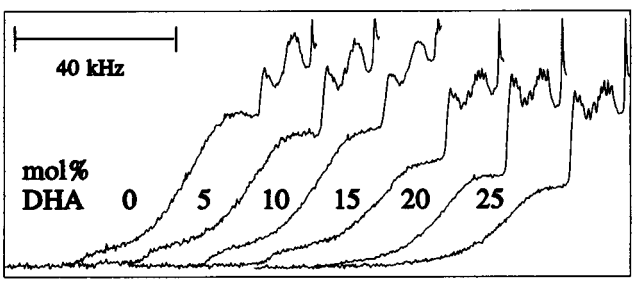

FIGURE 3 ^{2}H-NMR spectra of DPPC-d_{62} in PC/PS mixtures with 15 mol % DP and 0–25 mol % DHA at 30°C.

of gel phase lipid from 93% at 0 mol % DHA, to 60% at 25 mol % DHA. Because the gel phase was induced by the presence of DP, the reduction of percent L_β seen in DPPC-d_{62} and DPPS-d_{62} samples could be due to a changed stoichiometry of DP-phospholipid interactions whereby a portion of PC or PS is released from DP-phospholipid complexes into liquid-crystalline phase. In this case, no increase of DP in the L_α phase would be observed. Alternatively, the PUFAs can reduce the relative amount of all lipids, including DP, in the gel phase, resulting in the PUFA concentration-dependent increase of DP in the liquid-crystalline phase. The use of DP-d_{62} as a NMR label can distinguish between these possibilities. A comparison of samples containing 25 mol % DP with and without 25 mol % DHA is shown for a series of temperatures between 37 and 55°C using either the DPPC-d_{62} (Fig. 4) or DP-d_{62} (Fig. 5) label. At 37°C only ~90% of DPPC-d_{62} was in gel phase (Fig. 4 *A*), whereas practically all of DP-d_{62} was in gel domains (Fig. 5 *A*). At each of the temperatures shown, the presence of DHA reduced the relative amounts of both DPPC-d_{62} (Fig. 4) and DP-d_{62} (Fig. 5) in L_β phase. This directly shows that DHA increased the solubility of DP in the liquid-crystalline domains.

The effects of FAs at the bilayer-water interface were examined using choline headgroup-deuterated DPPC-d_4 (Fig. 6). In these spectra, the outer symmetric pair of peaks

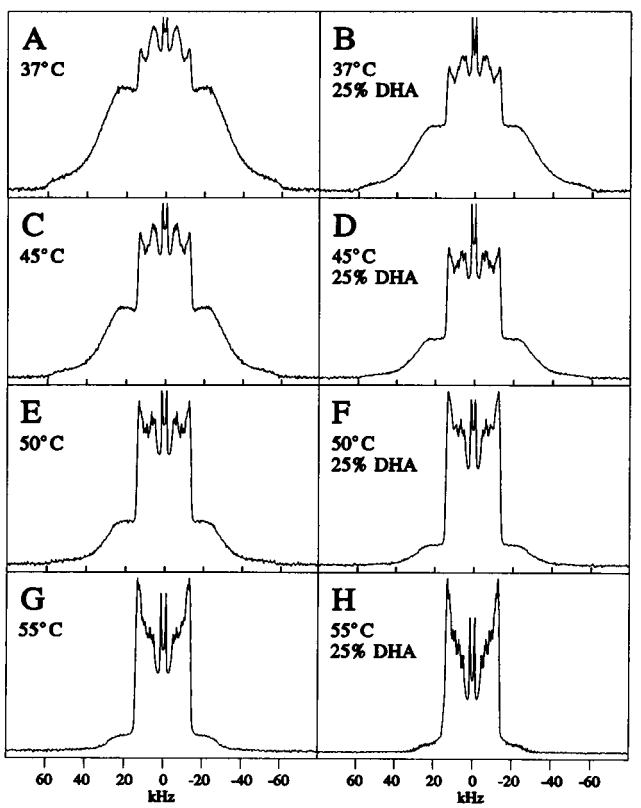

FIGURE 4 ^{2}H-NMR spectra of DPPC-d_{62} in PC/PS mixtures with 25 mol % DP in the absence (*left*) or presence (*right*) of 25 mol % DHA at 37–55°C.

