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

Edward M. Goldberg and Raphael Zidovetzki*

Department of Biology, University of California, Riverside, California 92521

Received August 6, 1997; Revised Manuscript Received January 14, 1998

ABSTRACT: The synergistic effects of diacylglycerol (DAG) and fatty acid (FA) in activating protein kinase C have been investigated by correlating their individual and combined effects on enzymatic activity and on membrane structure in phosphatidylcholine/phosphatidylserine (4:1) lipid mixtures using a combination of specific enzymatic assays and ^{31}P and ^{2}H NMR. Addition of DAGs and unsaturated FAs to the bilayers synergistically increased the tendency of the lipids to form nonbilayer phases with a concomitant increase in PKC activity until a maximum was achieved. Further increases in the DAG/FA concentration led to the formation of the nonbilayer lipid phases under the conditions of the PKC activity assays and correlated with decreased activity. The nonbilayer lipid phases still supported PKC activity, although with less than 50% efficiency as compared with the bilayer lipids. Long-chain saturated FA increased DAG-induced PKC activity by causing a lateral phase separation of gel (L_β) and liquid-crystalline (L_α) domains. Due to the preferential partitioning of DAGs into liquid-crystalline domains, the local DAG concentration increased in these domains, leading to an increase in PKC activity. Because a wide range of lipophilic compounds is capable of altering curvature stress, and therefore the tendency for nonbilayer phase formation in cellular membranes, these compounds would be expected to modulate PKC activity and the activities of a number of other membrane-associated enzymes that are sensitive to biophysical properties of lipid membranes.

The mammalian protein kinase C (PKC)[1] family of serine/threonine kinases consists of at least 12 isoforms (*1*) which are distributed nonuniformly throughout various tissues and are particularly concentrated in the nervous system (*2*). PKCs are implicated in the processes of learning and memory (*3*) and are involved in modulating an extensive number of cellular functions, including apoptosis (*4*), exocytosis, extracellular signal transduction, cell growth, and cell differentiation (*5*). PKC is the major receptor for the bryostatin and phorbol ester tumor promoters, and it performs a pivotal role in cellular cross-talk, directly phosphorylating a number of important signaling proteins, including phospholipase D (*6*), epidermal growth factor (*7*), DNA-regulatory proteins (*8*), and the tumor suppressor protein p53 (*9*).

Current models of activation for the conventional PKC isoforms involve the association of the inactive cytosolic protein with membranes containing acidic phospholipids in a Ca^{2+}-dependent manner, which causes a conformational change in the protein and its association with the lipid membrane (*10*−*13*). The association is strongly facilitated by the presence of phorbol ester or the natural cofactor 1,2-*sn*-diacylglycerol (DAG) which is produced as a result of stimulus-generated activation of phospholipase C or D. PKC activation is also modulated by the structure of the lipid bilayer, specifically as a result of stress, or destabilization of the lipid bilayer (*11*, *14*−*19*).

It has been recently shown that the presence of esterified or free polyunsaturated fatty acids increases bilayer curvature strain (*20*, *21*) and reduces phase transition temperatures for the gel-to-liquid-crystalline[2] and bilayer-to-nonbilayer phase transitions (*22*, *23*). Also, unesterified unsaturated fatty acids (FAs) in combination with DAG greatly enhance PKC activity in a synergistic manner (*24*−*28*). This study was designed to investigate the role of the lipid bilayer structure in the mechanism of the synergistic activation of PKC by DAG and fatty acids using ^{2}H NMR and PKC activity assays. The data show that the synergism exhibited by DAG and fatty acids in activating PKC is due to the synergistic effect of these molecules in inducing curvature strain in the bilayers and promoting the tendency to form nonbilayer lipid phases.

MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC), diperdeuteriopalmitoylphosphatidylcholine (DPPC-d_{62}), dipalmitoylphosphatidylserine (DPPS), diperdeuteriopalmitoylphosphatidylserine (DPPS-d_{62}), 1,2-dioleoyl-*sn*-glycerol (DO), and phosphatidylcholine extracts from bovine liver (BLPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Palm-

* To whom correspondence should be addressed. Telephone: (909) 787-5628. Fax: (909) 787-4286. E-mail: zidovet@ucrac1.ucr.edu.

[1] Abbreviations: AA, arachidonic acid; BLPC, bovine liver phosphatidylcholine; DAG, diacylglycerol; DHA, docosahexaenoic acid; diC_8, 1,2-*sn*-dioctanoyl-3-glycerol; DO, 1,2-*sn*-dioleoyl-3-glycerol; DPPC, dipalmitoylphosphatidylcholine; DPPC-d_{62}, diperdeuteriopalmitoylphosphatidylcholine; DPPS, dipalmitoylphosphatidylserine; DPPS-d_{62}, diperdeuteriopalmitoylphosphatidylserine; FA, fatty acid; OA, oleic acid; OA-d_2, oleic acid-9,10-d_2; PA, palmitic acid; PA-d_{31}, perdeuteriopalmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C; PS, phosphatidylserine.

[2] In this paper, we use "gel" for L_β and "liquid-crystalline" for L_α phases.

S0006-2960(97)01935-1 CCC: $15.00 © 1998 American Chemical Society
Published on Web 04/03/1998

itic (PA), oleic (OA), arachidonic (AA), and docosahexaenoic acids (DHA) were purchased from Nu-Check Prep (Elysian, MN). Perdeuteriopalmitic acid (PA-d_{31}) and 1,2-*sn*-dioctanoylglycerol (diC_8) were purchased from Serdary Research Laboratories (London, ON), and oleic acid-9,10-d_2 (OA-d_2) was from Cambridge Isotope Laboratories (Andover, MA). All lipids showed a single spot when checked for purity by thin-layer chromatography and visualized by spraying with 33% sulfuric acid, air-drying, and heating at 110 °C until the developed color had reached its maximum. Additionally, fatty acids were checked spectrophotometrically for the presence of the products of oxidation. Oxidation products constituted ≤0.5% of the fatty acids. Recombinant human protein kinases C α and β_{II} were purchased from PanVera (Madison, WI).

Multilamellar lipid dispersions were prepared by first dissolving the lipid mixtures 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 mole:mole:mole) with other lipids added as mentioned in the text. Lipid mixtures for PKC activity assays were placed under vacuum (<1 mTorr) for at least 8 h and then hydrated, vortexed, and quick-frozen in liquid nitrogen. Immediately prior to the assay, substrate and then enzyme solutions were added to samples, with final sample concentrations of 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 40 μM $CaCl_2$, 40 μM histone 1 (histone IIIs from Sigma, St. Louis, MO), 40 μM adenosine triphosphate (ATP, Sigma), 0.6 μCi [γ-^{32}P]ATP (Dupont NEN), and 10 ng of PKC and a total volume of 75 μL. The total phospholipid concentration was 4 mM with addition of other lipids as noted.

Lipid mixtures for NMR were dissolved in benzene/methanol (20:1 v/v), quickly frozen with liquid nitrogen, and then lyophilized under vacuum (<1 mTorr) for at least 8 h. Samples were hydrated with a 40 mM MOPS (pH 7.4), 65 mM $MgCl_2$ solution prepared in ^{2}H-depleted H_2O (Sigma). The samples were fully hydrated and were 1:2 (w/v) lipid/buffer. Lower hydration and the consequent higher lipid concentration of the NMR samples were required to prevent the orientation of the lipid bilayers in the magnetic field during NMR measurements. Assuming a Mg^{2+}−PS binding constant of 1.6×10^3 M and a stoichiometry of 2:1 (*29*), 65 mM Mg^{2+} was needed to achieve the equivalent saturation of PS for NMR samples, simulating the PKC assay conditions. To exclude the possible effects of higher Mg^{2+} concentrations, we also have prepared NMR samples with 90% hydration (25 mM PS) and 15 mM Mg^{2+}. The results from these samples were identical to those where the low-hydration, high-Mg^{2+} samples were used, except that the ^{2}H NMR spectral shape showed sample orientation. In different samples, DPPC, DPPS, OA, and PA were substituted with DPPC-d_{62}, DPPS-d_{62}, OA-d_2, or PA-d_{31}. A uniform lipid suspension was obtained by five freeze−thaw cycles (*30*, *31*).

