Correlation between Protein Kinase C α Activity and Membrane Phase Behavior

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ABSTRACT Lipid activation of protein kinase C α (PKC α) was studied by using a model mixture containing 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS), and 1,2-dimyristoyl-*sn*-glycerol (1,2-DMG). This lipid mixture was physically characterized by differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR), and ^{31}P-nuclear magnetic resonance (^{31}P-NMR). Based on these techniques, a phase diagram was constructed by keeping a constant DMPC/DMPS molar ratio of 4:1 and changing the concentration of 1,2-DMG. This phase diagram displayed three regions and two compounds: compound 1 (C_1), with 45 mol% 1,2-DMG, and compound 2 (C_2), with 60 mol% 1,2-DMG. When the phase diagram was elaborated in the presence of Ca^{2+} and Mg^{2+}, at concentrations similar to those used in the PKC α activity assay, the boundaries between the regions changed slightly and C_1 had 35 mol% 1,2-DMG. The activity of PKC α was studied at several temperatures and at different concentrations of 1,2-DMG, with a maximum of activity reached at 30 mol% 1,2-DMG and lower values at higher concentrations. In the presence of Ca^{2+} and Mg^{2+}, maximum PKC α activity occurred at concentrations of 1,2-DMG that were close to the boundary in the phase diagram between region 1, where compound C_1 and the pure phospholipid coexisted in the gel phase, and region 2, where compounds C_1 and C_2 coexisted. These results suggest that the membrane structure corresponding to a mixture of 1,2-DMG/phospholipid complex and free phospholipid is better able to support the activity of PKC α than the 1,2-DMG/phospholipid complex alone.

INTRODUCTION

Protein kinase C (PKC) is a family of enzymes that has attracted great interest because of its regulatory character and because it has been involved in the modulation of a wide variety of cellular processes, such as cell signaling and tumor promotion (reviewed in Newton, 1995, and in Nishizuka, 1995). Some of the isozymes that form this family are activated by diacylglycerols (DAGs), phosphatidylserine (PS), and Ca^{2+}. We will address our attention to the α-isoenzyme belonging to the group of "conventional" PKCs. Because these PKC isoenzymes are activated in the cell through their interaction with membranes, it can be speculated that this activation is produced by allosteric effects of PS, DAG, and Ca^{2+} on the enzyme or that the modulation of the membrane structure by PS, DAG, and Ca^{2+} is also important for the activation process (reviewed by Zidovetzki and Lester, 1992, and Newton, 1993).

There is a great deal of information which suggests that the membrane structure is modulated by the presence of DAGs. For example, it has been shown that DAGs may produce structural changes in membranes, such as lateral phase separations (Ortiz et al., 1988; De Boeck and Zidovetzki, 1989; Heimburg et al., 1992; López-García et al., 1994b; Goldberg et al., 1994; Dibble et al., 1996), nonbilayer phases (Das and Rand, 1986; Cheng and Hui, 1986; De Boeck and Zidovetzki, 1989; Heimburg et al., 1992; López-García et al., 1994a), and dehydration of the membrane interface (López-García et al., 1993, 1994a). Significantly, the dehydration produced by DAG is more drastic for phosphatidylserine than for phosphatidylcholine (López-García et al., 1994a). These effects may be responsible for facilitating membrane fusion (Siegel et al., 1989; Ortiz et al., 1992; Nieva et al., 1989; van Gorkom et al., 1992; Sánchez-Migallón et al., 1995) and perhaps for the activation not only of PKC but also of other enzymes, such as phospholipases (Dawson et al., 1983; Roldan and Fragio, 1994; Zidovetzki et al., 1992), CTP:phosphocholine cytidyltransferase (Arnold and Cornell, 1996), and tyrosine kinase (Arnold and Newton, 1996).

In addition, it has been widely suggested that PKC may be modulated by DAGs. For example, it has been suggested that the activity of PKC increases in correlation with the tendency of lipids to give nonbilayer phases, as may occur in regions of high bilayer curvature produced by molecules such as diacylglycerols or phosphatidylethanolamines, which have small polar headgroups (Das and Rand, 1986; Epand, 1985; Epand and Bottega, 1988; Goldberg et al., 1994). It has also been pointed out that the presence of DAGs will increase the spacing between phospholipid headgroups because of the interposition of the small groups of DAGs (Das and Rand, 1986; Cunningham et al., 1989; Epand, 1987; Bolen and Sando, 1992; Slater et al., 1994). It is possible that the dehydration of the membrane surface induced by DAGs might facilitate immersion of the protein

Received for publication 18 February 1998 and in final form 8 October 1998.

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0006-3495/99/02/916/12 $2.00

in the membrane (López-García et al., 1993). Finally, it has recently been suggested that membrane heterogeneity, with the coexistence of rich and poor phases in DAGs, may contribute to activation of PKC (Dibble et al., 1996).