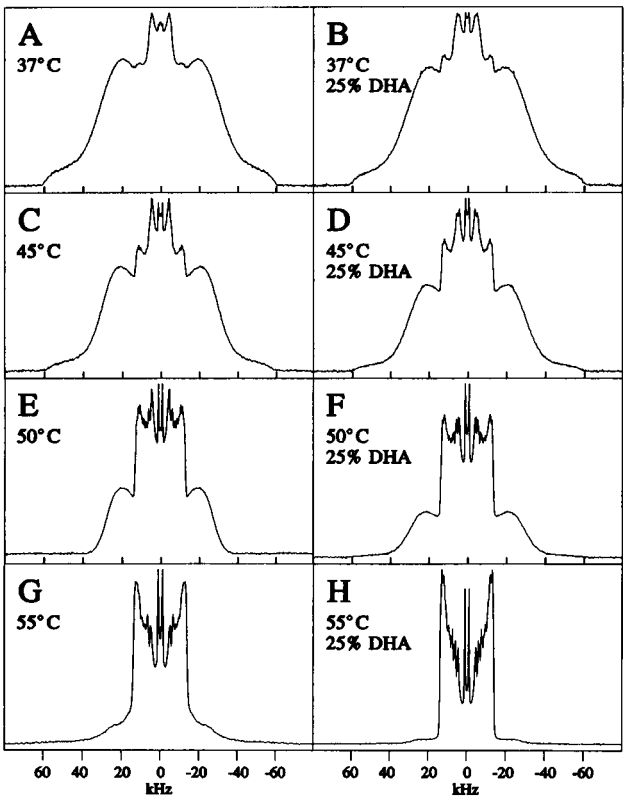

FIGURE 5 ^{2}H-NMR spectra of DP-d_{62} in PC/PS mixtures with 25 mol % DP-d_{62} in the absence (*left*) or presence (*right*) of 25 mol % DHA at 37–55°C.

corresponds to the deuterons located at the α-carbon of the choline moiety and the inner pair corresponds to the deuterons at the β-carbon (Scherer and Seelig, 1987). Fig. 6 *A* displays the typical liquid-crystalline spectrum seen in PC/PS mixtures (Goldberg et al., 1995) without DP or FAs. Addition of 15 mol % DP (Fig. 6 *B*) caused a broad component to appear in the spectrum, representing a large fraction of the DPPC-d_4 probe residing in gel phase domains, consistent with DPPC-d_{62} and DPPS-d_{62} spectra. Samples with 25 mol % DHA (Fig. 6 *C*) also exhibited the liquid-crystalline shape, although a counter-directional change in the quadrupole splittings was observed, with increased $\Delta\nu_\alpha$ and decreased $\Delta\nu_\beta$. The combination of 15 mol % DP and 25 mol % DHA (Fig. 6 *D*) caused a similar counter-directional quadrupole splitting change and a reduction of the gel phase component as compared with 15 mol % DP alone, again showing that DHA reduced the fraction of the gel phase component. When 15 mol % DP was fully solubilized into the liquid-crystalline phase at 60°C, it also caused a counter-directional change in the headgroup quadrupole splittings (see Table 1) whereas little to no difference was observed at 30°C, when DP primarily occupies gel domains. The magnitude of the counter-directional change induced by DP at 60°C was similar to previous observations with other DAGs (Goldberg et al., 1995). The combination of 15 mol % DP and 25 mol % DHA caused changes similar

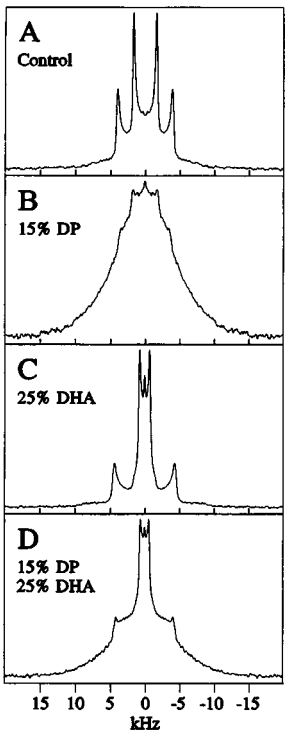

FIGURE 6 ^{2}H-NMR spectra of DPPC-d_4 in PC/PS mixtures in the absence or presence of 15 mol % DP and/or 25% mol % DHA at 30°C.