Protein Kinase C Activity Assays. PKC activity was measured by phosphorylation of the exogenous substrate histone 1 according to Sando and Chertihin (*32*), with modifications. Samples were temperature-equilibrated with substrate and ATP shortly before performing the assay which was then initiated by addition of PKC. The 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, England). The papers were then washed four times with 500 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.

NMR Measurements. ^{2}H NMR and ^{31}P NMR spectra were acquired at 11.74 T (corresponding to 500.13 MHz for ^{1}H, 76.77 MHz for ^{2}H, and 202.49 MHz for ^{31}P frequencies) on a General Electric GN500 spectrometer. ^{31}P NMR spectra were obtained with a phase-cycled Hahn echo with a (70°−τ−70°)n pulse sequence (22 μs 70° pulse) and full phase cycling (*33*). The 70° pulse was used instead of the more common 90° pulse 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. ^{2}H NMR spectra were acquired with a high-power probe (Doty Scientific, Columbia, SC) using the standard quadrupole echo sequence (*34*): $90°_{\varphi}-\tau-90°_{\psi}-\tau-$acquire($\varphi$). The spectral width was 500 kHz; the refocusing time τ was 60 μs, with a 90° pulse of 3.5 μs, a recycle time of 200 ms, and the following phase cycling: $\varphi = x, -x, x, -x$; $\psi = y, y, -y, -y$. The echos were phased using zero-order phase correction to minimize intensity in the out-of-phase channel. The spectra were then folded about the symmetry axis and then unfolded, averaging the intensities for each pair of points across the symmetry axis. Spectra were de-Paked by numerical deconvolution according to Sternin et al. (*35*), producing spectra equivalent to those that would be obtained from a planar membrane with its bilayer normal aligned parallel to the applied static magnetic field. This procedure enhances resolution and facilitates 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.

Phase Composition Analyses. Spectra exhibiting multiple phases were analyzed by spectral subtraction similar to the method of Morrow and Davis (*36*). All spectra were normalized to contain the same spectral area. The total spectral intensity of a multiphase spectrum is a superposition of intensities from each individual phase. The coefficient of subtraction necessary to remove a particular phase component from the multiple-phase spectrum indicated the fraction of lipid occupying that phase. Single-phase spectra for isotropic and liquid-crystalline phases were obtained from samples exhibiting the appropriate phase, and pure hexagonal (H_{II}) and gel phase spectra were obtained from samples exhibiting H_{II}/isotropic and L_α/L_β components, respectively, after digitally subtracting out the unwanted phase. To compensate for differences in the width of isotropic peaks and changes in quadrupole splittings of hexagonal and L_α phases for different samples, single-phase spectra could be modulated by a width constant (keeping total spectral area fixed) to best match the spectrum being analyzed.

RESULTS

The ^{2}H NMR spectra of DPPC-d_{62} (20 mol %) in phosphatidylcholine (PC)/PS mixtures in the presence or absence of DAG and/or FA at 30 °C are shown in Figure 1. The ^{2}H NMR spectrum of randomly dispersed, fully hydrated, deuterated lipids is a superposition of the powder

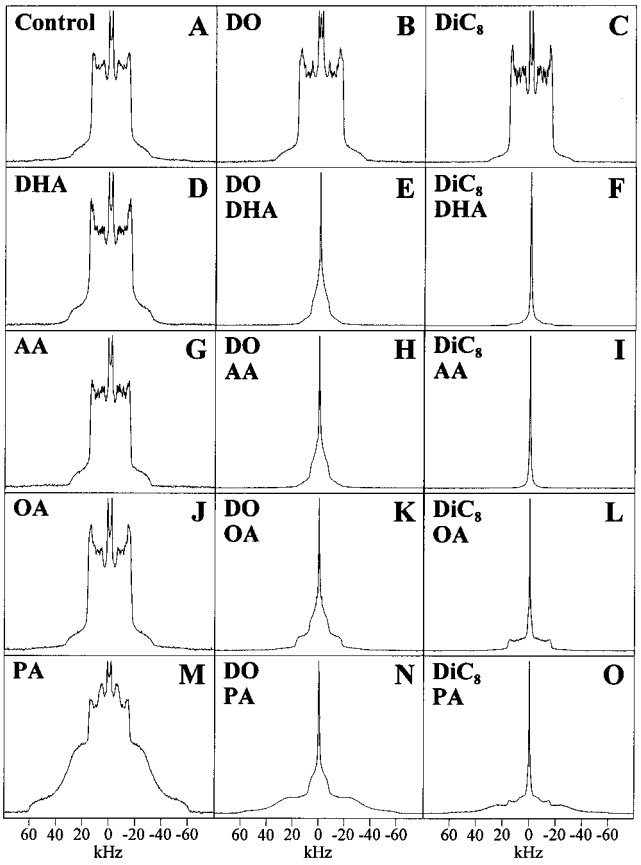

FIGURE 1: ^{2}H NMR spectra of DPPC-d_{62} in PC/PS mixtures in the absence or presence of 25 mol % DO or diC$_8$ and 25 mol % FA at 30 °C.

patterns arising from each deuterated position in the sample. In the case of liquid-crystalline bilayers, 11 distinct peaks can be discerned from DPPC-d_{62}, which is perdeuterated along both acyl chains. The distance between symmetric peaks defines the peak-to-peak quadrupole splitting ($\Delta\nu$) which is related to the order parameter S_{CD} by the equation

$$\Delta\nu^i = {}^3/_4 \frac{e^2Qq}{h} S_{CD}{}^i$$

where $e^2Qq/h = 167$ kHz is the static quadrupolar coupling constant (*37*) for a deuteron in a C−D bond. S_{CD} reflects both the range of motion of the C−D bond and its average orientation with respect to the bilayer normal. In the case of lipids with saturated acyl chains, the average orientation of each carbon segment is parallel to the bilayer normal, and changes in S_{CD} are due to differences in the range of motion of the carbon segments, with the highest-order parameter (and consequently the largest quadrupole splitting) corresponding to the motionally restricted region near the glycerol backbone, and monotonically decreasing along the acyl chain toward the terminal methyl group. The outermost peak corresponds to carbon segments 2−8, with each progressive peak representing carbon segments further along the chain as described (*38*). Lipids which are below the gel-to-liquid-crystalline transition temperature exhibit a broad shape with indistinct signals from individual methylene segments. The inverted hexagonal phase (H_{II}), which is characterized by the cylindrical packing of lipids, exhibits a spectrum with a shape that is similar to that of the liquid-crystalline bilayer,

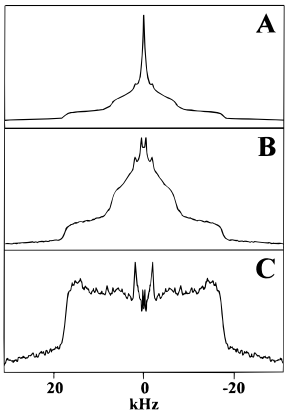

FIGURE 2: Determination of the phase composition of the ^{2}H NMR spectra of DPPC-d_{62} in PC/PS mixtures with 25 mol % DO and 25 mol % OA at 30 °C: (A) initial spectrum, (B) spectrum after subtraction of the 10.3% isotropic phase signal, and (C) spectrum after further subtraction of the 53% hexagonal phase signal.

but the peak splittings are reduced by a factor of 2 due to diffusion around the additional axis of motional averaging introduced by the cylindrical distribution of lipids in the H_{II} phase (*39*). The isotropic phase is seen as a narrow single peak at the center of the spectrum and can be due to lipids forming micelles, small vesicles, or the cubic phase.

Figure 1 shows that the liquid-crystalline phase of the bilayer was largely maintained upon addition of either 25 mol % unsaturated FA (Figure 1A,D,G,J) or 25 mol % DAG (Figure 1B,C). Sharper resolution of the spectrum on Figure 1C is due to a small residual orientation relative to the external magnetic field of the samples with high diC$_8$ content in the L_α phase. The sample orientation did not affect the quadrupole splittings. The presence of 25 mol % PA caused a lateral separation of gel and liquid-crystalline domains as indicated by the superposition of the spectral profiles associated with each of these phases (Figure 1M). The addition of both DAG and FA caused a dramatic effect on membrane structure. In each of the samples containing DO and FA (Figure 1E,H,K,N), the hexagonal phase was observed prominently, in addition to an isotropic component. In mixtures containing DO and PA (Figure 1N), nonbilayer phases coexisted with the gel phase, although no liquid-crystalline lipid was observed. In the case of diC$_8$ and FA (Figure 1F,I,L,O), no hexagonal phase was observed, although a prominent isotropic peak was present with each FA. Like the case with DO, the gel phase was observed in samples with diC$_8$ and PA (Figure 1O).