In this work we will examine the phase behavior of DMPC/DMPS/1,2-DMG mixtures in the absence and presence of the same Mg^{2+} and Ca^{2+} concentrations used for PKC activation. This phase behavior will be correlated with the PKC activity observed at different diacyglycerol concentrations and temperatures.

MATERIALS AND METHODS

Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and its perdeuterated homolog, DMPC-d_{54}, were purchased from Avanti Polar Lipids (Birmingham, AL). 1,2-Dimyristoyl-*sn*-glycerol (1,2-DMG) and its perdeuterated homolog, 1,2-DMG-d_{54}, were prepared from their respective phosphatidylcholines by the action of phospholipase C (*Bacillus cereus*; Boehringer-Mannheim, Barcelona) in ether:water (4:1, v/v) at 4°C for 5 h before being extracted from the ether phase. The purity of each diacylglycerol was determined by thin-layer chromatography on silica gel 60 plates (Macherey-Nagel, Dürren, Germany), using chloroform:acetone:methanol (94.5:5.0:0.5, v/v) as a solvent. Trypsin was obtained from Boehringer-Mannheim. Water was twice distilled and deionized in a Millipore system from Millipore Ibérica (Madrid).

Expression and purification of protein kinase C α

The recombinant baculovirus encompassing the full-length cDNA for the porcine protein kinase C α was kindly provided by Dr. Robert M. Bell of the Duke University Medical Center (Durham, NC). Porcine PKC α was expressed in Sf9 insect cells by infection with a high-titer recombinant baculovirus and purified to homogeneity from the cytosolic fraction. Purification was performed as previously described (Burns et al., 1990), with slight modifications. A 2-liter culture of Sf9 insect cells at 3×10^6 cells/ml was infected with the recombinant baculovirus. Cells were harvested 60 h postinfection (cell viability of 70%), pelleted at $1500 \times g$ for 10 min, and suspended in homogenization buffer (20 mM Tris pH 7.5, 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.001% leupeptin, 100 μM $NaVO_3$, 50 mM NaF). The pellet was disrupted by sonication (6×10 s), and the resulting lysate was centrifuged at $100{,}000 \times g$ for 60 min. The supernatant was applied in a batch mode to a 100-ml diethylaminoethyl Sephacel column equilibrated with elution buffer (20 mM Tris pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM β-mercaptoethanol), and bound proteins were eluted by the application of a linear gradient (0–0.5 M NaCl) at a flow rate of 1 ml/min. The fractions containing PKC were then applied to a 10-ml protamine agarose column, at a flow rate of 0.4 ml/min, and equilibrated with elution buffer before being eluted with a linear gradient from 0.3 to 1.5 M NaCl. Fractions containing PKC α activity were pooled and loaded onto a 10-ml phenyl-Sepharose column, and PKC was then eluted in the same buffer with a descending linear salt gradient (1–0 M NaCl). Pure PKC α was obtained, as seen by a silver-stained sodium dodecyl sulfate-polyacrylamide gel. The protein was stored at −80°C in the presence of 10% glycerol and 0.05% Triton X-100.

Determination of protein kinase C activity

Lipids to be used for the reaction were dried under a stream of N_2, and the last traces of organic solvent were removed by keeping the samples under vacuum for 2 h. The lipids were suspended in 20 mM Tris-HCl (pH 7.5), 0.05 mM EGTA and vortexed vigorously. The multilamellar vesicles formed gave more reproducible results in the kinase assay and were used for all of the assays described. Liposomes were added to the reaction to give a final concentration of total phospholipid of 480 μM, and the diacylglycerol was varied from 0 to 45%. A 50-μl sample of the dispersed lipids was added to the reaction vial (final volume 250 μl), containing 20 mM Tris-HCl (pH 7.5), 0.2 mg/ml histone III-S, 20 μM [γ-^{32}P]ATP (300,000 cpm/nmol), 5 mM $MgCl_2$, and 200 μM $CaCl_2$. The reaction mixture was incubated for 2 min at the desired temperature, and the reaction was started by the addition of 25 μl (0.5 μg) of the diluted enzyme. The reactions performed between 15°C and 20°C were terminated after 30 min, or after 10 min for those at temperatures above 20°C, by adding 1 ml of ice-cold trichloroacetic acid and 1 ml of ice-cold bovine serum albumin (0.05%), in that order. After precipitation on ice for 30 min, the protein precipitate was collected on a 2.5-cm glass fiber filter (Sartorius) and washed with 10 ml of ice-cold 10% trichloroacetic acid. The incorporation of ^{32}P into histone was measured by scintillation counting. The activity in the absence of phospholipids was been subtracted in each experiment. Data are means of triplicate determinations (± SD). The linearity of the assay was confirmed by charting the time course for the histone phosphorylation up to 30 min.

