Effects of Diacylglycerols and Ca^{2+} on Structure of Phosphatidylcholine/Phosphatidylserine Bilayers

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ABSTRACT The combined effects of the diacylglycerols (DAGs) with the various acyl chains and Ca^{2+} on the structure of phosphatidylcholine/phosphatidylserine (4:1 mole/mole) bilayers were studied using ^2H– and ^{31}P NMR. The following DAG- and Ca^{2+}-induced bilayer perturbations were identified. 1) Increased tendency to form nonbilayer lipid phases was induced by diolein or stearoylarachidonoylglycerol, and was synergistically enhanced by the addition of Ca^{2+}. 2) "Transverse" bilayer perturbation was induced by dioctanoylglycerol. The addition of this DAG caused increased ordering of the phospholipid acyl side chains in the region adjacent to the headgroup, with the concomitant *decrease* of the order toward the bilayer interior. 3) Separation of the phosphatidylcholine and phosphatidylserine bilayer components was induced by combinations of relatively high (1:5 mole/mole to phosphatidylserine) Ca^{2+} and 25 mol% (to the phospholipids) of diolein, stearoylarachidonoylglycerol, or oleoylacetylglycerol. 4) Lateral phase separation of the bilayers on the regions of different fluidities was induced by dipalmitin. These physicochemical effects were correlated with the effects of these DAGs and Ca^{2+} on the activity of protein kinase C. The increased tendency to form nonbilayer lipid phases and the transverse bilayer perturbations correlated with the increased protein kinase C activity, whereas the actual *presence* of the nonbilayer lipid phases, as well as the separation of the phosphatidylcholine and phosphatidylserine components, was associated with the *decrease* in the protein kinase C activity. The lateral phase separation of the bilayer on gel-like and liquid crystalline regions did not have an effect on the activity of the enzyme. These results demonstrate the importance of the physicochemical properties of the membranes in the process of activation of protein kinase C.

INTRODUCTION

The regulatory enzyme, protein kinase C (PK-C),[1] is activated in vivo by endogenous lipid second messengers, 1,2-*sn*-diacylglycerols (DAGs), which are produced as a result of a stimulus-induced activation of phospholipase C (Berridge, 1987). Most current models of PK-C activation involve association of the enzyme with the lipid membranes (Nelsestuen and Bazzi, 1991; Bell and Burns, 1991; Zidovetzki and Lester, 1992), where it binds to acidic lipids, primarily phosphatidylserine, in a Ca^{2+}-dependent manner. The efficiency of this physical process would be expected to depend on physicochemical properties (e.g., phase, fluidity, acyl side chain order) of the lipid membranes.

Although the lipid bilayer structure has long been implicated as an important factor in determining protein-lipid interactions and activity of some intrinsic membrane proteins and membrane-active enzymes, notably the phospholipases (Wilschut et al., 1978; Menashe et al., 1981; Jain and Jahagirdar, 1985; Romero et al., 1987; Gheriani-Gruszka et al., 1988; Sen et al., 1991; Zidovetzki et al., 1992), the role of these properties in the activity of PK-C has not yet been clarified. DAGs also induce structural changes in lipid membranes, including alterations of membrane curvature, modification of surface charge (Ohki et al., 1982), and promotion of bilayer to nonbilayer phase transition (Dawson et al., 1984; Das and Rand, 1986; Epand, 1985; Epand and Bottega, 1988; Siegel et al., 1989). In one of the first studies addressing the membrane effects of DAGs, Dawson et al., (1984) correlated DAG-induced formation of hexagonal phases with activation of intracellular phospholipases A_2 and C. Das and Rand, (1986) using x-ray diffraction, also found that DAG induced hexagonal lipid phase and, additionally, caused dehydration of the bilayers and spreading of the phospholipid polar groups. These changes were interpreted as destabilization of bilayers and were proposed to be responsible for the ability of DAGs to activate PK-C and phospholipases. Both the degree of unsaturation and the length of the DAG fatty acid chain affect its capacity to activate PK-C (Kishimoto et al., 1980; Cabot and Jaken, 1984; Lapetina et al., 1985; Bonser et al., 1988). It was also demonstrated that variations in the hydrophobic part of the activating phospholipids affect both the lipid-dependent activation of PK-C and the action of DAG (Snoek et al., 1988). Thus, a number of studies indicate that PK-C activation by DAG may involve a specific DAG-induced perturbation of the structure of membrane phospholipid bilayer. In addition, many agents that affect lipid membrane structure are also

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[1]*Abbreviations used in this article:* DAG, diacylglycerol; diC_8, 1,2-*sn*-dioctanoylglycerol; DP, dipalmitin; DPPC, dipalmitoylphosphatidylcholine; DPPS, dipalmitoylphosphatidylserine; DP-d_{62}, diperdeuteriopalmitoylglycerol; DPPC-d_{62}, diperdeuteriopalmitoylphosphatidylcholine; DPPS-d_{62}, diperdeuteriopalmitoylphosphatidylserine; PdBu, phorbol dibutyrate; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PK-C, protein kinase C; OAG, 1-oleoyl, 2-acetyl-*sn*-glycerol; SAG, 1-stearoyl, 2-arachidonoyl-*sn*-glycerol.

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PK-C-active, which is consistent with the biological properties of the membranes being a factor in the PK-C activation process (Epand and Lester, 1990).

In the previous works from this laboratory, we characterized perturbations of phosphatidylcholine (PC) bilayers by a series of saturated and unsaturated DAGs and correlated these effects with DAG-induced modulation of the activities of phospholipases A_2 from different sources (De Boeck and Zidovetzki, 1989; 1992; Zidovetzki et al., 1992). We now have characterized the effects of the DAGs on PC/phosphatidylserine (PS) bilayers in the presence and absence of Ca^{2+} and correlated these effects with DAG-induced activation of PK-C.

MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC), diperdeuteriopalmitoylphosphatidylcholine (DPPC-d_{62}), dipalmitoylphosphatidylserine (DPPS), diperdeuteriopalmitoylphosphatidylserine (DPPS-d_{62}), diperdeuteriopalmitoylglycerol (DP-d_{62}), and phosphatidylcholine (PC) extracts from bovine liver were purchased from Avanti Polar Lipids (Birmingham, AL). 1,2-*sn*-dioctanoylglycerol (diC_8), 1-stearoyl, 2-arachidonoyl-*sn*-glycerol (SAG), and 1-oleoyl, 2-acetyl-*sn*-glycerol (OAG) were obtained from Serdary Research Laboratories (London, Ontario). Dipalmitin (DP) was from Sigma Chemical Co. (St. Louis, MO).

Multilamellar lipid dispersions were prepared by first dissolving phospholipid or the phospholipid-DAG mixture in chloroform. The solvent was then evaporated with a stream of dry nitrogen, and the sample was placed under a vacuum (<1 mtorr) for at least 8 h. The thin film thus formed was hydrated with 25 mM Tris [2-amino-2-(hydroxymethyl)-propane-1,3-diol] (pH 7.4) buffer solution, with $CaCl_2$ as required, prepared in ^2H-depleted H_2O (Sigma) for ^2H NMR experiments or in H_2O for ^{31}P NMR experiments. The samples were always fully hydrated and were typically 1:10 (w/v) in lipid/water. A uniform lipid suspension was obtained by three freeze-thaw cycles (Westman et al., 1982; Mayer et al., 1985). The composition of the phospholipids was: bovine liver PC/DPPC/DPPS = 3:1:1 (mol/mol/mol), giving the molar ratio of PC/PS as 4:1. For the ^2H NMR experiments either DPPC-d_{62} or DPPS-d_{62} was substituted for the corresponding nondeuterated lipid.