Spectrum subtraction was used to determine the fractions of lipid in each phase for the spectra in Figure 1, as illustrated in Figure 2 for a sample containing 25 mol % DO and 25 mol % OA. The original spectrum is displayed in Figure 2A, and after subsequent subtraction of 10.3% of a pure isotropic spectrum, the superposition of the remaining liquid-crystalline and hexagonal phases is displayed in Figure 2B. Figure 2C shows the result after further subtraction of 53% of a pure hexagonal spectrum, depicting the final 37% liquid-crystalline component. The background noise is higher in this final panel because 63% of the total intensity has been deleted. Further confirmation of the presence of the three lipid phases in this sample was realized using ^{31}P NMR, where isotropic (0 ppm) and hexagonal (4.2 ppm, left peak) peaks are well separated (Figure 3). The phase composition

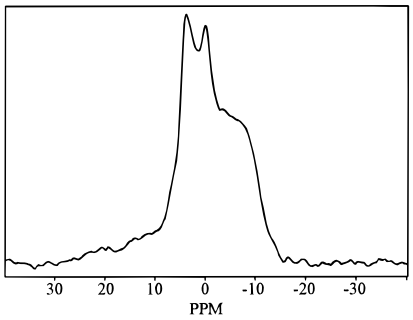

FIGURE 3: ^{31}P NMR spectrum of a PC/PS mixture containing 25 mol % DO and 25 mol % OA at 30 °C.

Table 1: Phase Compositions of Samples Containing 25 mol % DAG and/or 25 mol % FA[a]

DAG	FA	L_β	L_α	H_{II}	iso	Figure 1 panel
none	none	0	100	0	0	A
none	DHA	0	100	0	0	D
none	AA	0	100	0	0	G
none	OA	0	100	0	0	J
none	PA	71	29	0	0	M
DO	none	0	88	11	1	B
DO	DHA	0	0	87	13	E
DO	AA	0	0	89	11	H
DO	OA	0	37	53	10	K
DO	PA	75	0	19	6	N
diC_8	none	0	100	0	0	C
diC_8	DHA	0	10	28	62	F
diC_8	AA	0	0	0	100	I
diC_8	OA	0	76	0	24	L
diC_8	PA	63	18	5	14	O

[a] Values were determined from DPPC-d_{62} spectra and are ±0.5% for the isotropic phase and ±2% for the other phases.

of the spectrum in Figure 3 obtained by spectral subtraction analysis was similar to that of the corresponding ^{2}H NMR spectrum in Figure 2. The phase composition for each of the spectra shown in Figure 1 is listed in Table 1. The degree of membrane perturbation by the presence of different FAs can be inferred from the amount of nonbilayer lipids present in the series of spectra from samples containing 25 mol % DO and 25 mol % FA. In the absence of FAs, 88% of the lipids were in the liquid-crystalline phase. Upon addition of 25 mol % PA, 25% of the lipids were in hexagonal or isotropic phases. This number increased to 63% with OA, which was probably due to the presence of the double bond. The perturbations caused by the presence of AA or DHA, with four or six double bonds, respectively, were sufficient to cause the complete transition of these samples to nonbilayer phases (Table 1). Due to the distinctly different "transverse" perturbation (*40*) caused by the shorter acyl chains of diC_8, the majority of the nonbilayer phase lipid in the presence of this DAG was in an isotropic rather than in a hexagonal phase (Table 1). The order of efficiency of the different FAs in inducing nonbilayer phases was consistent between the two DAGs, although in most cases diC_8-containing samples had a smaller fraction of the nonbilayer phase lipid. DiC_8 with PA produced only 19% nonbilayer phase, whereas with OA, this increased slightly to 24%. The polyunsaturated FAs were much more efficient in that AA and DHA caused 90 and 100% transitions, respectively. Samples with labeled DPPS, but otherwise having the same compositions as those used for Figure 1, produced nearly identical

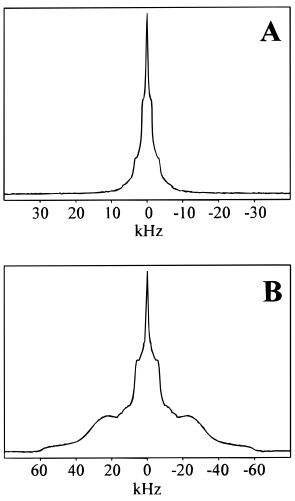

FIGURE 4: ^{2}H NMR spectra of deuterated FAs in PC/PS/25 mol % DO mixtures at 30 °C (A) with 25 mol % OA-d_2 and (B) with 25 mol % PA-d_{31}.

results (data not shown), indicating that PC and PS partition similarly among these phases under the conditions of this study.

We have further investigated the phase distribution of FAs in samples comprised of multiple phases by using deuterated OA or PA as ^{2}H NMR labels. The spectrum of OA-d_2, deuterated at carbons 8 and 9, in a PC/PS mixture containing 25 mol % DO and 25 mol % OA-d_2, is displayed in Figure 4A. Spectral subtraction was used to determine the fraction of isotropic lipid as 20.5%, and integration of the de-Paked spectra indicated that 74.5% of the OA was in the hexagonal phase, leaving only 5% in the L_α phase as compared with the 38% L_α phase found for DPPC-d_{62} in chemically identical samples (Figure 1K and Table 1). Spectral subtraction was used to determine the distribution of PA in each phase for PC/PS samples containing 25 mol % DO and 25 mol % PA-d_{31} (Figure 4B). The results showed that PA was distributed among the phases in a manner similar to that of PC and PS with the bulk (71.5%) being in the L_β phase and the remainder in nonbilayer phases (25% H_{II} and 3.5% isotropic).

PKC activity measurements of PC/PS samples with 0−25 mol % DO and 0−25 mol % FA are shown in Figure 5. In each case, the effect of FA alone was small when compared to the activity due to DO without FA. However, with each of the unsaturated FAs, addition of 25 mol % FA to samples containing only 5% DO caused a marked increase in activity, illustrating the synergistic effects of the combination of DAG and unsaturated FA. In the case of arachidonic acid, the synergistic effect was already prominent with 5 mol % DO and FA (Figure 5B). A similar result was also apparent with DO and PA, although PA was not as efficient as the unsaturated FAs. Figure 6 displays similar measurements for samples containing diC_8 and FAs. Again, synergism was observed for each of the FAs, with PA being much less effective than the unsaturated FAs.

In each of the panels in Figure 5, a distinct maximum for PKC activity was observed which was located at or near the 15 mol % DAG/15 mol % FA point. Identical results were obtained using PKC β_{II} (data not shown). Qualitatively similar although less pronounced behavior was observed in the case of diC_8 and unsaturated FAs (Figure 6A−C). The