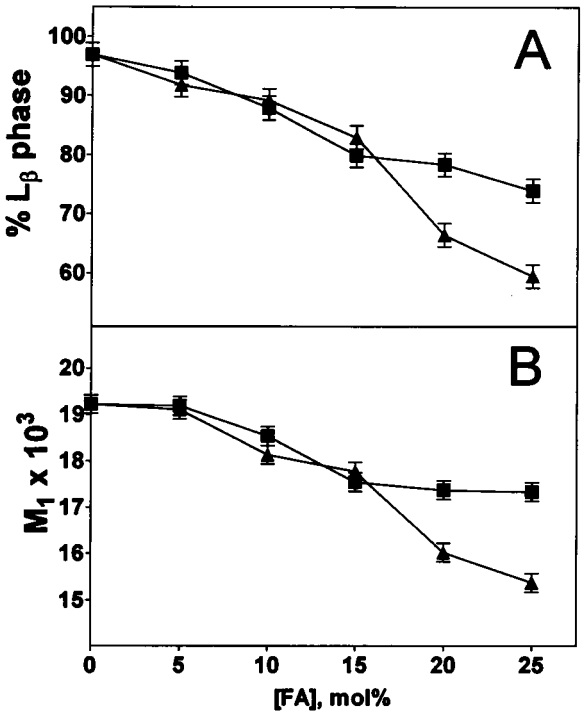

FIGURE 7 Effects of AA (■) or DHA (▲) on percent gel phase (*A*) and first moment (*B*) as determined from DPPC-d_{62} spectra of PC/PS mixtures with 15 mol % DP and 0–25 mol % FA.

in magnitude to DHA alone; thus with regard to the quadrupole splittings at the choline headgroup, the effects were not additive. PA showed a similar effect as DP such that at 30°C relatively little change in quadrupole splittings was observed, whereas at 60°C $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ values were similar to those obtained with 15 mol % DP at 60°C (Table 1).

The dependencies of percent L_β lipid and M_1 values on PUFA concentration in the presence of 15 mol % DP at 30°C are displayed in Fig. 7. Values were determined by analysis of spectra from DPPC-d_{62}-containing samples. With 15 mol % DP, nearly all DPPC-d_{62} was found in L_β phase (93 ± 3%), whereas increasing AA concentration to 25 mol % reduced percent L_β to 74 ± 3% and increasing DHA to 25 mol % was even more effective (59 ± 3% L_β). The M_1 values for DPPC-d_{62} in L_β phase are similar to those determined by Davis (1979) for pure DPPC in L_β phase near the phase transition temperature. The first moment decreased as a greater fraction of lipid converted to L_α phase, as expected (Davis, 1979; Nichol et al., 1980; Jarrell et al., 1981; Davis, 1983). The trends for both percent L_β and M_1 are similar, although the percent L_β determination is expected to be more precise (see Materials and Methods) and it directly represents a simple physical parameter of the lipid system.