^{31}P- and ^2H NMR spectra were acquired at 11.74 T (corresponding to 500.13 MHz ^1H, 202.49-MHz ^{31}P, and 76.78-MHz ^2H frequencies) on a General Electric GN500 spectrometer. ^{31}P NMR spectra were obtained with a phase-cycled Hahn echo with a (70°-τ-70°)n pulse sequence (22-μs 70° pulse) and full phase cycling (Rance and Byrd, 1983). The 70° pulse was used instead of the more common 90° pulse, in order to compensate for the limited radio-frequency power. The pulse spacing was 30 μs, and the recycle delay was 1.5 s. Gated broad-band proton decoupling of 10 W was used. ^2H NMR spectra were acquired with a high-power probe (Doty Scientific, Columbia, SC) using the standard quadrupole echo sequence (Davis et al., 1976). The spectral width was 0.5–1 MHz, and refocusing time was 60 μs with a 90° pulse of 3.2 μs.

Two types of assays were used to measure PK-C activity: phosphorylation of the exogenous substrate histone 1, and binding to [^3H]phorboldibutyrate ([^3H]PdBu) (Amersham Corporation, Arlington Heights, IL, 20 Ci/mmol) as described previously (Lester, 1989, 1990a). Briefly, a mixture of PK-C isozymes was purified as described by Lester et al. (1990a). The lipids for the assays were prepared as described above for the NMR experiments, except that the final PS concentration was 100 μM, and Ca^{2+} concentrations were adjusted to the same molar ratios to PS as in the corresponding NMR experiments. In the histone 1 phosphorylation experiments $MgCl_2$ (5 mM), [^{32}P]ATP (18 μM, 1000 cpm/pmol) (Amersham, 5000 Ci/mmol), and histone 1 (Sigma, 16 μg/ml) were all added immediately before performing the assay, which was initiated by addition of PK-C (30 ng). Additional assays were performed at a 10-fold higher lipid concentration (1 mM). Assay time was 10 min at 35°C, and the reaction was terminated and the samples processed as previously described (Lester, 1990). [^3H]PdBu binding assays were performed as described above, except that 20 nM [^3H]PdBu was added. Total binding was determined in the absence of DAGs, and nonspecific binding was determined in the presence of a large excess of unlabeled PdBu (5 μM). Samples were incubated and processed as previously described (Lester, 1989; 1990).

RESULTS

The ^2H NMR spectra of DPPC-d_{62} or DPPS-d_{62} in PC/PS mixtures in the absence or presence of 25 mol% DAGs are shown in Fig. 1. A ^2H NMR spectrum of fully hydrated lipid bilayers above gel-to-liquid crystalline phase transition (T_c) is a superposition of the powder patterns arising from different deuterons of the perdeuterated acyl chains. The order parameter S_{CD} for each segment is related to the observed peak-to-peak quadrupole splittings ($\Delta\nu$) by the equation:

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

Where (e^2qQ/h) = 170 kHz is the quadrupole coupling constant of a deuteron in CD bond (Burnett and Muller, 1971). Previous studies demonstrated the existence of the order parameter profile along the acyl chains of the lipids in liquid crystalline phase with a plateau of high S_{CD} values corresponding to the side chain region of about eight CD_2 segments adjacent to the glycerol backbone (Seelig, 1977). The corresponding peaks overlap near the edge of a ^2H NMR spectrum (see peak #2, Fig. 1 *A*). Further from the glycerol backbone the lipid acyl chains are progressively more disordered, producing decreasing $\Delta\nu$ values and well resolved peaks with the smallest $\Delta\nu$ value corresponding to the terminal CD_3 group (peak #13, Fig. 1 *A*). Above T_c up to 13 peaks are resolved, and their tentative assignment to the CD_2 segments was given by Davis (1979).

Visual examination of the spectra in Fig. 1 shows that the basic bilayer structure of the PC/PS mixtures is maintained in the presence of 25 mol% DAGs. Moreover, nearly identical spectra were obtained using either DPPC-d_{62} (Fig. 1, *left*) or DPPS-d_{62} (Fig. 1, *right*) as a ^2H NMR label, indicating that the PC and PS components of the bilayers were well mixed both in the absence and in the presence of each of the DAGs studied. The presence of 25 mol% of DP caused the appearance of a broad component in both DPPC-d_{62} (Fig. 1 *K*) and DPPS-d_{62} (Fig. 1 *L*) spectra, superimposed in the narrower spectra with the well resolved peaks. The values of the quadrupole splitting of the narrow spectral components were virtually unaffected by the presence of DP (not shown). A similar effect of DP on DPPC and bovine liver PC bilayers was observed by us in a previous study and is indicative of lateral phase separation of gel phase-like DP-enriched phospholipid domains from the bulk phospholipids in the liquid crystalline phase (De Boeck and Zidovetzki, 1989). The absence of the change of the quadrupole splittings of the narrow spec-