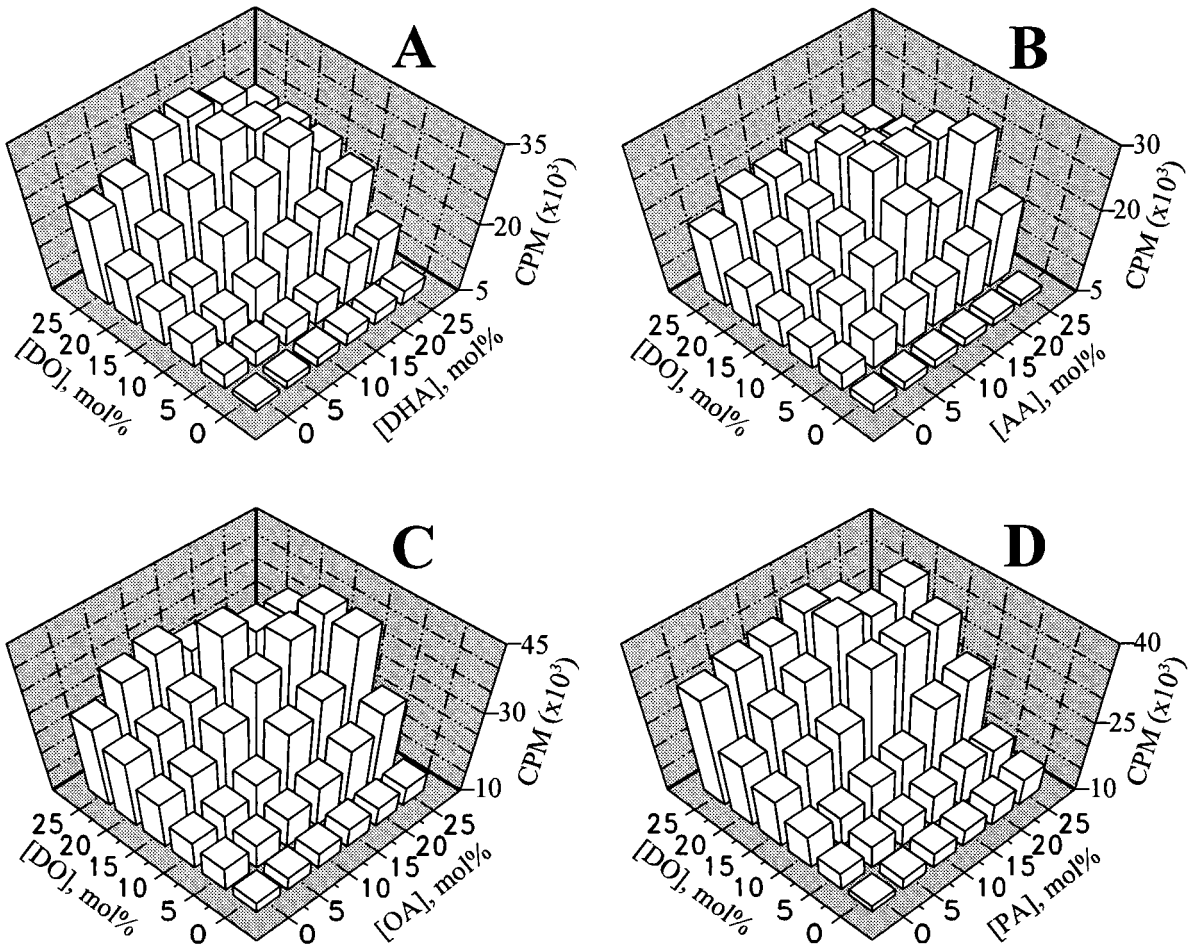

FIGURE 5: Dependence of PKC activity on the concentration of DO and different FAs: (A) DHA, (B) AA, (C) OA, and (D) PA.

PKC activities at 15 mol % DAG/15 mol % FA, obtained from a separate experiment, are presented in Table 2, along with the maximum activities observed for each DAG/FA combination, which were similar for either DAG with the unsaturated FAs. Similar local maxima of PKC activity were observed for diC_8 (Figure 6); however, their locations were less restricted to the 15 mol % DAG/15 mol % FA position on the graphs. The possibility that this phenomenon was due to the phase properties of the samples was investigated by NMR. ^2H NMR spectra of a series of samples with equal DO and AA concentrations, corresponding to the diagonal in Figure 5B, are shown in Figure 7A−E. Increasing the DO/AA concentration to 15 mol % did not induce nonbilayer lipid phases (Figure 7A−C). Further increases of DO and AA to 20 mol % reduced the amount of bilayer lipid present to 52% (Figure 7D), and the transition to nonbilayer phases was complete with 25 mol % DO and AA (Figure 7E). At these concentrations, PKC activity sharply decreased, coincident with the appearance of nonbilayer phases as demonstrated in Figure 7F. There was, however, a significant activity of the enzyme at 25 mol % DO/AA, showing that the nonbilayer lipid phase supported PKC activity, albeit to a lesser degree.

Figure 7F shows that the sharp increase in PKC activity was not accompanied by a change in the lipid phase composition. We have investigated the tendency of the corresponding samples to form nonbilayer phases at increased temperatures. The effects of 15 mol % DO and 15 mol % AA on bilayer structure, individually and in combination, at 30 and 60 °C are shown in Figure 8. Neither DO (Figure 8A,B) nor AA (Figure 8C,D) alone induced nonbilayer phases at temperatures of up to 60 °C. However, the presence of both 15 mol % DO and AA induced nonbilayer phases at 60 °C (Figure 8F), demonstrating that DO and AA synergistically increase the tendency of the lipids to form nonbilayer lipid phases. Similar results were obtained with diC_8 and with the other unsaturated FAs (data not shown).

The perturbations by DAGs and FAs could be observed at each carbon position within the liquid-crystalline bilayer interior by measuring the changes in the order parameters of perdeuterated molecules in the lipid mixtures. The relative order parameter changes due to 15 mol % DO, 15 mol % AA, and equimolar DO/AA concentrations of up to 15 mol % are shown in Figure 9A. Both AA and DO increased the order along the entire acyl chain; however, DO was much more potent than AA and had significantly greater ordering effects on the carbon segments toward the center of the hydrophobic interior. Addition of AA to DO-containing samples attenuated the effect of DO. DiC_8 caused a transverse perturbation as described previously (*40*, *41*), which increased the order in the top half of the acyl chains and decreased order parameters for carbon segments 10−16 (Figure 9B). This effect was previously described by us as being due to the shortness of this DAG which allowed for a greater range of motion in the lower portion of the phospholipids. The presence of 15 mol % diC_8 and 15 mol % AA together yielded a profile which was nearly identical with the plot for 15 mol % diC_8 alone (Figure 9B).

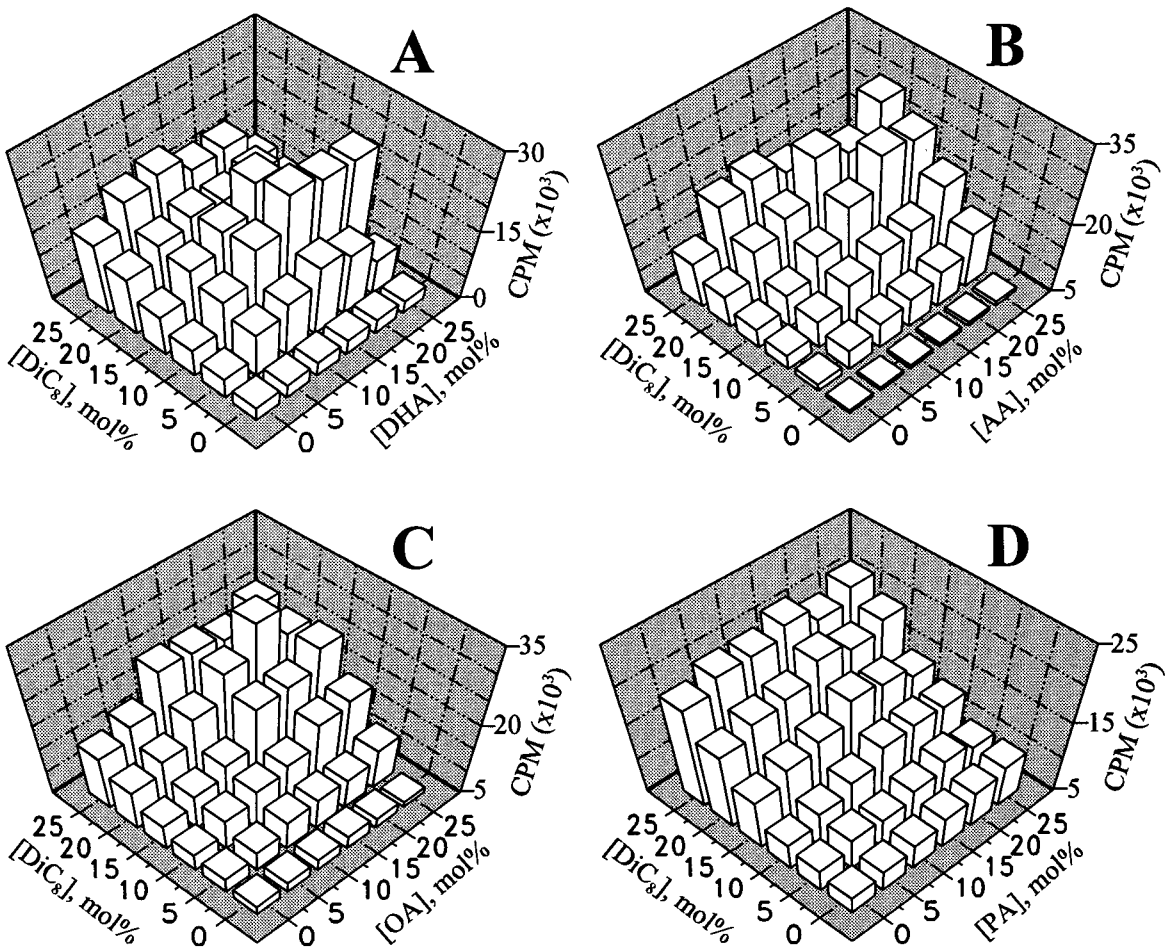

FIGURE 6: Dependence of PKC activity on the concentration of diC_8 and different FAs: (A) DHA, (B) AA, (C) OA, and (D) PA.