Differential scanning calorimetry

Samples containing 3 μmol of phospholipid and the appropriate amount of 1,2-*sn*-DMG were dried under a stream of N_2, and the last traces of organic solvent were removed by keeping the samples under vacuum for 2 h. Multilamellar vesicles were formed by incubating the dried lipid on 1 ml of 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA for 15 min at a temperature above that of the phase transition with occasional and vigorous vortexing. Samples were incubated at the mentioned temperature for an additional 30 min and left to cool slowly to 20°C in a water bath at a cooling rate of ~0.3°C/min. Another set of samples were scanned in the presence of the same buffer without EGTA; however, after the lipids were dispersed for 15 min as above, $CaCl_2$ and $MgCl_2$ were added to keep lipid concentrations identical to those of the activity assays. These samples were then incubated for 60 min more at a temperature above transition and left to cool down as mentioned for the calcium-free samples. Samples were centrifuged at $16{,}000 \times g$ for 30 min, and the pellets were transferred to small aluminum pans. Calorimetric scans were then run after 2 h of incubation at 20°C for the calcium-free samples or after overnight incubation at the same temperature for the samples containing cations. Thermograms were recorded with a Perkin-Elmer (Norwalk, CT) DSC-7 calorimeter and a sample pan containing the same buffer as a reference. The differential scanning calorimetry (DSC) instrument was calibrated using indium as standard. The samples were scanned over a temperature range from 15°C to 70°C, at a heating rate of 4°C/min and a sensitivity of 1 mCal/s (occasionally, a 0.5°C/min rate was used, although this provided no better resolution of the thermograms). The scans were repeated until identical profiles were obtained. Unless otherwise stated, the third scan was used for transition calculations unless otherwise stated. To determine the acyl migration of 1,2-DMG to 1,3-DMG after the experiments were carried out, the different samples were treated as previously described (López-García et al., 1994b), with no more than 7% of total diacylglycerol being represented by 1,3-DMG.

^{31}P-NMR

Thirty micromoles of a DMPC/DMPS mixture (4:1) and the appropriate amount of 1,2-*sn*-DMG were mixed in chloroform and evaporated to dryness under a stream of oxygen-free N_2. The remaining traces of solvent were removed by storage for 5 h under high vacuum. Afterward, 0.4 ml of 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA was added to the dry lipid mixtures, and the samples were heated at a temperature above the transition temperature for 30 min with occasional and vigorous vortexing. The samples were transferred to a conventional 10-mm NMR tube. ^{31}P-NMR

spectra were recorded in the Fourier transform mode, using a Bruker AC-200 spectrometer (81 MHz) interfaced with an Aspect 3000 computer (Bruker, Rheinstetten, Germany). The temperature was kept at ±0.5°C with a standard Bruker B-VT-1000 variable temperature control unit. The $\Delta\sigma$ values were calculated as three times the chemical shift difference between the high-field peak and the position of the peak recorded for lipid molecules in isotropic motion (Seelig, 1978). All chemical shift values are quoted in parts per million (ppm) compared with micelles of pure lysophosphatidylcholine (0 ppm); positive values refer to low-field shifts. All spectra were obtained in the presence of a gated broad-band decoupling pulse (10 W input power during acquisition time), and accumulated free induction decays were obtained from as many as 2000 transients. A spectral width of 25 kHz, a memory of 8000 data points, a 1-s interpulse time, and a 90° radio frequency pulse (11.5 μs) were the parameters used to record the spectra. Before Fourier transformation, exponential multiplication was applied, resulting in a 100-Hz line broadening.

Fourier transform infrared spectroscopy

Samples for Fourier transform infrared spectroscopy (FTIR) containing 5 μmol of total phospholipid (DMPC/DMPS or DMPC-d_{54}/DMPS, 4:1) and the appropriate amount of 1,2-*sn*-DMG or 1,2-*sn*-DMG-d_{54} in chloroform were dried under a stream of oxygen-free N_2, and the last traces of solvent were removed by dessication under high vacuum for more than 3 h. After the addition of 1 ml of 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, multilamellar liposomes were formed by using a bench vibrator and keeping the samples at a temperature above the transition temperature for at least 30 min. Mixing was continued until a homogeneous and uniform suspension was obtained. After centrifugation, the pellets were transferred to the infrared cell. A sample that contained 15 mol% was prepared in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}, in the same way as samples used for DSC, which also contained these cations.

Infrared spectra were recorded using a Philips PU9800 Fourier transform spectrometer equipped with a deuterated triglycine sulfate detector. Samples were examined in a thermostatted Specac 20710 cell (Specac, Kent, England) equipped with CaF_2 windows and 25-μm Teflon spacers. The samples were equilibrated at 15°C for 30 min in the infrared cell before the spectra were recorded. Each spectrum was obtained by collecting 100 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization, using a sample shuttle accessory to obtain average background spectra. The sample chamber of the spectrometer was continuously purged with dry air to prevent atmospheric water vapor from obscuring the bands of interest. Samples were scanned between 15°C and 50°C at 1°C intervals, with a 1-min delay between scans and using a circulation water bath interfaced to the spectrometer computer. Spectral subtraction was performed interactively with the Spectra-Calc program (Galactic Industries Corp., Salem, NH).