PKC activity was measured in samples with the same lipid compositions as those used for NMR experiments. The relative PKC activity in the presence of PC/PS mixtures with and without 15 mol % DP and 0–25 mol % FAs is presented in Fig. 8. Neither 15 mol % DP nor 25 mol % PA, individually or in combination, caused any significant increase in PKC activity, which concurs with other studies indicating that long-chain saturated FAs and DAGs are poor PKC activators (Sekiguchi et al., 1988; Lester, 1990; Goldberg et al., 1994, Schachter et al., 1996). Either AA or DHA alone slightly increased PKC activity at high molar concentrations, although 15 mol % DP in conjunction with either PUFA caused a dramatic increase in the enzyme activity. Under these conditions a significant amount of DP was

TABLE 1 Effects of DP and FAs on headgroup quadrupole splittings

	$\delta\Delta\nu_\alpha$ (30°C)	$\delta\Delta\nu_\beta$ (30°C)	$\delta\Delta\nu_\beta/\delta\Delta\nu_\alpha$ (30°C)	$\delta\Delta\nu_\alpha$ (60°C)	$\delta\Delta\nu_\beta$ (60°C)	$\delta\Delta\nu_\beta/\delta\Delta\nu_\alpha$ (60°C)
15 mol% DP	−136	−324	2.38	1058	−1438	−1.40
25 mol% DHA	1449	−2433	−1.68	1254	−2591	−2.07
25 mol% PA	385	−1259	−3.27	1037	−1610	−1.55
15 mol% DP plus 25 mol% DHA	1024	−2668	−2.61	1514	−2415	−1.60

The $\delta\Delta\nu$ values are given in Hz. The estimated error of $\Delta\nu$ determination was ±50 Hz.

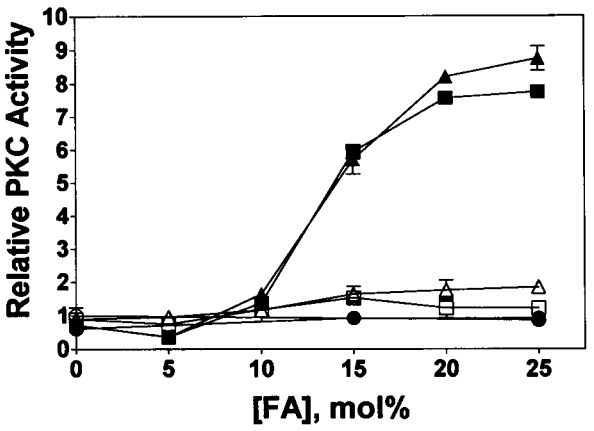

FIGURE 8 PKC activity measured in PC/PS mixtures in the presence (*closed symbols*) or absence (*open symbols*) of 15 mol % DP and 0–25 mol % PA (● and ○), AA (▲ and △), or DHA (■ and □).

solubilized into the liquid-crystalline phase as observed by ^{2}H-NMR.

DISCUSSION

This project was originated to understand the biophysical causes behind the observed synergism of various DAGs and FAs in activating PKC. Specifically, this study explores the mechanism behind the variation in PKC-activating efficiency observed with different DAGs. In previous studies, saturated long-chain DAGs such as DP were unable to induce PKC activation (Kishimoto et al., 1980; Mori et al., 1982; Go et al., 1987; Goldberg et al., 1994), whereas *cis*-unsaturated or saturated short-chain DAGs induced PKC activity to a greater or lesser extent, dependent upon the species of DAG (Goldberg et al., 1994). The variation among 1,2-*sn*-DAGs in their capacity to activate PKC signifies that, although apparently the enzyme is in contact primarily with the bilayer surface rather than with the hydrophobic core (Mosior and Newton, 1995), the differences in the DAG acyl chains, and the resulting changes in DAG-induced membrane structural perturbations, play a critical role in optimizing PKC-lipid interactions.