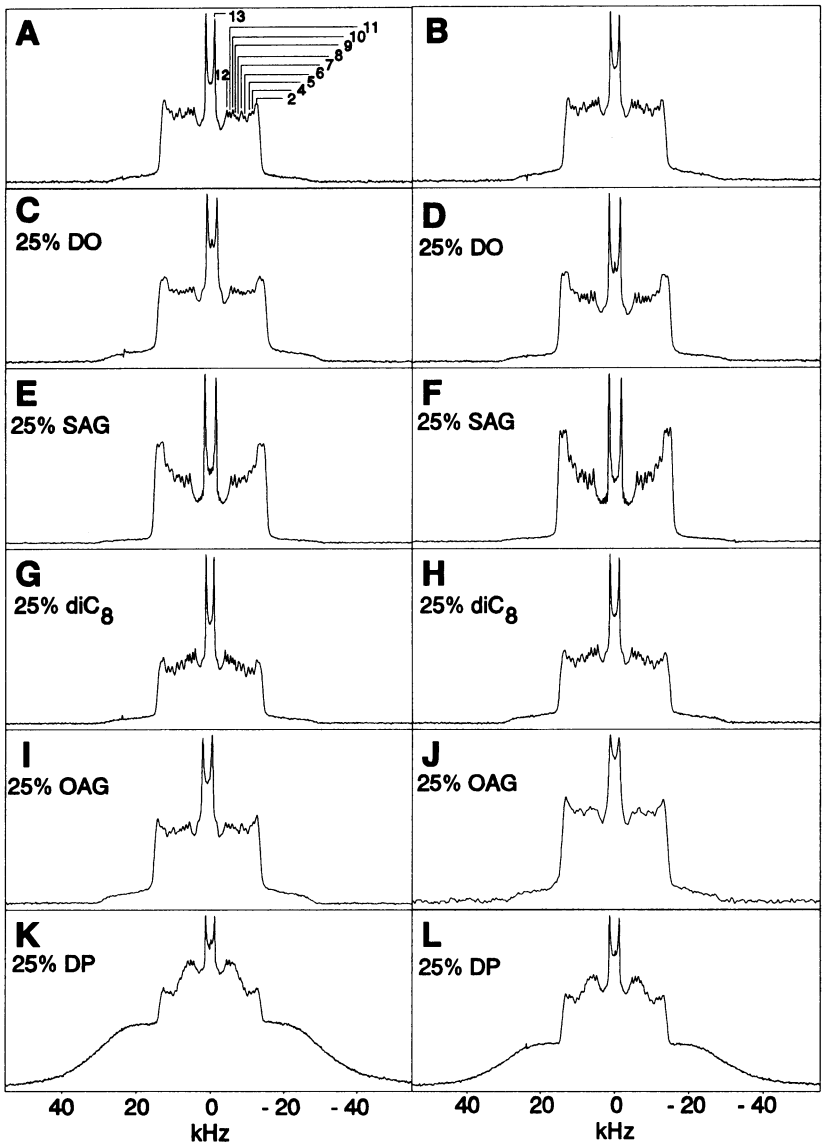

FIGURE 1 ^{2}H NMR spectra of DPPC-d_{62} (*left*) or DPPS-d_{62} (*right*) in PC/PS mixtures in the absence (*A*, *B*) or presence (*C-L*) of 25 mol% DAG at 37°C. The peaks are labeled on panel *A*; peaks 1 and 3 were resolved from peak 2 at higher temperatures.

tral components indicates that at 37°C DP is not miscible with PC/PS in the liquid crystalline phase. Similarity of the spectra obtained in the presence of DP with PC or PS probes (Fig. 1, *K* and *L*) indicates that DP interacts similarly with both lipid components. We further investigated miscibility of DP in PC/PS mixtures by using DP-d_{62} as a ^{2}H NMR probe. Under conditions identical with those of the experiments shown in Fig. 1, DP-d_{62} gave only broad ^{2}H NMR spectra in PC/PS mixtures, showing that, indeed, it does not mix with the lipids in the liquid crystalline phase (Fig. 2 *A*). Increasing the temperature resulted in a gradual decrease of the spectral intensities of the broad component for both DPPC-d_{62} and DPPS-d_{62} labels, indicating, as in our previous work (De Boeck and Zidovetzki, 1989), formation of one phase at about 60°C (Fig. 2 *C*). Under the same conditions DP-d_{62} also gave a narrow spectrum with well resolved peaks, which is consistent with miscibility with the phospholipids at this temperature (Fig. 2 *B*). Moreover, the quadrupole splittings of DP-d_{62} were nearly identical to those of DPPC-d_{62} at 60°C, but were considerably larger than the splittings of DPPC-d_{62} in the samples without DP at the same temperature (Fig. 3).

The effects of DO or SAG on the order parameters of DPPC-d_{62} in PC/PS mixtures are shown in Fig. 4. In all cases the order parameters of DPPS-d_{62} were nearly identical to those of DPPC-d_{62} for the corresponding samples (not shown). DO and SAG caused an increase of the order parameters along the phospholipid acyl side chains (Fig. 4). The relative order parameter increase was more pronounced toward the end of the chains (Fig. 4). This type of order parameter increase was previously described as a result of decrease in temperature, or addition of phosphatidylethanolamine or cholesterol to PC bilayers (Lafleur et al., 1990). As was discussed in our previous publication (De Boeck and Zidovetzki, 1989), such an effect suggests tighter packing and decreased amplitude of fluctuations of the acyl side chains in the presence of these DAGs, and it is probably a result of the interspacing of the DAG mole-

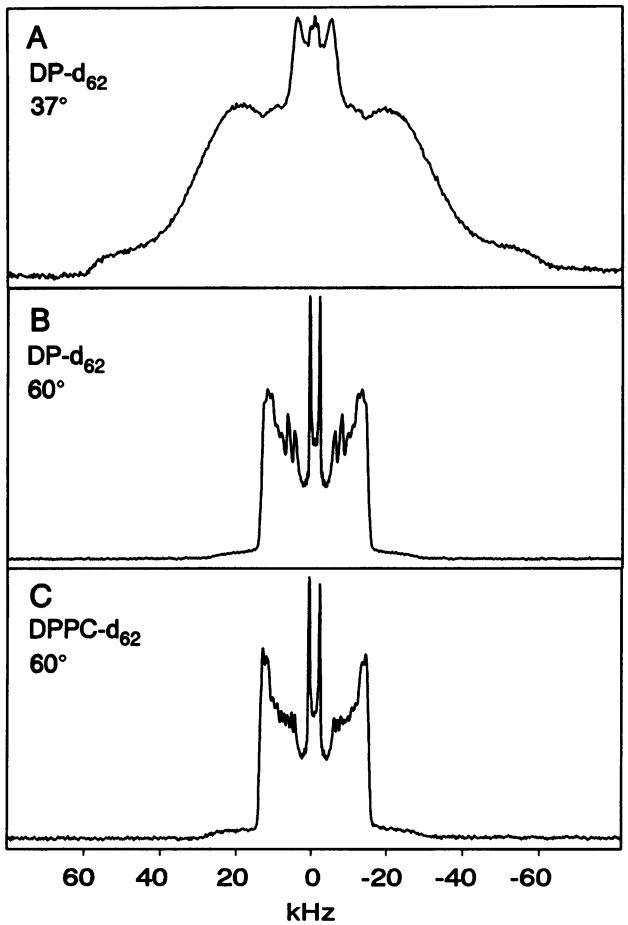

FIGURE 2 ^2H NMR spectra of DP-d_{62} (*A*, *B*), or DPPC-d_{62} (*C*) in PC/PS/25 mol% DP mixtures at different temperatures. See Fig. 1 *K* for the spectrum of DPPC-d_{62} in this mixture at 37°C.

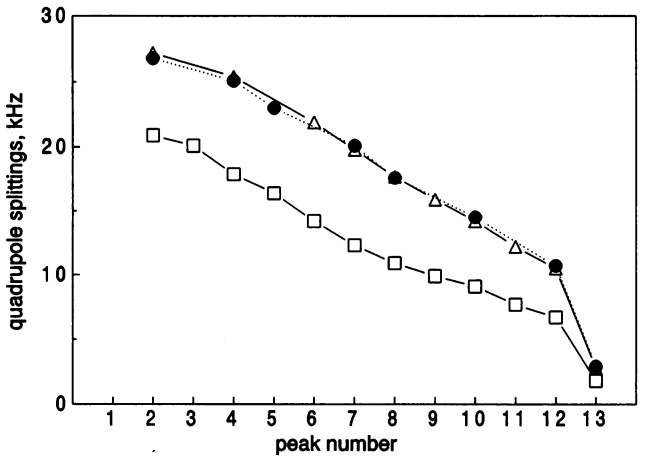

FIGURE 3 Plots of quadrupole splittings versus peak number of DPPC-d_{62} or DP-d_{62} in PC/PS mixtures at 60°C. The size of the symbols corresponds to the experimental error. (□) DPPC-d_{62} in PC/PS mixture without DP; (△) DPPC-d_{62} in PC/PS/25 mol% DP mixture; (●) DP-d_{62} in PC/PS/25 mol% DP-d_{62} mixture.