RESULTS

Calorimetric studies

DSC was used to study the perturbation exerted by 1,2-DMG in aqueous dispersions of DMPC/DMPS (4:1 molar ratio) both in the absence and in the presence of Ca^{2+} and Mg^{2+}. Thermograms recorded in heating scans of the different mixtures examined in the absence of Ca^{2+} and Mg^{2+} are shown in Fig. 1, in which it can be seen that in the absence of 1,2-DMG both DMPC and DMPS at a 4:1 molar ratio mix well, with a cooperative main transition from the gel to the liquid crystalline phase, an onset temperature of the gel to liquid-crystalline phase transition (T_c) of 24.4°C,

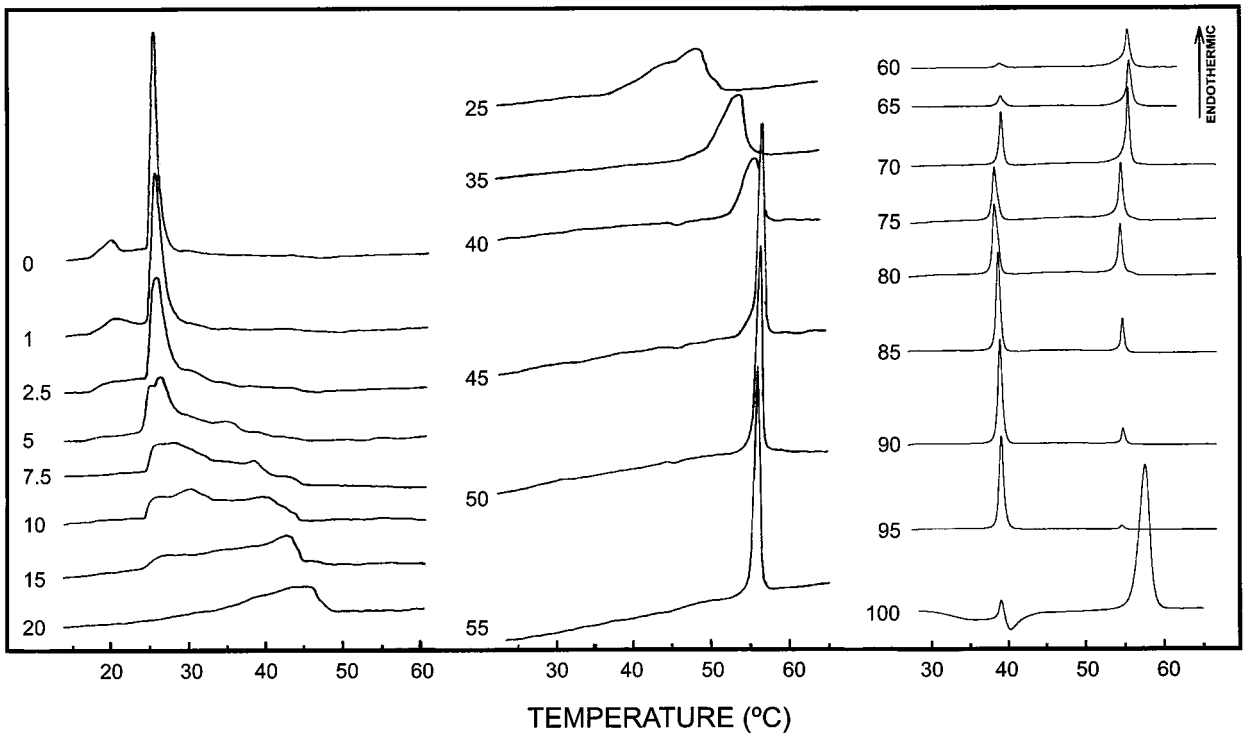

FIGURE 1 DSC heating thermograms of aqueous dispersions of mixtures of DMPC/DMPS at 4:1 molar ratio containing 1,2-DMG. The mol% of 1,2-DMG in the mixture is indicated on each of the thermograms. The fifth scan is showed for DMPC/DMPS lipid mixtures at a 4:1 molar ratio containing 1,2-DMG ranging from 60 mol% to pure 1,2-DMG.

and pretransition at 17.2°C. These temperatures are closer to the transition of pure DMPC (23.5°C for the main transition and 14°C for the pretransition) than to that of pure DMPS (35°C for the transition). The addition of low concentrations of 1,2-DMG (such as 1 mol%) slightly broadened both the pretransition and the main transition. At concentrations of 2.5 mol% 1,2-DMG and higher, more drastic effects were observed, with the gradual disappearance of the pretransition and the appearance of additional peaks at temperatures between 35°C and 40°C. Complex patterns were observed at concentrations between 2.5 and 15 mol% of 1,2-DMG. The general tendency observed with increasing concentrations of 1,2-DMG was a progressive reduction in the area of the peak located at the same temperature as the main transition in the phospholipid sample without diacylglycerol, and a concomitant increase in the area of the peaks located at ~35°C-45°C within the transition interval. At 20 mol% 1,2-DMG, the transition peak was broad and asymmetrical, with a maximum at 46.5°C. A similar profile was observed at 25 mol% 1,2-DMG, with a maximum at 47°C, although a shoulder was clearly visible at lower temperatures. In the samples containing 35 and 40 mol% 1,2-DMG, only one peak, albeit broad, was observed, which was centered at 52°C and 54°C, respectively.