Table 2: Comparison of PKC Activities for Samples Containing DAG and/or FA

DAG	FA	15 mol % DAG/FA (cpm)	maximum activity[a] (cpm)
DO	none	9800 ± 100	13723 ± 211
DO	DHA	18360 ± 430	21684 ± 340
DO	AA	19180 ± 390	20028 ± 1034
DO	OA	20230 ± 130	23302 ± 418
DO	PA	15190 ± 660	16856 ± 226
diC_8	none	9910 ± 220	12676 ± 163[b]
diC_8	DHA	17530 ± 270	23507 ± 436
diC_8	AA	17840 ± 640	22413 ± 540
diC_8	OA	16410 ± 100	22772 ± 386
diC_8	PA	13730 ± 120	17145 ± 209

[a] Maximum activity observed over all DAG/FA concentrations. [b] Activity of 25 mol %. The maximum was not reached at this concentration.

DISCUSSION

Biophysical properties of membranes are known to modulate PKC activity in lipid bilayers (for reviews, see refs *11* and *19*). The presence of unsaturation in either the PC or PS component of PC/PS mixtures affects both the lipid-dependent activation of PKC and the action of DAG (*16*, *42*), suggesting that PKC activation by DAG may involve a specific DAG-induced perturbation in the structure of the membrane phospholipid bilayer. Sando and co-workers (*43*, *44*) showed that, by using short-chain PC and/or PS, it is possible to totally bypass the requirement of PKC for DAG, giving additional support to the hypothesis that the role of DAG may be to generate a particular "activating" structure. Indeed, DAG, which is produced from phosphatidylinositol or PC by removing the polar group with its associated water, is a very hydrophobic molecule which profoundly affects bilayer structure (*40*, *41*, *45*−*63*). Additionally, the fatty acyl chains of DAGs determine their PKC activating capacity which is greatly enhanced by unsaturation as demonstrated in sonicated vesicles (*24*, *28*, *64*−*68*) and planar bilayers (*23*, *32*, *40*, *69*). It has recently been shown that PKC primarily interacts with the membrane surface and does not penetrate more than 8 Å into the hydrophobic core (*70*). As the differences among DAGs are solely due to their acyl chain composition, the hydrophobic portion must determine the differences in the effects of various DAGs that are sensed by PKC at the membrane surface.

In general, lipophilic molecules which promote the hexagonal phase also increase PKC activity (*14*, *15*, *71*, *72*). FAs have also been shown to alter bilayer structure and modulate PKC activity. Unsaturated FAs reduce the L_β−L_α and L_α−H_{II} transition temperatures, whereas saturated FAs do not (*22*, *73*). Accordingly, several reports have shown that addition of unsaturated FAs strongly enhances DAG-induced PKC activity, although saturated FAs have little effect (*24*−*28*, *74*−*76*). Thus, the PKC-activating properties of DAGs and FAs can be associated with the increased propensity for hexagonal phase formation.

The relationship between the propensity for the inverted hexagonal phase (see ref *77* for a review of the structure of this phase) and a wide range of biological phenomena has

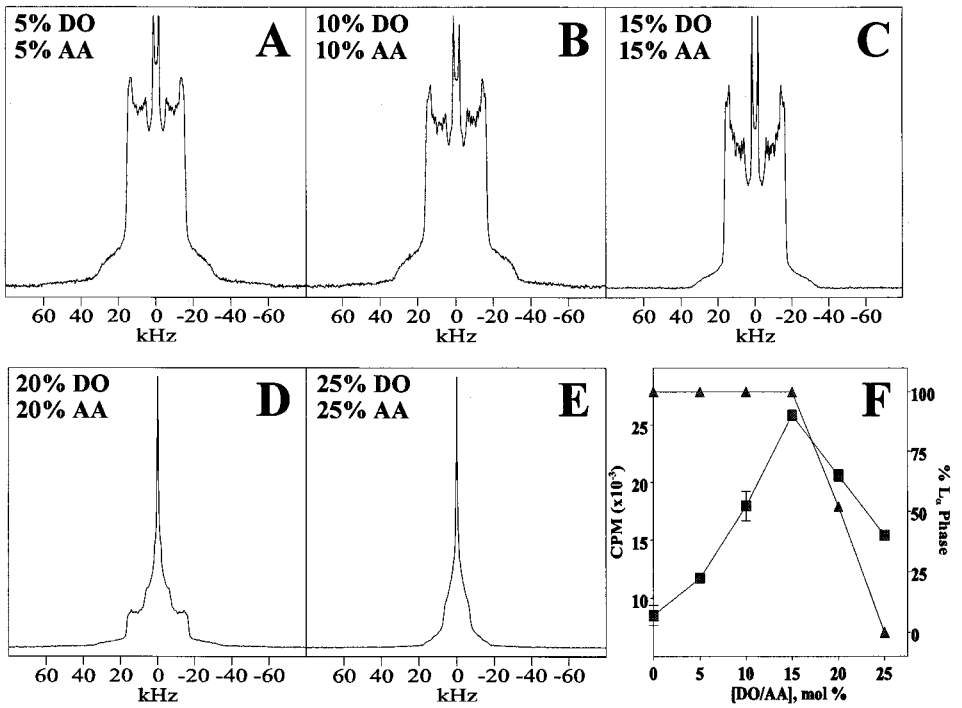

FIGURE 7: ^{2}H NMR spectra of DPPC-d_{62} in PC/PS mixtures containing increasing concentrations of DO and AA at 30 °C: (A) 5 mol % DO and 5 mol % AA, (B) 10 mol % DO and 10 mol % AA, (C) 15 mol % DO and 15 mol % AA, (D) 20 mol % DO and 20 mol % AA, and (E) 25 mol % DO and 25 mol % AA. Effects of increasing DO and AA on PKC activity (■) and percentage of liquid-crystalline lipid (▲) (F).

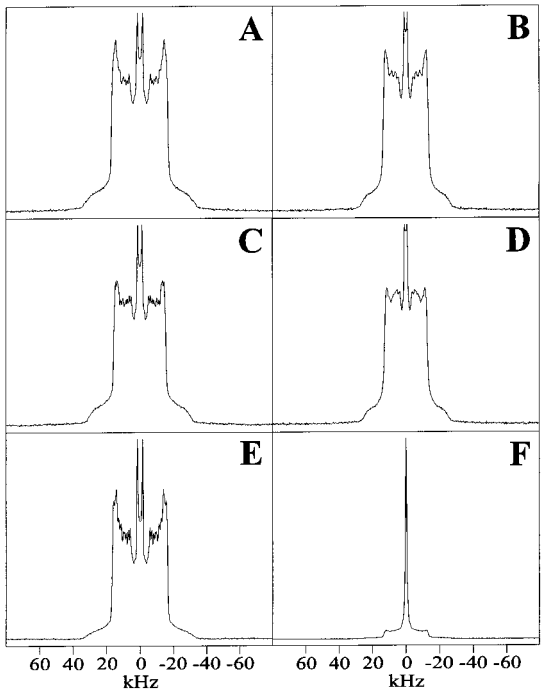

FIGURE 8: ^{2}H NMR spectra of DPPC-d_{62} in PC/PS mixtures at 30 °C (left) or 60 °C (right) containing 15 mol % DO (A and B), 15 mol % AA (C and D), or both (E and F).