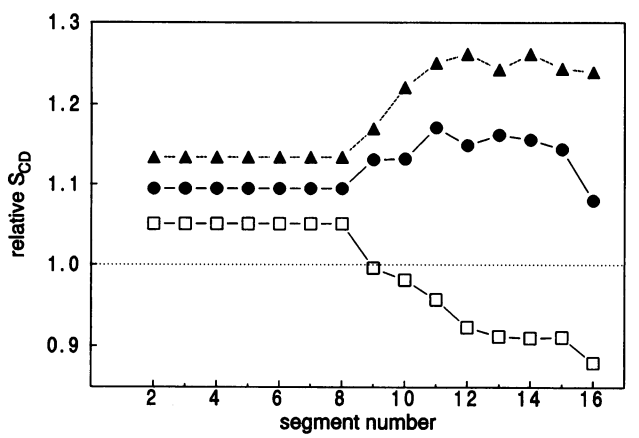

FIGURE 4 Effect of 25 mol% DAGs on order parameter profile of DPPC-d_{62} in PC/PS/DAG mixtures at 37°C. A relative S_{CD} is the ratio of segmental order parameters obtained in the presence of DAG to the corresponding values obtained in the absence of DAG. (□) with diC_8; (●) with DO; (▲) with SAG.

cules between the phospholipid molecules allowing for the tighter contact between the chains. In contrast, the presence of 25 mol% diC_8 resulted in an increase of the order parameters in the region of the lipid side chains adjacent to the phospholipid glycerol backbone with a concomitant decrease of the order parameters in the lower regions (Fig. 4). A similar effect was observed by us for diC_8 in DPPC and bovine liver PC (De Boeck and Zidovetzki, 1992) and was termed a "transverse" bilayer perturbation. A model of the diC_8 molecule in phospholipid bilayers (Zidovetzki and Lester, 1992) illustrates that the increase of the order parameters on the top of the chains is caused by tighter contact between the phospholipid side chains. Because of the short length of the diC_8 side chains, a free space is formed toward the middle of the bilayer, which is filled by increasing amplitude of the fluctuations of the phospholipid side chains. Addition of OAG did not have a significant effect on the order parameters of the phospholipid side chains (not shown).

Upon increase in temperature in the presence of 25 mol% DO, we observed formation of an "isotropic" nonbilayer lipid phase (Fig. 5, *left*). Similar spectra were obtained with DPPC-d_{62} (Fig. 5, *left*) or DPPS-d_{62} (not shown) indicating that the nonbilayer lipid phase is not formed preferentially with either PC or PS lipid component. A similar response was caused by the presence of 25 mol% SAG (not shown). Only spectra indicative of the bilayer phase were obtained with either 25 mol% diC_8 or OAG up to 60°C by both ^2H- and ^{31}P NMR measurements (not shown).

Addition of 5 mM Ca^{2+} (1:5 molar ratio to PS) to the PC/PS bilayers resulted in only a small effect on the ^2H NMR spectra of the bilayers (Fig. 6, *A* and *B*) and a small uniform increase in the $\Delta\nu_S$ of the side chains (not shown). A similar picture was observed by us previously in PC/phosphatidylglycerol (PG) mixtures (Zidovetzki et al., 1989).

Dramatic changes in the structures of PC/PS mixtures were observed in the presence of both DAG and Ca^{2+}. In the

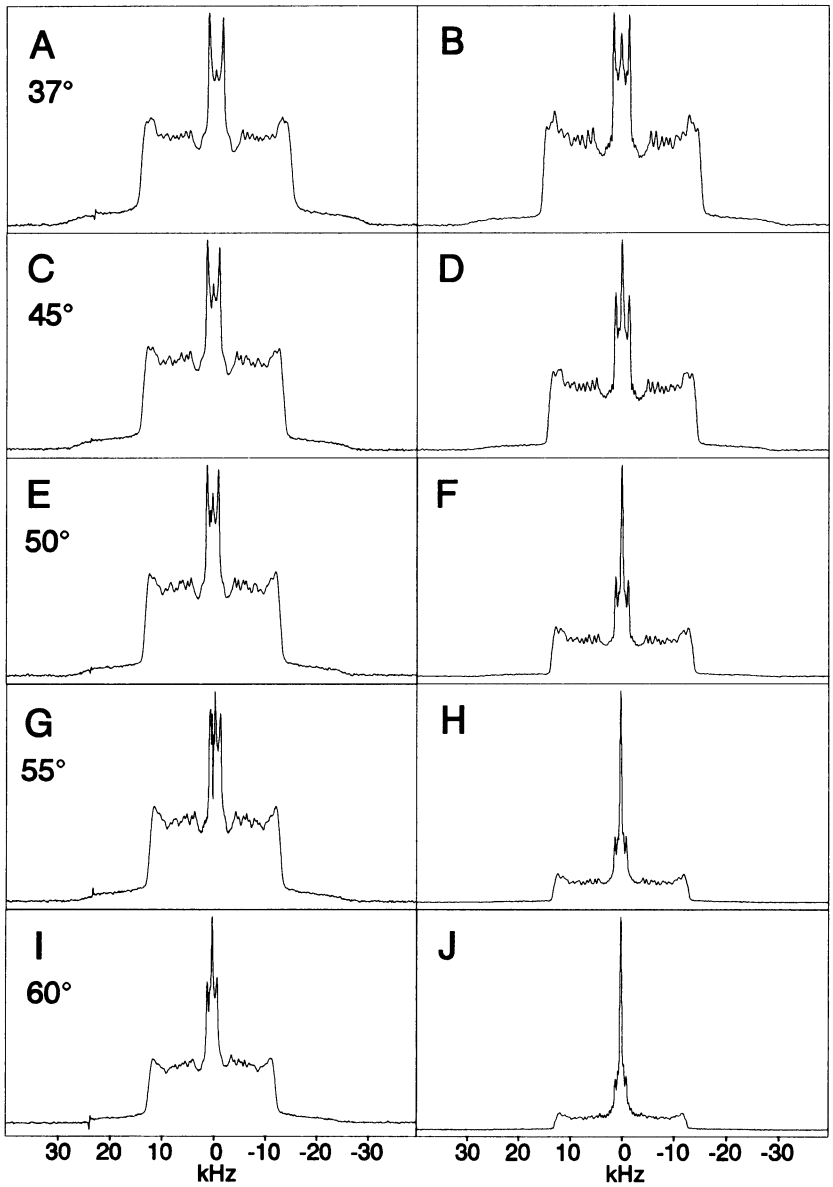

FIGURE 5 Effect of Ca^{2+} on the ^2H NMR spectra of DPPC-d_{62} in PC/PS/25 mol% DO mixtures at different temperatures. (*Left*) no Ca^{2+}; (*right*) with Ca^{2+} (at 1:10 molar ratio to PS).

case of DO and SAG, the spectra showed appearance of a central peak, which was especially prominent when observing the PS component (Fig. 6, *D* and *F*), indicating the presence of a nonbilayer lipid phase. ^{31}P NMR spectra of the samples indicated that the nonbilayer phase corresponds to the lipids in the "isotropic" (micelle or cubic), but not in the inverted hexagonal, phase (not shown). An even more complex picture was observed in the case of OAG, where the spectrum of the PS component was a superposition of the spectrum corresponding to the bilayer phase with an addition of a large central peak and a broad component (Fig. 6 *J*). ^{31}P NMR spectrum of this sample showed that this PC/PS/OAG/Ca^{2+} mixture consists of a complex mixture of phases, probably including gel-like and "isotropic" lipid phases (not shown).