At 45 mol% the profile was relatively similar to that observed at 40 mol%, with only one peak being found (centered at 55°C), although it was much narrower than the peak seen at 40 mol%. This pattern of only one narrow peak was also observed at 50 and 55 mol%, the peak being centered at 55°C at both compositions. At 60 mol% a new peak appeared, with a T_c of 38°C. This corresponded to pure 1,2-DMG and coexisted with the 55°C peak. It should be added that the thermograms of the samples ranging from 60 to 100 mol% 1,2-DMG correspond to the fifth scan, so that they show only the transition of the metastable crystalline α-form to the fluid phase (see Heimburg et al., 1992, and López-García et al., 1994b). After incubation at room temperature for more than 1 h, a transition corresponding to the β-form of 1,2-DMG was observed in the first scan. Because this transition presented a T_c of 55°C and therefore could not be separated from the peak caused by the phospholipid/diacylglycerol complex observed at approximately the same temperature (see below), we chose to present the fifth scan of each sample, in which the transition of the α-form was well separated from that of the phospholipid/diacylglycerol complex. In the 60–95 mol% range of 1,2-DMG, the peak corresponding to pure 1,2-DMG progressively increased in size as the concentration of the diacylglycerol rose, whereas the size of the peak corresponding to the phospholipid/diacylglycerol mixture decreased.

To establish any correlation between the phase behavior of the DMPC/DMPS/1,2-DMG system and PKC α activity, these calorimetric experiments were repeated in the presence of 200 μM $CaCl_2$ and 5 mM $MgCl_2$, i.e., at the concentrations at which they were used in the enzymatic assays of protein kinase C. It has previously been stated that the PS/Ca^{2+} binding stoichiometry is 2:1 mol/mol (Ekerdt and Papahadjopoulos, 1982; Feigenson, 1986; López-García et al., 1993, 1994a). The concentration of calcium used in this work corresponded to a molar DMPS/Ca^{2+} ratio of ~1:2. In the case of systems formed only of PS/DAG, this amount of calcium has been shown to be sufficient to affect all of the PS molecules and therefore to abolish the transition (López-García et al., 1994a). In contrast, the phase transition could be still observed in the samples used here for the DSC and for the activity assays that included DMPC, and therefore it could reasonably be concluded that Ca^{2+} was not at saturating concentration. In fact, the profile of the transition of the DMPC/DMPS at a 4:1 molar ratio shown in Fig. 2 looks more like that previously observed for a 4:1 or 6:1 PS/Ca^{2+} molar ratio in the absence of phosphatidylcholine (López-García et al., 1994a), and it was certainly different from the patterns seen at saturating Ca^{2+} concentrations at the same DMPC/DMPS 4:1 molar ratio (Silvius and Gagné, 1984).

Nevertheless, the effect of these ions on the phospholipid mixture DMPC/DMPS (4:1 molar ratio) in the absence of

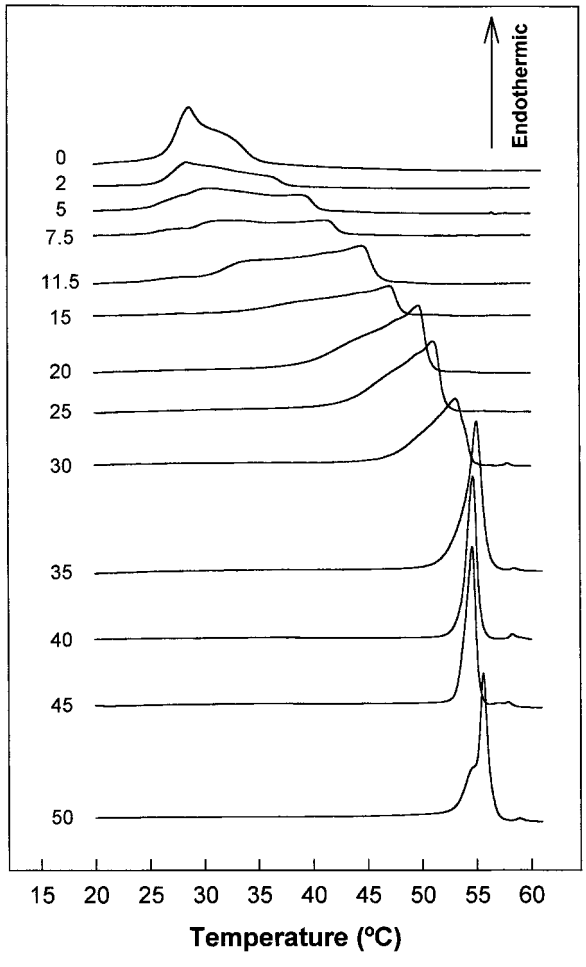

FIGURE 2 Differential scanning calorimetric heating thermograms for DMPC/DMPS lipid mixtures at a molar ratio of 4:1 containing different molar percentages of 1,2-DMG as indicated, in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}.