The biophysical mechanism behind PKC activation has been investigated for over a decade, and although many data have been accumulated, a clear understanding remains elusive. The prevailing models all include, to a greater or lesser extent, biophysical mechanisms that would be expected to depend on physicochemical properties of the lipid membrane, such as lipid phase, coexistence of multiple domains, lipid hydration, headgroup spacing, curvature stress, fluidity, order, and surface charge. The use of different membrane systems such as detergent mixed micelles, small high-curvature sonicated vesicles, or essentially planar large vesicles, would also be expected to alter these properties and would particularly alter the effects of lipophilic modulators on bulk lipid properties. In support of this, the efficiency of different PKC cofactors has been shown in many cases to vary depending upon the lipid system used (Bazzi and Nelsestuen, 1987; Walker et al., 1990; Sandermann and Duncan, 1991). In the case of DP, it was shown to not activate PKC in PC/PS bilayers (Goldberg et al., 1994). However, in sonicated vesicles DP does cause activation (Sekiguchi et al., 1988) although not as efficiently as other DAGs. These differences may be due to profound differences in the physicochemical structure among the lipids in micelles, sonicated and nonsonicated vesicle forms (Dill and Flory, 1981; Mitaku et al., 1983; Parmar et al., 1984). To better understand the biologically relevant mechanisms behind PKC activation, the use of nonsonicated lipid dispersions is recommended, which provides a better model of the physicochemical properties of biological membranes (Boni, 1992; Arnold and Cornell, 1996).

Among the lipophilic agents naturally present in biological membranes are unesterified fatty acids. The concentration of total unesterified fatty acids in certain biological membranes can be as high as 8% in rat liver plasma membranes (Ray et al., 1969) and 5% in the sarcolemmal membranes of cardiac muscle after myocardial ischemia (Chien et al., 1984). Previous studies on the effects of FAs on lipid bilayer structure showed that FAs strongly modulate important membrane properties such as permeability (Schramm et al., 1967; Wojtczak, 1976), fusion (Ahkong et al., 1973; Kantor and Prestegard, 1978), membrane-bound enzyme activity (Orly and Schramm, 1975; Hanski et al., 1979; Andreasen and McNamee, 1980), and cell adhesion (Hoover et al., 1977). It has also been documented that DAGs and FAs synergize in PKC activation (Verkest et al., 1988; Lester, 1990; Shinomura et al., 1991; Chen and Murakami, 1992; Schachter et al., 1996), but the mechanism of this synergism is not clear. This work was designed to test the hypothesis that synergism between DP and FAs in PKC activation reflects their effect on bilayer structure.

Our results on the effect of DP on PC/PS bilayer structure agree with the previous publications showing that long-chain saturated DAGs induce coexistence of gel and liquid-crystalline lipid phases in lipid bilayers of different composition (De Boeck and Zidovetzki, 1989; Heimburg et al., 1992; Goldberg et al., 1994; López-García et al., 1994; Schorn and Marsh, 1996). The results were interpreted as a formation of complex-like interactions between a saturated DAG and phospholipids, resulting in the lateral phase separation of these gel-like complexes from the noncomplexed phospholipids that remained in the liquid-crystalline phase (De Boeck and Zidovetzki, 1989; Heimburg et al., 1992; Goldberg et al., 1994; Quinn et al., 1995). As a result, no DAG is present in the liquid-crystalline phase. In the present study we investigated the modulation of this process by FAs.

Previous investigations into the biophysical effects of FAs on membranes, such as the partitioning properties of FAs and their effects on lipid order, have been performed to explain their effects on cellular functions (Klausner et al., 1980; Karnovsky et al., 1982; Anel et al., 1993). It was found that saturated and *trans*-unsaturated FAs have similar

biological and partitioning effects, which are different from those of *cis*-unsaturated FAs. Anel and co-workers (1993) showed that diphenylhexatriene polarization in liquid-crystalline bilayers is increased by saturated FAs and decreased by unsaturated FAs, concluding that unsaturated FAs decrease lipid acyl chain order with increasing unsaturation. In contrast, we have observed that both saturated and unsaturated FAs increased ordering of the phospholipid acyl chains. The use of nonperturbing ^{2}H-labeled probes is an improvement over fluorescent probes and ^{2}H-NMR is particularly well suited for studying lipid chain order, whereas steady-state fluorescence does not distinguish between membrane order and fluidity, lending more confidence to the ^{2}H-NMR results. Our results with palmitic acid are also in good agreement with the NMR study by Pauls et al. (1983), who showed that PA increases order along the acyl chain and induces gel phase in DPPC bilayers. It has also been shown that in the gel domains, saturated long-chain FAs form 1:2 stoichiometric complexes with PC molecules (Cevc et al., 1988).