In the case of diC_8, the ^2H NMR (Fig. 6, *G* and *H*) and ^{31}P NMR (not shown) spectra corresponded to the bilayer phase of both PC and PS components. Similar results were obtained when DPPC-d_{62} (Fig. 6 *G*) or DPPS-d_{62} (Fig. 6 *H*) was used as a ^2H NMR probe. The combined effects of diC_8 and Ca^{2+} on the acyl chain order parameters were similar to the effect of diC_8 alone. The combined effects of DP and Ca^{2+} resulted only in the broadening of the peaks of the narrow spectral component (Fig. 6, *K* and *L*).

We further investigated the synergistic effects of DO and Ca^{2+} by varying the concentrations of both cofactors. The effect was observable, when decreasing either DO or Ca^{2+} concentrations, as a tendency to form nonbilayer lipid phases at higher temperatures. Thus, in the presence of 25 mol% DO and 2.5 mM Ca^{2+} (1:10 molar ratio to PS), the nonbilayer lipid phase was observable already at 45°C, with the intensity similar to the one obtained without Ca^{2+} at 60°C (Fig. 5 *D*). Further increase of the temperature resulted in the increase of the relative amount of

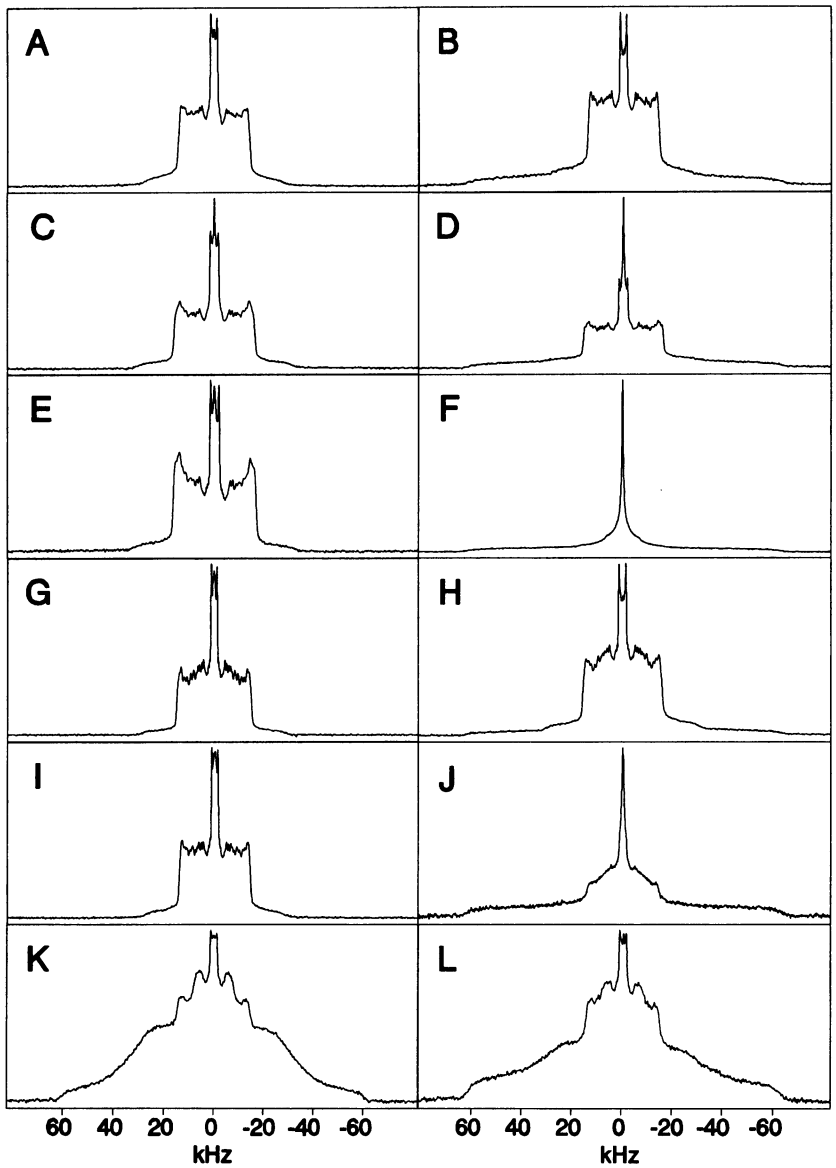

FIGURE 6 Combined effects of 25 mol% DAG and Ca^{2+} (1:5 molar ratio to PS) on ^2H NMR spectra of DPPC-d_{62} (*left*) or DPPS-d_{62} (*right*) in PC/PS mixtures at 37°C. (*A, B*) no DAG; (*C, D*) with DO; (*E, F*) with SAG; (*G, H*) with diC_8; (*I, J*) with OAG; (*K, L*) with DP.

the nonbilayer phase, reaching at 60°C a much higher ratio to the bilayer phase than in the absence of Ca^{2+} at the same temperature (Fig. 5 *J*). Similarly, addition of 15 mol% DO and 5 mM Ca^{2+} to the PC/PS bilayers resulted in the formation of a nonbilayer lipid phase at 45°C; the relative amount of this phase increased upon the increase of temperature (Fig. 7). In the absence of Ca^{2+}, the presence of 15 mol% DO did not cause formation of such phases up to the temperature of 60°C.

To correlate the observed effects of DAGs and Ca^{2+} on the lipid bilayer structure with their role as cofactors in activation of PK-C, we performed specific assays of PK-C activity under conditions similar to those used in the NMR experiments. Because the enzymatic assays use lower lipid concentrations than those required for the NMR experiments, the molar *ratios* of PC, PS, DAG, and Ca^{2+} were kept constant in both PK-C assays and the NMR measurements. It was shown by Hannun and Bell (1990) in micellar systems, and was observed by Lester with the unsonicated lipid system (Lester, unpublished observations) that PK-C responds to the mole ratio of lipid activators and inhibitors rather than to the total bulk concentration of these molecules. Moreover, due to the high affinity of Ca^{2+} for PC/PS bilayers (Feigenson, 1989), under our conditions the invariant Ca^{2+}/PS ratio criterion is equivalent to invariant free Ca^{2+} concentration, which was shown to define the phase behavior of these systems (Feigenson, 1989). The validity of using the molar ratios as the criterion was tested in both PK-C assays, where increasing lipid and Ca^{2+} concentrations by a factor of 10 resulted in the same relative potencies of the DAGs to increase PK-C activity (data not shown), and in NMR experiments where *decreasing* the concentrations by a factor of 5 did not change the picture of the synergistic effects of DAGs and Ca^{2+}. The example of such an NMR measurement at the lower concentrations of the lipids and Ca^{2+} is shown in

Fig. 8, where the synergism of DO and Ca^{2+} is prominent at 1 mM Ca^{2+}, but at the same molar ratio of Ca^{2+} to PS (1:5) as in other NMR measurements.