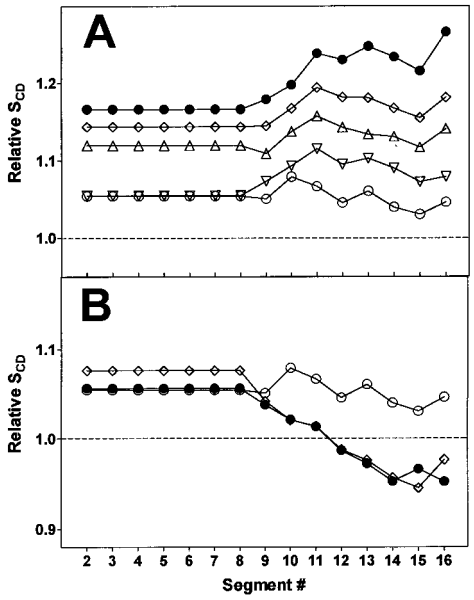

FIGURE 9: Effects of DO (A) or diC$_8$ (B) with or without AA on the relative order parameters of DPPC-d_{62} in PC/PS mixtures at 30 °C: (●) 15 mol % DAG, (○) 15 mol % AA, (▽) 5 mol % DAG and 5 mol % AA, (△) 10 mol % DAG and 10 mol % AA, and (◇) 15 mol % DAG and 15 mol % AA.

been reviewed (*78*). The relevant physical property is probably the increase in bilayer stress induced by the inclusion of nonbilayer-forming lipids that have a small radius of spontaneous curvature. The molecular packing stress and associated bilayer defects reach a maximum at the onset of the bilayer−H_{II} transition (*79*−*84*). The stress model of the bilayer−H_{II} transition further predicts that the lateral stress is increased in bilayers which contain nonbilayer-phase-preferring lipids (*85*). Some evidence of such increased stress has been reported. Chen et al. (*86*), using frequency-domain fluorescence measurements, found that the values of local orientational order parameters increase significantly at the bilayer-to-hexagonal phase transition temperature and that the curvature-related stress in the lipid bilayer increases with the content of the nonbilayer phase-promoting lipids. Frequency-domain fluorescence intensity decay measure-

ments have also shown that the addition of dioleoyl-PE (a lipid with a high intrinsic curvature) to dioleoyl-PC bilayers increases lateral stress in the region near the center of the bilayers (*87*). DAGs have a small radius of spontaneous curvature, and indeed, a number of studies reported the induction of nonbilayer lipid phases by DAGs in a temperature-, concentration-, and phospholipid-dependent manner (*40*, *45*, *48*, *59*, *88*, *89*; see ref *19* for review).

It has been specifically shown that lipid mixtures with greater negative monolayer curvature also support greater PKC activity (*69*). The lipid bilayer frustration due to the presence of nonbilayer-promoting lipids is reflected in the order parameters of the liquid-crystalline bilayer (*90*). Due to their small headgroup size, DAGs (*40*) and FAs (this study and our unpublished results) intercalate between phospholipids, allowing for tighter contact between the acyl chains and thereby increasing phospholipid order parameters. This results in increased lateral pressure and contributes to negative curvature stress, which is directly related to the promotion of H_{II} and isotropic phases (*21*, *90*, *91*). Additionally, long acyl chains intercalate deep into the core of the bilayer, generating torque that increases with unsaturation (*21*, *92*−*95*). Thus, unsaturated DAGs and FAs increase curvature stress both by decreasing headgroup spacing and by increasing acyl chain repulsion. The combined curvature stress induced by DAGs and FAs at 25 mol % was sufficient to induce hexagonal and/or isotropic phases in PC/PS mixtures as observed in Figure 1; however, individually, neither diC_8 nor FAs were capable of inducing nonbilayer phases at concentrations of up to 25 mol %, and only a small amount of these phases was observed with DO. The efficiency of the various FAs in enhancing nonbilayer phases followed their degree of unsaturation. With DO, both polyunsaturated FAs caused complete transition to nonbilayer phases, whereas monounsaturated oleic acid induced only 63% nonbilayer phase (Table 1). Examination of the phase compositions of PA-containing samples showed that the amounts of gel phase were nearly identical with or without DO. PA has been shown previously to induce gel domains in DPPC bilayers by forming 1:2 stoichiometric complexes with phospholipids (*96*). Thus, palmitic acid is not expected to directly cause nonbilayer phases. These facts suggest that the nonbilayer phases in samples with PA were promoted by the lateral phase separation of liquid-crystalline and gel domains. DO decreases the $L_\beta-L_\alpha$ phase transition temperature of DPPC (*48*), showing that, as expected, it preferentially partitions into lipids in the liquid-crystalline phase. Therefore, when a significant portion of the lipids are in the gel phase due to the presence of PA, the preferential partitioning of DO to the liquid domains would cause a rise in local DO concentration in excess of the approximately 30 mol % required for hexagonal phase formation by DAG alone (*48*, *89*; E. M. Goldberg and R. Zidovetzki, unpublished observations). A similar argument applies to the samples containing diC_8; however, this DAG was less efficient at promoting nonbilayer phases (see Table 1). At lower concentrations of DAG and FA, neither 15 mol % DO nor 15 mol % AA induced nonbilayer phases at temperatures of up to 60 °C, whereas in the presence of both DO and AA, 60% of the lipids were in nonbilayer phases (Figure 8). This indicates that at lower temperatures, in the absence of nonbilayer lipid phases, the stability of the bilayers is reduced.

Consequences for PKC Activity. The increased tendency to form nonbilayer phases brought about by the presence of DAGs and FAs in the membrane resulted in an increase in PKC activity, as seen in Figures 5 and 6. At concentrations of DO and FA below the threshold for nonbilayer phase induction, increased activity could be brought about by increasing either DO or FA as long as a minimal amount of DAG was present as a necessary cofactor for PKC activation. Increasing the FA concentration was not as efficient as increasing DO, which is to be expected considering that DO is a stronger perturbant as shown by the effects on order parameters (Figure 9) and on the H_{II} transition temperature (*22*, *45*). Similar results were observed with diC_8 and FAs, indicating that, although the shorter length of the acyl chains of diC_8 may influence the specific perturbations generated by this DAG, the results on curvature strain and PKC activity are similar to those with DO. With either DAG, PA was much less effective than the unsaturated FAs in activating PKC (Figures 5D and 6D), correlating with the reduced DAG−PA synergism in inducing nonbilayer lipid phases (Figure 1).

When DAG and FA concentrations were increased past a certain point (15 mol % in the case of DO and AA, Figure 7), nonbilayer phases formed with a concurrent sharp decline in PKC activity (Figure 7F). This is seen as a ridge or a region of local maximum activity in the plots of Figures 5 and 6 and usually occurred at or near the 15 mol % DAG/15 mol % FA point with the PKC activity plots reflecting the phase compositions of the lipid mixtures. It is also noteworthy that there is a maximum rate of PKC activity that can be sustained by the lipid bilayer. Whereas the different DAG/unsaturated FA combinations vary in their efficiency in promoting PKC activity, they all reach the same maximum PKC activity (Table 2), although this happens at different concentrations of the additives. The difference in efficiency of these lipids in activating PKC correlated with their capacity to induce nonbilayer phases, and maximum PKC activity corresponded to the onset of nonbilayer phase formation and was independent of the choice of DAG and unsaturated FA. The comparison of panels E and F of Figure 7 shows that nonbilayer lipid phases support PKC activity less efficiently than the bilayers, agreeing with Boni and Rando (*97*). The decline of PKC activity observed at high DAG/FA concentrations (Figures 5 and 6) was due to the increased presence of the nonbilayer lipid phases. In support of this, it should be noted that, with diC_8 and PA, relatively little isotropic phase had formed even at up to 25 mol % concentrations (Figure 1O) and no local maximum was observed at these concentrations. At higher concentrations, however, PKC activity declined as expected (data not shown).