The relative changes in order parameters along the length of the DPPC-d_{62} acyl chains induced by FAs exhibited a biphasic behavior, with order increasing up to carbons 10–12 and then decreasing (Fig. 2). With addition of DAGs (Goldberg et al., 1994), as with phosphatidylethanolamine, cholesterol, or decreasing temperature (Lafleur et al., 1990), relative order increases continuously throughout the carbon chain. The effect is due to the lower sensitivity of the tightly packed acyl chains near the bilayer/water interface to these perturbants (Cheng et al., 1994). However, depending on the characteristics of the perturbant, its effects on the ordering of the bilayer can also be different at the surface versus the hydrophobic core, which in turn would alter curvature stress (Gawrisch and Holte, 1996), as was shown to be the case in the presence of *cis* double bonds when the order decreases preferentially toward the hydrophobic core (Holte et al., 1995; Separovic and Gawrisch, 1996). Thus, there is a balance between the competing effects of a fatty acid, ordering throughout the acyl chain due to the small headgroup and disordering due to polyunsaturation felt preferentially in the lower portion of the acyl chain. The effect of these forces is observable as changes in order parameters along the acyl chain.

The addition of fatty acids modified the effects of DP in PC/PS bilayers with respect to both order parameters and phase properties. The presence of PUFAs, but not PA, reduced the relative amount of phospholipids and DP in gel phase. The presence of PUFAs apparently weakens the DP-phospholipid interactions resulting in the increased miscibility of DP with the phospholipids in the liquid-crystalline phase.

The effects of FAs on the DPPC-d_{62} order parameter profile of DP-containing samples were different for saturated and unsaturated FAs, where the comparison was made at 60°C, when all the components are in L_α phase (Fig. 2 *B*). The order parameters shown for a sample containing both PA and DP indicate an approximately additive effect as compared with PA and DP alone. However, the presence of PUFAs decreased order throughout the chains in DP-containing samples, and the relative effect was observed most strongly toward the hydrophobic core. Thus, although PUFAs increased order by themselves, they counteracted the ordering effects of DP. This suggests that the bilayer becomes more sensitive to the disordering effects of the double bonds in the PUFAs, when the bilayer is already tightly packed due to the presence of DP with its small headgroup.

We have previously correlated the change in headgroup quadrupole splittings in PC/PS bilayers with the PKC activity-inducing effects of various DAGs (Goldberg et al., 1995), suggesting that they are related, perhaps via the degree of hydration at the bilayer surface. The DAG-induced changes in splittings were observed as counter-directional shifts with $\Delta\nu_\alpha$ increasing and $\Delta\nu_\beta$ decreasing. The counter-directional changes were used to define a molecular voltmeter model (Scherer and Seelig, 1989) in which membrane-bound ions assert a Coulombic force on the P^+–N^- dipole of the headgroup, repelling or attracting the positive choline moiety with respect to the membrane surface and causing changes in the average C-D bond angles with the molecular axis of rotation, which in the case of the headgroup of a lipid molecule in a bilayer corresponds to the bilayer normal (McLaughlin et al., 1975).