1,2-DMG was already significant (Fig. 2), with a broad transition seen to start at 26.3°C. At percentages of 1,2-DMG from 2 to 11.5%, this peak broadened further, and the low-temperature components decreased in area, just as those corresponding to high temperature increased. However, a gradual sharpening of the high-temperature component was observed from 11.5 to 35 mol%, with a cooperative peak observed at 35 mol% 1,2-DMG. It should be noted that a sharp peak was also obtained in the absence of these ions (Fig. 1) but at a 1,2-DMG percentage of 45 mol%. It is clear, then, that the presence of calcium and magnesium contributed to the formation of a complex, compound 1 (C_1) (see below), at the lower concentrations of 1,2-DMG. A profile consisting of a sharp peak was also maintained at concentrations of 1,2-DMG between 35 and 45 mol%. Experiments in the presence of Ca^{2+} and Mg^{2+} were performed only up to 50 mol% 1,2-DMG, because this was considered to be the interesting range for the correlation of activity and physical properties.

FTIR studies

To confirm and distinguish the origin of the different transitions observed in the thermograms obtained by DSC, several infrared spectroscopy experiments were performed. A sample that contained 15 mol% 1,2-DMG was studied in the absence (Fig. 3 *A–C*) and in the presence (Fig. 3 *D–F*) of 200 μM Ca^{2+} and 5 mM Mg^{2+}. Perdeuterated DMPC (DMPC-d_{54}) and perdeuterated 1,2-DMG (1,2-DMG-d_{54}) were used in combination with other protonated lipids to distinguish the different lipids present in each mixture.

Fig. 3 *A* shows the results for the sample containing 15 mol% 1,2-DMG-d_{54} in DMPC/DMPS (4:1 molar ratio) in the absence of Ca^{2+} and Mg^{2+}. The maximum of phospholipid CH_2 asymmetrical stretching vibration changed with the temperature, with a broad transition starting at ~25°C and ending at ~48°C. This range of temperatures was quite similar to that found in DSC for the sample containing the same lipid composition (Fig. 1). When the behavior of the 1,2-DMG-d_{54} was observed through its C^2H_2 stretching vibration, it was noted that this transition had two components. The first had an onset similar to that of the phospholipids and the second an onset at a temperature higher than that of the phospholipids (at ~42°C), indicating that 1,2-DMG was distributed in at least two different phases in this composition, with most of it located in the phase with a higher temperature transition and the main transition of the diacylglycerol coincident with the upper part of the wide transition of the phospholipid. It seems, then, that there may be a phase separation between a phase that melts at a lower temperature and is composed mainly of phospholipid, with a small amount of 1,2-DMG, and another phase containing most of the 1,2-DMG and a fraction of the phospholipids. The broad transition of the phospholipid probably corresponds to the broad peak observed in DSC for the sample containing 5 mol% 1,2-DMG in DMPC/DMPS (Fig. 1). Fig. 3 *B* shows the result of a sample with the same relationship, but using perdeuterated DMPC and perdeuterated 1,2-DMG, i.e., it is possible to observe the CH_2 of DMPS alone. In this case the only protonated acyl chain belonged to DMPS, and it was observed that DMPC and DMPS mixed well. 1,2-DMG could not be clearly distinguished, but it is worth noting that it accounted for only 15 mol% of the total lipid, so that the C^2H_2 stretching vibration belonged essentially to DMPC-d_{54} (~4.5 times greater than 1,2-DMG-d_{54}). Fig. 3 *C* depicts the results obtained with the same lipid ratio, but with DMPC as the only perdeuterated lipid in the mixture. There seems to be a slight phase separation when the phase transition temperatures of DMPC (perdeuterated) and DMPS plus 1,2-DMG (protonated) are compared. Two observations should be made in this respect. First, it should be taken into account that perdeuterated DMPC has a phase transition that is ~4°C lower than that of protonated DMPC (~4°C lower). On the other hand, 1,2-DMG had a significant weight in the protonated lipids, because the overall composition was 68 mol% DMPC, 17 mol% DMPS, and 15 mol% 1,2-DMG, i.e., DMPS and 1,2-DMG in this case were in a ratio close to 1:1. Looking at the behavior of both stretching vibration maxima, a phase separation seemed to take place between DMPS and DMPC-d_{54}. However considering that 1,2-DMG represented almost half of the protonated chains, the shift of the CH_2 stretching vibration maximum was probably due to the heterogeneous distribution of 1,2-DMG in at least two separated phases. Taking into account all of the data presented in Fig. 3 *A–C*, it is reasonable to think that there is most probably no phase separation between DMPC and DMPS (compare Fig. 3, *A* and *B*), but 1,2-DMG is heterogeneously distributed between at least two separated phases, mainly in the phase melting at higher temperature (see Fig. 3 *A*).