Thus, our results indicate that the observed synergism between DAGs and FAs in PKC activation is due to their synergistic effect on lipid bilayer structure, specifically to the increased tendency to form nonbilayer lipid phases, corresponding to increased frustration of the bilayer structure. However, the actual presence of nonbilayer phases is detrimental to PKC activity. The synergistic effects of DAG and unsaturated fatty acids were apparent already at concentration of 5 mol % for both additives (see especially

Figures 5B and 6A). The total level of DAG in most cells was found to be ≤2 mol %. However, local levels of DAG may be considerably higher depending on the rates of its enzymatic production and lateral diffusion. Moreover, in some cells, the total level of DAG can exceed 5 mol %. Such high DAG levels were reported in PC12 cells (*98*), human epidermal A431 cells (*99*), human endothelial cells (*100*), and transformed 3T3 cells (*101*). The concentration of total unesterified fatty acids in certain biological membranes can be as high as 8% in rat liver plasma membranes (*102*) and 5% in the sacrcolemmal membranes of cardiac muscle after myocardial ischemia (*103*). Thus, the strong synergistic effects observed at concentrations of 5 mol % for both DAGs and FAs are within, or close to, physiological concentrations of DAGs and fatty acids in biological membranes.

Because many cellular membranes exist under conditions of high curvature stress, being close to the hexagonal phase transition (*104*, *105*), the physicochemical properties of these membranes are expected to be sensitive to changes in DAG and FA concentrations, as well as other membrane components and lipophilic agents which may modulate curvature stress. Therefore, membrane-associated enzymes that are sensitive to curvature strain would be highly responsive to changes in DAG and FA concentrations. The enzymes modulated by lipid bilayer structure include Mg^{2+}-ATPase, mitochondrial ubiquinol−cytochrome *c* reductase, H^+-ATPase (*106*), and lipid metabolic enzymes mycoplasmic diglycosyl diacylglycerol synthase, phospholipases A_2, phospholipases C, phosphocholine cytidylyltransferase, phosphatidate phosphohydrolase, and diacylglycerol kinase (see ref *107* for a review). These lipid metabolic enzymes produce many of the nonbilayer-forming lipid second messengers which regulate each other and PKC. PKC, in turn, regulates the activity of such lipid metabolic enzymes as phospholipases A_2, C, and D (*6*, *108*, *109*), thereby increasing the potential for cross-talk among the lipid metabolic, and especially between the metabolic and nonmetabolic enzymes. It follows that a broad range of cellular activities may be modulated by the biophysical characteristics of the plasma membrane, which is a function of lipid composition as determined by dietary fat intake and the activity of lipid metabolic enzymes. Understanding the cellular functions of membrane lipid structure will lead to a better comprehension of the mechanisms involved in signal transduction and cellular signaling cross-talk as well as the effects of lipophilic drugs and the roles of dietary essential fatty acids in the pathologies and treatments of a variety of diseases and malnutritive conditions.

ACKNOWLEDGMENT

We thank J. J. Sando for her indispensable help in refining the PKC assay procedure and D. B. Borchardt for his help in optimizing NMR experiments. The NMR work was performed with a GN500 spectrometer, funded by National Science Foundation Grant DMB840491 and National Institutes of Health Grant BRSG2507.

REFERENCES

1. Dekker, L. V., and Parker, P. J. (1994) *Trends Biochem. Sci. 19*, 73−77.
2. Wetsel, W. C., Khan, W. A., Merchenthaler, I., Rivera, H., Halpern, A. E., Phung, H. M., Negro-Vilar, A., and Hannun, Y. A. (1992) *J. Cell Biol. 117*, 121−133.
3. Fagnou, D. D., and Tuchek, J. M. (1995) *Mol. Cell. Biochem. 149/150*, 279−286.
4. Lucas, M., and Sánchez-Margalet, V. (1995) *Gen. Pharmacol. 26*, 881−887.
5. Nishizuka, Y. (1986) *Science 233*, 305−312.
6. Kiss, Z. (1996) *Chem. Phys. Lipids 80*, 81−102.
7. Ido, M., Sekiguchi, K., Kikkawa, U., and Nishizuka, Y. (1987) *FEBS Lett. 219*, 215−218.
8. Buchner, K. (1995) *Eur. J. Biochem. 228*, 211−221.
9. Baudier, J., Delphin, C., Grunwald, D., Khochbin, S., and Lawrence, J. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11627−11631.
10. Mosior, M., and McLaughlin, S. (1991) *Biophys. J. 60*, 149−159.
11. Zidovetzki, R., and Lester, D. S. (1992) *Biochim. Biophys. Acta 1134*, 261−272.
12. Nakamura, S.-I., and Nishizuka, Y. (1994) *J. Biochem. 115*, 1029−1034.
13. Newton, A. C. (1995) *J. Biol. Chem. 270*, 28495−28498.
14. Epand, R. M. (1987) *Chem.-Biol. Interact. 63*, 239−247.
15. Epand, R. M., and Lester, D. S. (1990) *Trends Pharmacol. Sci. 11*, 317−320.
16. Bolen, E. J., and Sando, J. J. (1992) *Biochemistry 31*, 5945−5951.
17. Slater, S. J., Kelly, M. B., Taddeo, F. J., Ho, C., Rubin, E., and Stubbs, C. D. (1994) *J. Biol. Chem. 269*, 4866−4871.
18. Dibble, A. R. G., Hinderliter, A. K., Sando, J. J., and Biltonen, R. L. (1996) *Biophys. J. 71*, 1877−1890.
19. Zidovetzki, R. (1997) *Curr. Top. Membr. 44*, 255−283.
20. Epand, R. M., Fuller, N., and Rand, R. P. (1996) *Biophys. J. 71*, 1806−1810.
21. Gawrisch, K., and Holte, L. L. (1996) *Chem. Phys. Lipids 81*, 105−116.
22. Epand, R. M., Epand, R. F., Ahmed, N., and Chen, R. (1991) *Chem. Phys. Lipids 57*, 75−80.
23. Giorgione, J., Epand, R. M., Buda, C., and Farkas, T. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9767−9770.
24. Lester, D. S. (1990) *Biochim. Biophys. Acta 1054*, 297−303.
25. Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K., and Nishizuka, Y. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5149−5153.
26. Chen, S. G., Kulju, D., Halt, S., and Murakami, K.. (1992) *Biochem. J. 284*, 221−226.
27. Chen, S. G., and Murakami, K. (1992) *Biochem. J. 282*, 33−39.
28. Schachter, J. B., Lester, D. S., and Alkon, D. L. (1996) *Biochim. Biophys. Acta 1291*, 167−176.
29. Portis, A., Newton, C., Pangborn, W., and Papahadjopoulos, D. (1979) *Biochemistry 18*, 780−790.
30. Westman, J., Boulanger, Y., Ehrenberg, A., and Smith, I. C. P. (1982) *Biochim. Biophys. Acta 685*, 315−328.
31. Mayer, L. D., Hope, M. J., Cullis, P. R., and Janoff, A. S. (1985) *Biochim. Biophys. Acta 817*, 193−196.
32. Sando, J. J., and Chertihin, O. I. (1996) *Biochem. J. 317*, 583−588.
33. Rance, M., and Byrd, R. A. (1983) *J. Magn. Reson. 52*, 221−240.
34. Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M., and Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390−394.
35. Sternin, E., Bloom, M., and MacKay, A. L. (1983) *J. Magn. Reson. 55*, 274−282.
36. Morrow, M. R., and Davis, J. H. (1988) *Biochemistry 27*, 2024−2032.
37. Burnett, L. J., and Muller, B. H. (1971) *J. Chem. Phys. 55*, 5829−5831.
38. Davis, J. H. (1979) *Biophys. J. 27*, 339−358.
39. Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353−418.
40. Goldberg, E. M., Lester, D. S., Borchardt, D. B., and Zidovetzki, R. (1994) *Biophys. J. 66*, 382−393.
41. De Boeck, H., and Zidovetzki, R. (1992) *Biochemistry 31*, 623−630.