The addition of FAs to PC/PS bilayers also induced a counter-directional change in headgroup splittings, increasing $\Delta\nu_\alpha$ and decreasing $\Delta\nu_\beta$, as the concentration of FA was increased. The pK_a of FAs in sonicated vesicles has been shown to be ~7.6 for both saturated and unsaturated species (Hamilton and Cistola, 1986); thus in our measurements at pH 7.4, approximately one-half of the FA molecules carry a negative charge. Negatively charged lipids cause an increase (decrease) in $\Delta\nu_\alpha$ ($\Delta\nu_\beta$) with $\delta(\Delta\nu_\beta)/\delta(\Delta\nu_\alpha) \approx -1$ (Scherer and Seelig, 1987), whereas the value observed in the case of FAs was -1.7. This is probably due to a compound effect caused by the presence of both ionized and un-ionized FAs, with the latter acting more like other uncharged amphiphiles that exhibit higher $\delta(\Delta\nu_\beta)/\delta(\Delta\nu_\alpha)$ values in the range of -2.7 to -3.4 (Bechinger and Seelig, 1991; Goldberg et al., 1995). It was also noted that at 60°C with only liquid-crystalline phase present, the non-PKC-activating PA was approximately one-half as efficient as DHA in changing quadrupolar splitting values (Table 1). Because the pK_a is the same for saturated and unsaturated FAs (Hamilton and Cistola, 1986), this difference is not due to a difference in the fraction of ionized FA molecules. As the effect of the charge on headgroup quadrupole splittings is also dependent on the position of the charge relative to the choline moiety (Marassi and Macdonald, 1992), the results indicate that PA is positioned in the bilayer differently than AA or DHA. Thus, the hydrophobic portion of even a relatively simple lipid such as a FA determines its effect on membrane physical parameters at the bilayer surface. Addition of DP to PC/PS bilayers caused little change in headgroup splittings at 30°C, which was due to the virtual

absence of DP in the liquid-crystalline domains. When the temperature was raised to 60°C, DP altered headgroup splittings in a manner similar to that of PKC-activating DAGs (Goldberg et al., 1995).

In the absence of DP, we observed a small but significant activation of PKC by PUFAs, but not by PA, agreeing with the previous studies, which also found that long-chain saturated FAs do not activate PKC whereas *cis*-unsaturated FAs do in the absence of DAG in sonicated vesicles (Murakami et al., 1986; Lester, 1990). The synergistic effects of FAs and DP in activating PKC was strongly dependent on the presence of double bonds in the FAs. The activating capacity of DP was insignificant and was not enhanced by PA. Addition of PUFAs to the DP-containing samples resulted in a dramatic activation of PKC, agreeing with the results of Shinomura et al. (1991), who showed that, in conjunction with DAGs, unsaturated FAs act synergistically to increase activity whereas saturated FAs leave activity unchanged. Examining the differences in FAs with respect to their activation of PKC, Epand and co-workers (1991) studied phosphatidylethanolamine bilayers containing unesterified fatty acids, finding that long-chain saturated fatty acids have little effect on the bilayer-to-hexagonal transition temperature, whereas long-chain PUFAs (e.g., DHA) efficiently reduce this temperature. Other studies from the same group reported that the reduction of L_α-H_{II} temperature correlates with increased PKC activity (Epand, 1985; Epand, 1987; Senisterra and Epand, 1993), suggesting that, in the present case, AA and DHA were capable of activating PKC because of their membrane-destabilizing effects whereas PA did not activate PKC because it stabilizes bilayers. It has been previously suggested that FAs might modulate PKC activity through such effects on the bulk lipid properties (Lester, 1990; Zidovetzki and Lester, 1992; Kinnunen, 1996).

PKC acts equally efficiently whether the lipid cofactors are in liquid-crystalline or gel phase (Senisterra and Epand, 1993). The observation that DP in gel phase does not activate PKC suggests that the strong interactions between DP and phospholipids in these domains preclude DP from interacting with PKC in the required fashion. This would not be expected with non-gel-inducing DAGs such as unsaturated or short-chain saturated DAGs.

CONCLUSIONS

In this study, we have shown the relationships between PKC activation and several physicochemical parameters of the lipid bilayer, modulated by FAs and DP, including lipid acyl chain order, headgroup orientation, fraction of lipid in the liquid-crystalline phase, and partitioning of DP between L_α and L_β domains. The biophysical description of this system explains significant factors in the synergistic modulation of PKC activity by diacylglycerols and fatty acids. The simplest explanation of the data is that DP in gel phase is not accessible by PKC because DP strongly interacts with gel phase phospholipids. The PUFAs solubilize a portion of DP into liquid-crystalline domains, which makes DP available for efficient interaction with PKC. PKC activation takes place at the membrane surface and is thus dependent on the surface effects of both DP and FA, and these effects, in turn, are directly modulated by their acyl chain composition.

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