To obtain detailed knowledge of the phospholipid organization in the same conditions as used for the enzymatic activity, a sample containing DMPC and DMPS (4:1, molar ratio) and 15 mol% 1,2-DMG was studied by FTIR in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+} (Fig. 3 *D–F*). It can be seen that the presence of these cations induced modifications in the organization of the lipids. When 1,2-DMG was the only deuterated component (Fig. 3 *D*), its transition was also biphasic, indicating once again a heterogeneous distribution between two phases, with the most of the 1,2-DMG being found in the component melting at the higher temperature. However, the transition of the deuterated lipid (i.e., 1,2-DMG) was nearly coincident with that of the protonated lipids, in this case predominantly represented by DMPC. On the other hand, when 1,2-DMG and DMPC were deuterated, and therefore DMPS was the only protonated lipid (Fig. 3 *E*), it was clear that a phase separation existed, so that at least some of the phosphatidylserine molecules had their transition shifted toward higher temperatures, as is to be expected from their interaction with Ca^{2+} and Mg^{2+} (Jacobson and Papahadjopoulos, 1975). Finally, when the only deuterated component was DMPC (Fig. 3 *F*), it could be seen that DMPC was more clearly separated from the protonated components (DMPS and 1,2-DMG) in the presence (Fig. 3 *F*) than in the absence (Fig. 3 *C*) of