The effects of the DAGs and Ca^{2+} on the activity of PK-C are summarized in Table 1. A noticeable feature of the data in the Table 1 is that activation of the enzyme is induced by high concentrations of DAGs in the absence of Ca^{2+}. In all cases (except for DP) the activity of PK-C was monotonously increasing with increasing DAG concentration, as demonstrated for DO (Fig. 9).

Interestingly, with SAG and with 25 mol% DO or OAG, the addition of Ca^{2+} (1:5 molar ratio to PS) actually caused a *decrease* in the PK-C activity (Table 1). We further investigated this phenomenon by measuring the dependence of PK-C activity on Ca^{2+} concentrations at 15 and 25 mol% DO (Fig. 10) and SAG (not shown). For 15 mol% DO, Ca^{2+} at lower concentration (1:10 molar ratio to PS) caused an increase of PK-C activity; further additions of Ca^{2+} caused a small decrease of the PK-C activity (Fig. 10). The effect of Ca^{2+} with 15 mol% SAG was qualitatively similar, except that the decrease of PK-C activity with increasing Ca^{2+} concentration was more pronounced (Table 1). In contrast, in the presence of 25 mol% DO, already at Ca^{2+}/PS molar ratio of 1:10, there was a strong decrease in the PK-C activity, and further additions of Ca^{2+} resulted in a linear decrease of the activity (Fig. 10). Similar dependence of PK-C activity on Ca^{2+} concentration was observed in the case of SAG (not shown).

Phorbol ester competition binding assays demonstrated that the efficiency of the DAGs to displace [^{3}H]PdBu from PK-C correlated with their efficiency as PK-C activators (Table 2).

TABLE 1 Dependence of PK-C activity on the DAG species and the presence or absence of CA^{2+}

	PK-C activity, μmol/mg·min			
	15 mol% DAG		25 mol% DAG	
DAG*	no Ca^{2+}	with Ca^{2+}‡	no Ca^{2+}	with Ca^{2+}‡
DO	0.37 ± 0.01	0.58 ± 0.03	0.73 ± 0.04	0.53 ± 0.06
SAG	0.59 ± 0.02	0.08 ± 0.01	0.79 ± 0.02	0.03 ± 0.01
diC_8	0.17 ± 0.01	0.25 ± 0.03	0.38 ± 0.02	0.35 ± 0.03
OAG	0.13 ± 0.01	0.20 ± 0.02	0.27 ± 0.03	0.18 ± 0.01
DP	≤0.01	≤0.01	≤0.01	≤0.01

* The PK-C activity without DAGs was less than 0.01.
‡ At a molar ratio of 1:5 to PS.

TABLE 2 Inhibition of [^{3}H]PdBu binding to PK-C by DAGs (25 mol%) in the absence of Ca^{2+}

DAG	[^{3}H]PdBu Bound
	pmol/mg
Control	82 ± 3
DO	4 ± 1
SAG	≤1
diC_8	7 ± 2
OAG	6 ± 3
DP	76 ± 11

DISCUSSION

The present work was initiated to extend our previous studies on the role of DAG-induced perturbations of PC bilayers in the mechanism of activation of phospholipases A_2 (De Boeck and Zidovetzki, 1989, 1992; Zidovetzki et al., 1992) to include the lipid system that contains the cofactors required for the activation of another membrane-associated enzyme, PK-C. Our results have shown that at high DAG content, maximal activation of PK-C is obtained without Ca^{2+}. Walker et al. (1990) and Epand et al. (1992) were also able to achieve PK-C activation without Ca^{2+} by changing the composition of the phospholipid cofactors. Both groups suggested that localized alterations in the physical state of the cellular membrane would facilitate PK-C association/activation in a Ca^{2+}-independent manner. Our observation that unsaturated and short chain-saturated DAGs activate PK-C, whereas the long-chain DP does not, agrees with the studies of Kishimoto et al. (1989) and Sekiguchi et al. (1988), who used the bilayer form of the lipids in their PK-C assays, but not with the results of Schaap et al. (1990), who used the mixed micelle assays and observed significant activation of PK-C by DP. This discrepancy emphasizes the importance of using multibilayers in studying the effect of modulators on PK-C activity (Boni, 1992). Much of the data available on the PK-C activation were obtained in the presence of detergents, using the mixed-micelle system, which eliminates the effect of the physical properties of the lipids on the activation process. These studies have an inherent assumption that the relevant recognition sites are the same in mixed micelle, sonicated vesicles, or essentially planar plasma membranes. Some recent studies, however, questioned this assumption, suggesting that the mechanism of activation of PK-C is different depending on the nature of the physical state of the lipid cofactors (i.e., mixed micelle, sonicated or nonsonicated vesicles) (Snoek et al., 1988; Lee and Bell, 1989; Bazzi et al., 1992; Sando et al., 1992). These differences may be due to the profound differences in the physicochemical structure among the lipids in micelles, sonicated and nonsonicated vesicle forms (Dill and Flory, 1981). We used nonsonicated lipid dispersions, which provide a better model to investigate the dependence of PK-C activity, cofactor requirements, and substrate specificity on the physicochemical properties of the membranes (Boni, 1992). To clearly observe the biophysical effects, rather high concentrations of DAGs were used. The obtained information, however, should be useful in understanding the effects of low physiological DAG concentrations (Zidovetzki and Lester, 1992).

Using this model membrane system, we have observed the following types of the PC/PS bilayer perturbations, induced by the DAGs, or by DAG-Ca^{2+} combinations: 1) Interspacing of phospholipid molecules (induced by all

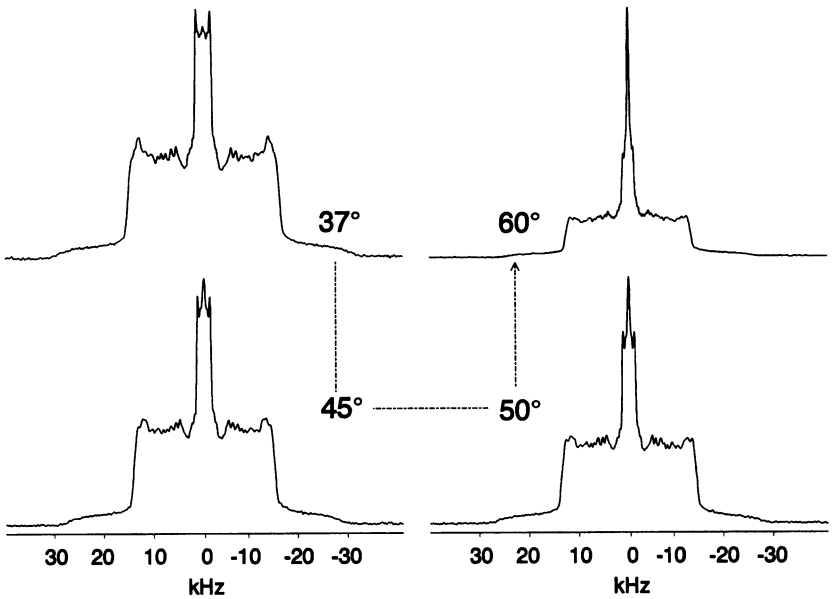

FIGURE 7 Combined effects of 15 mol% DO and Ca^{2+} (1:5 molar ratio to PS) on ^2H NMR spectra of DPPC-d_{62} in PC/PS mixture at different temperatures.