42. Snoek, G. T., Feijen, A., Hage, W., van Rotterdan, W., and de Laat, S. W. (1988) *Biochem. J. 255*, 629−637.
43. Walker, J. M., and Sando, J. J. (1988) *J. Biol. Chem. 263*, 4537−4540.
44. Walker, J. M., Homan, E. C., and Sando, J. J. (1990) *J. Biol. Chem. 265*, 8016−8021.
45. Epand, R. M. (1985) *Biochemistry 24*, 7092−7095.
46. Rand, R. P., Das, S., and Parsegian, V. A. (1985) *Chem. Scripta 25*, 15−21.
47. Smaby, J. M., and Brockman, H. L. (1985) *Biophys. J. 48*, 701−707.
48. De Boeck, H., and Zidovetzki, R. (1989) *Biochemistry 28*, 7439−7446.
49. Hamilton, J. A., Fujito, D. T., and Hammer, C. F. (1991) *Biochemistry 30*, 2894−2902.
50. Ortiz, A., Aranda, F. J., Villalain, J., San Martin, C., Micol, V., and Gómez-Fernández, J. C. (1992) *Chem. Phys. Lipids 62*, 215−224.
51. Smith, S. O., Kustanovich, I., Bhamidipati, S., Salmon, A., and Hamilton, J. A. (1992) *Biochemistry 31*, 11660−11664.
52. Bhamidipati, S. P., and Hamilton, J. A. (1993) *J. Biol. Chem. 268*, 2431−2434.
53. Rand, R. P., and Fang, Y. (1993) *Biochem. Soc. Trans. 21*, 266−270.
54. Goldberg, E. M., Lester, D. S., Borchardt, D. B., and Zidovetzki, R. (1995) *Biophys. J. 69*, 965−973.
55. López-García, F., Villalain, J., Gómez-Fernández, J. C., and Quinn, P. J. (1994) *Biophys. J. 66*, 1991−2004.
56. Nieva, J. L., Alonso, A., Basañez, G., Goni, F. M., Gulik, A., Vargas, R., and Luzzati, V. (1995) *FEBS Lett. 368*, 143−147.
57. Orädd, G., Lindblom, G., Fontell, K., and Ljusberg-Wahren, H. (1995) *Biophys. J. 68*, 1856−1863.
58. Quinn, P. J., Takahashi, H., and Hatta, I. (1995) *Biophys. J. 68*, 1374−1382.
59. Leikin, S., Kozlov, M. M., Fuller, N. L., and Rand, R. P. (1996) *Biophys. J. 71*, 2623−2632.
60. Basañez, G., Nieva, J. L., Rivas, E., Alonso, A., and Goni, F. M. (1996) *Biophys. J. 70*, 2299−2306.
61. Ruiz-Arguello, M. B., Basañez, G., Goni, F. M., and Alonso, A. (1996) *J. Biol. Chem. 271*, 26616−26621.
62. Schorn, K., and Marsh, D. (1996) *Biophys. J. 71*, 3320−3329.
63. Schorn, K., and Marsh, D. (1996) *Biochemistry 35*, 3831−3836.
64. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y. (1980) *J. Biol. Chem. 255*, 2273−2276.
65. Mori, T., Takai, Y., Yu, B., Takahashi, J., Nishizuka, Y., and Fujikura, T. (1982) *J. Biochem. 91*, 427−431.
66. Cabot, M. C., and Jaken, S. (1984) *Biochem. Biophys. Res. Commun. 125*, 163−169.
67. Go, M., Sekiguchi, K., Nomura, H., Kikkawa, U., and Nishizuka, Y. (1987) *Biochem. Biophys. Res. Commun. 144*, 598−605.
68. Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U., and Nishizuka, Y. (1988) *J. Biochem. 103*, 759−765.
69. Marignani, P. A., Epand, R. M., and Sebaldt, R. J. (1996) *Biochem. Biophys. Res. Commun. 225*, 469−473.
70. Mosior, M., and Newton, A. C. (1995) *J. Biol. Chem. 270*, 25526−25533.
71. Epand, R. M., and Bottega, R. (1988) *Biochim. Biophys. Acta 944*, 144−154.
72. Epand, R. M., Stafford, A. R., Cheetham, J. J., Bottega, R., and Ball, E. H. (1988) *Biosci. Rep. 8*, 49−54.
73. Ortiz, A., and Gómez-Fernández, J. C. (1987) *Chem. Phys. Lipids 45*, 75−91.
74. McPhail, L. C., Clayton, C. C., and Snyderman, R. (1984) *Science 224*, 622−625.
75. Shearman, M. S., Shinomura, T., Oda, T., and Nishizuka, Y. (1991) *FEBS Lett. 279*, 261−264.
76. Yoshida, K., Asaoka, Y., and Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6443−6446.
77. Seddon, J. M. (1990) *Biochim. Biophys. Acta 1031*, 1−69.
78. Epand, R. M. (1990) *Biochem. Cell Biol. 68*, 17−23.
79. Bentz, J., Ellens, H., Lai, M. Z., and Szoka, F. C., Jr. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5742−5745.
80. Ellens, H., Bentz, J., and Szoka, F. C. (1986) *Biochemistry 25*, 285−294.
81. Ellens, H., Bentz, J., and Szoka, F. C. (1986) *Biochemistry 25*, 4141−4147.
82. Gruner, S. M., Parsegian, V. A., and Rand, R. P. (1986) *Faraday Discuss. Chem. Soc. 81*, 29−37.
83. Hui, S.-W., and Sen, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5825−5829.
84. Sen, A., Isac, T. V., and Hui, S.-W. (1991) *Biochemistry 30*, 4516−4521.
85. Gruner, S. M. (1989) *J. Phys. Chem. 93*, 7562−7570.
86. Chen, S. Y., Cheng, K. H., and Van der Meer, B. W. (1992) *Biochemistry 31*, 3759−3768.
87. Cheng, K. H., Ruymgaart, L., Liu, L. I., Somerharju, P., and Sugar, I. P. (1994) *Biophys. J. 67*, 914−921.
88. Das, S., and Rand, R. P. (1984) *Biochem. Biophys. Res. Commun. 124*, 491−496.
89. Das, S., and Rand, R. P. (1986) *Biochemistry 25*, 2882−2889.
90. Lafleur, M., Bloom, M., Eikenberry, E. F., Gruner, S. M., Han, Y., and Cullis, P. R. (1996) *Biophys. J. 70*, 2747−2757.
91. Marsh, D. (1996) *Biophys. J. 70*, 2248−2255.
92. Baenziger, J. E., Jarrell, H. C., Hill, R. J., and Smith, I. P. C. (1991) *Biochemistry 30*, 894−903.
93. McCabe, M. A., Griffith, G. L., Ehringer, W. D., Stillwell, W., and Wassall, S. R. (1994) *Biochemistry 33*, 7203−7210.
94. Holte, L. L., Peter, S. A., Sinnwell, T. M., and Gawrisch, K. (1995) *Biophys. J. 68*, 2396−2403.
95. Separovic, F., and Gawrisch, K. (1996) *Biophys. J. 71*, 274−282.
96. Cevc, G., Seddon, J. M., Hartung, R., and Eggert, W. (1988) *Biochim. Biophys. Acta 940*, 219−240.
97. Boni, L. T., and Rando, R. R. (1985) *J. Biol. Chem. 260*, 10819−10825.
98. Altin, J. G., and Bradshaw, R. A. (1990) *J. Neurochem. 54*, 1666−1676.
99. van Veldhoven, P. P., and Bell, R. M. (1988) *Biochim. Biophys. Acta 959*, 185−196.
100. Whatley, R. E., Stroud, E. D., Bunting, M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1993) *J. Biol. Chem. 268*, 16130−16138.
101. Wolfman, A., and Macara, I. G. (1987) *Nature 325*, 359−361.
102. Ray, T. K., Skipski, V. P., Barclay, M., Essner, E., and Archibald, F. M. (1969) *J. Biol. Chem. 244*, 5528−5536.
103. Chien, K. R., Han, A., Sen, A., Buja, L. M., and Willerson, J. T. (1984) *Circ. Res. 54*, 313−322.
104. Rilfors, L., Hauksson, J. B., and Lindblom, G. (1994) *Biochemistry 33*, 6110−6120.
105. Rietveld, A. G., Chupin, V. V., Koorengevel, M. J., Wienk, J. L. J., Dowhan, W., and de Kruijff, B. (1994) *J. Biol. Chem. 269*, 28670−28675.
106. Yang, F. Y., and Hwang, F. (1996) *Chem. Phys. Lipids 81*, 197−202.
107. Cornell, R. B., and Arnold, R. S. (1996) *Chem. Phys. Lipids 81*, 215−227.
108. Bennett, C. F., and Crooke, S. T. (1987) *J. Biol. Chem. 262*, 13789−13797.
109. Nemenoff, R. A., Winitz, S., Qian, N. X., Van Putten, V., Johnson, G. L., and Heasley, L. E. (1993) *J. Biol. Chem. 268*, 1960−1964.

BI9719354