DAGs except DP); 2) "transverse" perturbation of the bilayer structure (induced by diC_8); 3) tendency to form nonbilayer lipid phases (induced by DO and SAG with or without Ca^{2+}); 4) tendency for PC/PS separation (induced by Ca^{2+} and DO, SAG or OAG)–note that the optimal PK-C activity correlates with the DAG-Ca^{2+} concentrations just below of those which cause PC/PS separation; 5) lateral separation of the bilayers on the gel-like and liquid crystalline phases, both containing PC and PS components (induced by DP and DP-Ca^{2+}). We will discuss below how each of these bilayer effects correlates with PK-C activity.

Interspacing of the phospholipids.

DAGs, which are miscible with the phospholipids in the liquid crystalline phase, "dilute" the phospholipids, thereby providing the spacing between the phospholipid headgroups. This effect, exhibited by all DAGs except DP, is probably a "minimal requirement" for the DAG-induced PK-C activation. The weakest PK-C activator, OAG, exhibited this effect only on the bilayer structure, whereas additional bilayer perturbations were induced by other activating DAGs. DP, which forms gel-like complexes with the phospholipids, laterally phase separates from the bulk lipids in the liquid crystalline phase and thus does not provide interspacing for the *bulk* lipids. This lack of miscibility of DP in the liquid crystalline phase provides an explanation for the inability of this DAG to displace [^3H]PdBu from PK-C. Thus, solubility in the liquid crystalline phase is a necessary requirement for a DAG to be a PK-C activator, as was suggested previously by Ortiz et al. (1988), who commented that the common theme in effects of unsaturated and short-chain saturated DAGs is miscibility with and, consequently, perturbation of the *bulk* phospholipid. The interspacing of the phospholipid molecules by DAGs as a factor in PK-C activation was also suggested by Lester (1990) and Bolen and Sando (1992).

"Transverse" perturbation of the bilayer.

This effect, induced by diC_8, consists of the DAG-induced increase of the order parameters of the phospholipid side chains in the region adjacent to the glycerol backbone, with the concomitant *decrease* of the order in the lower regions of the chains, close to the bilayer interior. This type of perturbation was previously observed by us with PC bilayers (De Boeck and Zidovetzki, 1992), and it correlated with the activation of bee and cobra venom phospholipases A_2 (Zidovetzki et al., 1992). As in the previous case, we may conclude that activation of PK-C by diC_8 correlates with the increased structural fluctuations in the bilayer interior, which allows for easier penetration of the enzyme into the bilayer, as previously suggested (Zidovetzki and Lester, 1992). This type of enzyme penetration may be different from that proposed to occur in the formation of the membrane-associated (particulate) PK-C form (Lester, 1992).

Tendency to form nonbilayer lipid phases.

This tendency was induced by DO and SAG and was synergistically enhanced by Ca^{2+}. Formation of the nonbilayer lipid phases by DAG-containing phospholipids was previously demonstrated in various DAG/phospholipid systems (Dawson et al., 1984; Epand, 1985; Das and Rand, 1986; Epand and Bottega, 1988; De Boeck and Zidovetzki, 1989; Gómez-Fernández et al., 1989; Siegel et al., 1989). A correlation of the enzymatic activity with the tendency to form nonbilayer lipid phases was demonstrated in the case of phospholipases A_2 (Dawson et al., 1984; Sen et al., 1991; Zidovetzki et al., 1992). Epand and co-workers (Epand,

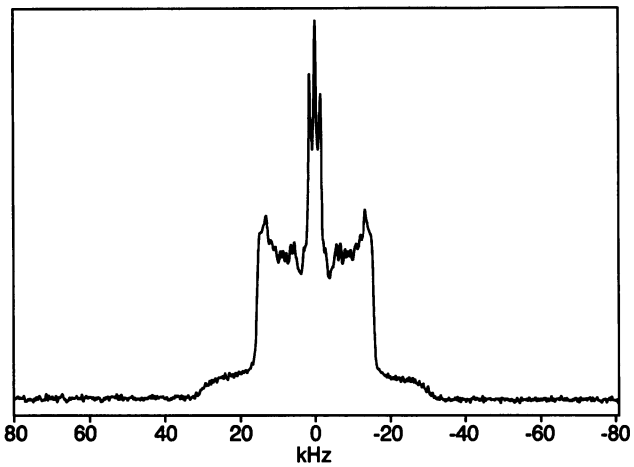

FIGURE 8 ^2H NMR spectrum of DPPC-d_{62} in PC/PS/25 mol% DO mixture with Ca^{2+} (1:5 molar ratio to PS) at 37°C. The lipid and Ca^{2+} concentrations are 5 times lower than those used to obtain the spectrum on Fig. 6 *C*.

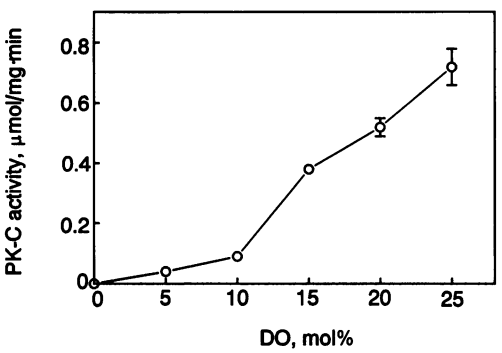

FIGURE 9 Dependence of PK-C activity on the concentration of DO added to PC/PS mixtures in the absence of Ca^{2+}.

1985; Epand and Bottega, 1988) postulated that DAG-induced *propensity* of the membranes to form nonbilayer lipid phases (but not necessarily the actual presence of these phases) can be associated with increased PK-C activity. The propensity to form nonbilayer lipid phases is observable as a decrease of the bilayer-nonbilayer phase transition temperature, and it reflects decreased stability of the bilayers at the temperature of PK-C activity assays. Indeed, we observed optimal PK-C activation under conditions of a minimal presence of the nonbilayer lipid phases, i.e., in cases of DO or SAG without Ca^{2+}, or 15 mol% SAG or DO and low (1:10 to PS) Ca^{2+} concentration. Addition of Ca^{2+} to the samples containing 25 mol% SAG or DO resulted in the formation of nonbilayer lipid phases at the temperature of the PK-C activity measurements and correlated with the *decreased* PK-C activity. A detrimental effect of altered bilayer phases on PK-C activation was also reported by Boni and Rando (1985).

Bolen and Sando (1992) observed much more efficient PK-C activation by DO when phospholipids with unsaturated acyl chains were used as lipid support. Even more

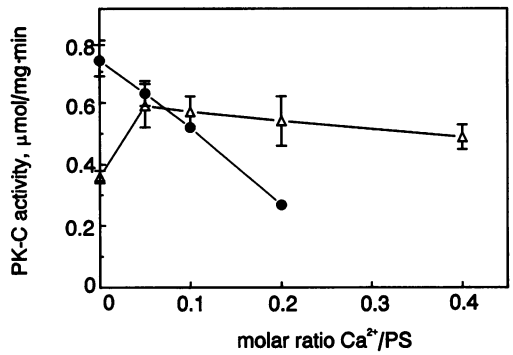

FIGURE 10 Dependence of PK-C activity on Ca^{2+} concentration in the presence of 15 mol% DO (△), or 25 mol% DO (●).

pronounced differences between the effects of DO on enzymatic activity as a function of phospholipid acyl chain unsaturation were observed in the case of phospholiase A_2: DO inhibited this enzyme with the fully saturated DPPC substrate, but activated it with the unsaturated substrate (Zidovetzki et al., 1992). These observations could be explained by the finding that DO increases the tendency to form nonbilayer lipid phases only when added to unsaturated phospholipids (De Boeck and Zidovetzki, 1989). It was previously concluded that the effect of unsaturation is due to some physical property of the lipid bilayer rather than to a specific protein-lipid interaction (Lester, 1990; Bolen and Sando, 1992). Because the activity was not affected by cholesterol (Bolen and Sando, 1992), this property is not the fluidity of the bilayers. Our results suggest that this property is related to the increased tendency of DO-containing membranes to form nonbilayer lipid phases. We have suggested previously that the tendency to form nonbilayer lipid phases may be associated with increased bilayer fluctuations (De Boeck and Zidovetzki, 1989; Zidovetzki and Lester, 1992). Because DO and SAG *increase* order parameters of the phospholipid acyl chains, such fluctuations would occur on a time scale slower than that of the ^2H NMR quadrupole splitting measurements.

DAG-Ca^{2+}-induced tendency for PC/PS separation.

This effect was induced by 25 mol% DO, SAG, or OAG and high (1:5 to PS) Ca^{2+} concentration. A similar type of effect was observed in an earlier study by Ohki et al. (1982) at much lower PC/PS ratio than the ratio used presently (4:1), which closely resembles the ratio of neutral to acidic lipids in many biological membranes. Ca^{2+} alone can cause lateral phase separation of PS (McLaughlin et al., 1981; Feigenson, 1986; Huang et al., 1993); however, our results show that the presence of DAG significantly reduces the Ca^{2+} requirement for this process. Moreover, DO lowers the threshold for molar ratio of PS to PC, sufficient for the PC/PS separation. A previous study established that a minimum of 30% PS should be present to allow Ca^{2+}-induced PC/PS separation (Silvius, 1990). In the presence of DO this threshold was found to be 25 mol%

by Bolen and Sando (1992), or 20 mol% in this study. In recent studies, using mixed micelle assay and saturating (in respect to PS) concentration of Ca^{2+}, Orr and Newton (1992a, b) suggested that DAG and Ca^{2+} increase PK-C affinity for PS by laterally separating this lipid and creating PS-enriched domains. Orr and Newton (1992a) proposed that PK-C cooperatively sequesters PS around its membrane-interacting domain. Bazzi and Nelsestuen (1991) also showed that PK-C clusters acidic lipids upon membrane binding. We found that even in the absence of PK-C, addition of a *subsaturating* (with respect to PS) amount of Ca^{2+} and DAG synergistically causes lateral separation of PS from PC in PC/PS multi-bilayers. This process may promote PK-C-induced aggregation of PS. Importantly, Lester (1990) and Bolen and Sando (1992) suggested that the ability of the acidic lipids to phase separate limits optimal PK-C activation. This agrees with our observations that under conditions where PS is phase-separated (high Ca^{2+}), the PK-C activity *decreases.* This effect was most prominent in the case of SAG (Fig. 6, *E* and *F*) and correlated with the largest drop of PK-C activity upon addition of Ca^{2+} in the presence of this DAG (Table 1). The only PK-C-activating DAG that did not cause PC/PS separation in the presence of Ca^{2+} was diC_8; it was also the only DAG for which the addition of Ca^{2+} to 1:5 molar ratio to PS did not cause a decrease in the PK-C activity.

It was shown that PS, but not other acidic lipids, exposes the pseudosubstrate autoinhibitory domain of PK-Cβ (Orr et al., 1992). Furthermore, Orr and Newton (1992b), by observing differences in the effects of PG and PS in PK-C activation, concluded that PK-C has at least one binding site that is specific for PS. Both of these effects can be at least partially due to the unique properties of PS with respect to its interaction with PC, DAG, and Ca^{2+} cofactors. Our results point out the different effects of Ca^{2+} on PC/PG (Zidovetzki et al., 1989) or PC/PS (present study) mixtures. Only in the latter case did saturating concentrations of Ca^{2+} cause the sequestration of the acidic lipid component. Thus, purely physicochemical differences between PC/PG and PC/PS mixtures may contribute to the PK-C requirement for PS, but not PG, lipid cofactor.

Lateral phase separation of the bilayers on gel-like and liquid crystalline phases.

This effect, induced by DP and DP-Ca^{2+}, is apparently not relevant for PK-C activation. This result was somewhat surprising, because such phase co-existence must be accompanied by the defects of the bilayer structure on the boundary between the gel-like and fluid domains. This type of defect was previously shown by us to be the most efficient in activating extracellular phospholipases A_2 (Zidovetzki et al., 1992). The observed lack of activation of PK-C by this type of bilayer defect supports the tentative conclusions of Snoek et al., (1988), who did not detect lateral phase separation in the PK-C-activating lipid bilayers. More recently, Senisterra and Epand (1993) also reported the lack of effect of phase boundaries on PK-C activation.

CONCLUSIONS

The simplest explanation of the available data is that the variations in the efficacy of different 1,2-*sn*-DAG species to activate PK-C are due to the differences in DAG-induced membrane bilayer perturbations. PK-C activation can be associated with increased fluctuations in the *bulk* ("fluid") lipid phase. Indeed, Cheng et al. (1991) suggested that the number of phospholipid conformational states in phosphatidylethanolamine/DO mixtures increases with increasing DO content. Souvignet et al. (1991) also suggested that DAG activates PK-C through local changes in the surrounding lipid phase organization. A diagnostic feature of such increased fluctuations is the increased tendency of the bilayers to form nonbilayer lipid phases. The facilitated penetration of PK-C into monolayers upon addition of DO observed by Lester et al. (1990b) may be a consequence of such increased bilayer fluctuations. It follows that the agents that attenuate the DAG-induced bilayer perturbations would inhibit PK-C activity. Indeed, we have recently found that the amphiphilic drug chloroquine, although not interacting strongly with the phospholipids, prevents SAG-induced formations of the nonbilayer lipid phases (Zidovetzki et al., 1993) and inhibits PK-C (Lester et al., unpublished observations).

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