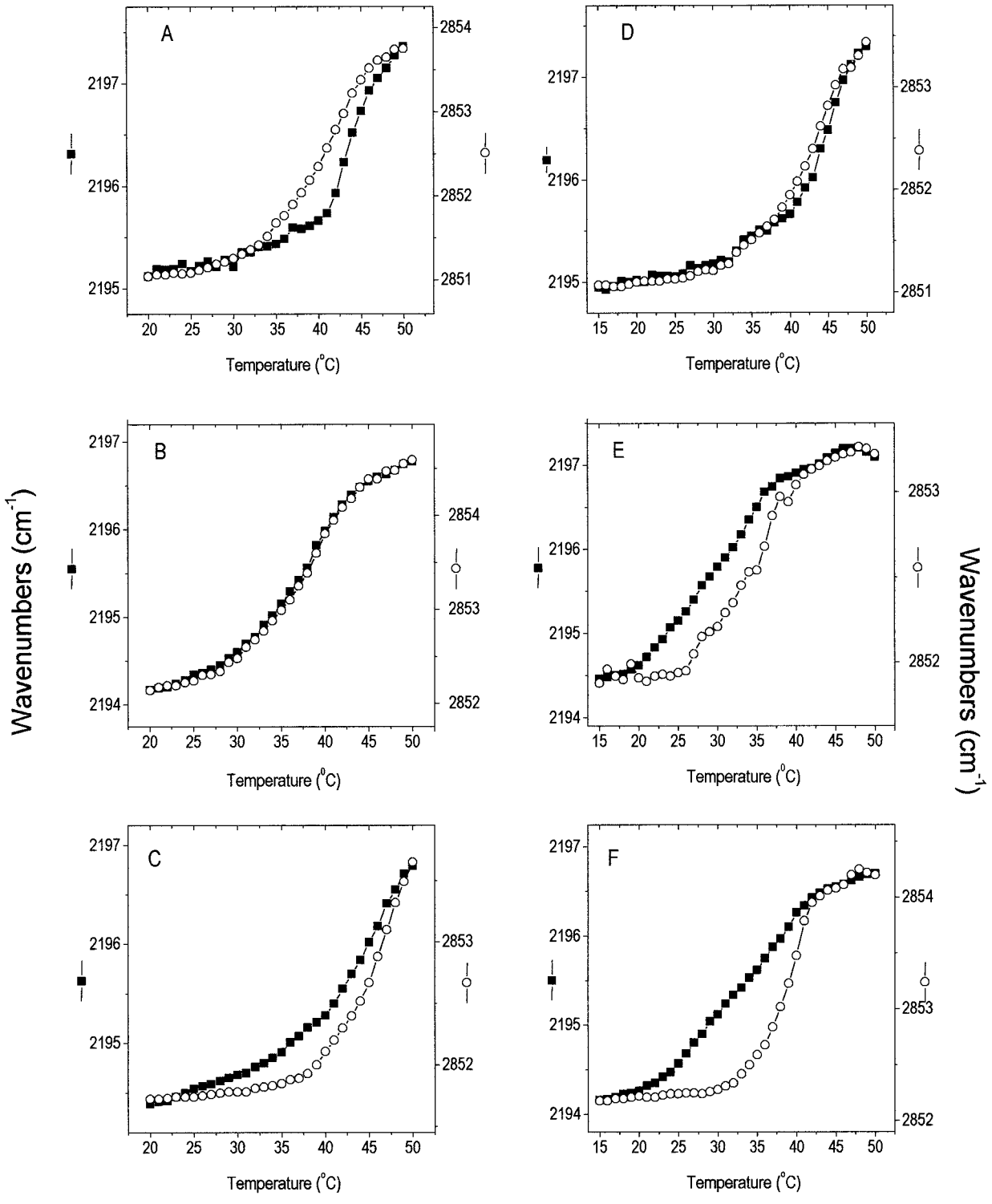

FIGURE 3 Temperature dependence of the frequency of the symmetrical C^2H_2 (■) stretching and symmetrical CH_2 (○) stretching of mixtures containing 15 mol% of 1,2-DMG in DMPC/DMPS (4:1 molar ratio) where the perdeuterated lipids lipids used are 1,2-DMG-d_{54} (*A* and *D*), DMPC-d_{54} and 1,2-DMG-d_{54} (*B* and *E*), and DMPC-d_{54} (*C* and *F*). Samples corresponding to *D*, *E*, and *F* contain 200 μM Ca^{2+} and 5 mM Mg^{2+}.

Ca^{2+} and Mg^{2+}. Because the mixture contained similar proportions of DMPS and 1,2-DMG, this separation may be taken as an indication of a phase separation of a substantial part of the DMPS molecules from DMPC, induced by the presence of Ca^{2+} and Mg^{2+}.

^{31}P-NMR spectroscopy

The effect of 1,2-DMG on the phase polymorphism of the DMPC/DMPS mixture (4:1 molar ratio) was investigated by ^{31}P-NMR spectroscopy. The ^{31}P-NMR spectra of aqueous

dispersions of phospholipid mixtures codispersed with different amounts of 1,2-DMG and recorded at various temperatures are presented in Fig. 4.

In the absence of Ca^{2+} and Mg^{2+}, at 20 mol% 1,2-DMG and 22°C, i.e., in the gel state, the mixture gave rise to an asymmetrical lineshape with a high-field peak and a low-field shoulder (Fig. 4 *A*), which is characteristic of an axially symmetrical chemical shift tensor and consistent with the arrangement of the phospholipids in a bilayer configuration. The lineshapes are broad, with a chemical shift anisotropy ($\Delta\sigma$) of ~58 ppm, as is to be expected for the gel state. At this temperature, all of the other mixtures studied showed the same broad asymmetrical lineshape indicative of the bilayer gel phase, although the mixture containing 88 mol% 1,2-DMG also presented a small isotropic component (see below). These data are in accordance with the DSC data described above (Fig. 1).

At 45°C the mixtures containing 20 mol% 1,2-DMG were in a fluid state, whereas the mixtures containing 45, 63, and 88 mol% 1,2-DMG were in the gel condition (Fig. 4). At 20 mol% 1,2-DMG the $\Delta\sigma$ was ~42 ppm, which is characteristic of a fluid lamellar (L_α) phase, whereas at higher mol% 1,2-DMG, such as the 45 mol% of Fig. 4 *B*, the $\Delta\sigma$ was characteristic of a gel phase (56 ppm). As can also be seen in Fig. 4, in the spectra taken at 45°C an isotropic peak overlapped with the anisotropic spectrum at some of the concentrations of 1,2-DMG studied, e.g., 20, 63, and 88 mol%. This isotropic peak was small at for all of the compositions in relation to the total integrated area of the spectral peak. The origin of this isotropic peak is uncertain, although it could arise from small particles or from certain areas of the membrane with a very high curvature (Heimburg et al., 1992; López-García et al., 1994b).

At 60°C and at increasing concentrations of 1,2-DMG, the anisotropic component decreased in area, while an isotropic component appeared and increased in intensity (Fig. 4). At 20 mol% 1,2-DMG, the spectrum was predominantly axially anisotropic, with a chemical shift anisotropy of ~30 ppm, which is characteristic of a fluid lamellar L_α phase. At 45 mol% 1,2-DMG, the isotropic peak was predominant, although a small proportion of the integrated area corresponded to an anisotropic component characteristic of a bilayer structure. At 63 and 88 mol%, nearly all of the ^{31}P-NMR peak corresponded to an isotropic peak. As mentioned above, the isotropic component probably corresponded to regions of the bilayer with high curvature or perhaps to a separate phase, either a cubic phase or an isotropic melt (Heimburg et al., 1992; López-García et al., 1994b).

At 20 mol% 1,2-DMG and in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}, the spectra obtained at temperatures above and below the phase transition corresponded to lamellar phases (Fig. 4 *E*). At 45 mol% (Fig. 4 *F*) and at 22 and 45°C (i.e., below of phase transition) lamellar phases were again detected, whereas at 60°C, which is above the phase transition, a nonlamellar organization was evident, with an isotropic component superimposed on another component. This gave rise to an axially anisotropic powder pattern, with a chemical shift anisotropy of a sign opposite that of the lamellar phase, which is characteristic of a phase of cylindrical symmetry, most probably corresponding to an inverted hexagonal H_{II} phase.

PKC activity

To determine the dependence of the PKC α activity on the phase behavior of the lipid mixtures studied in the presence

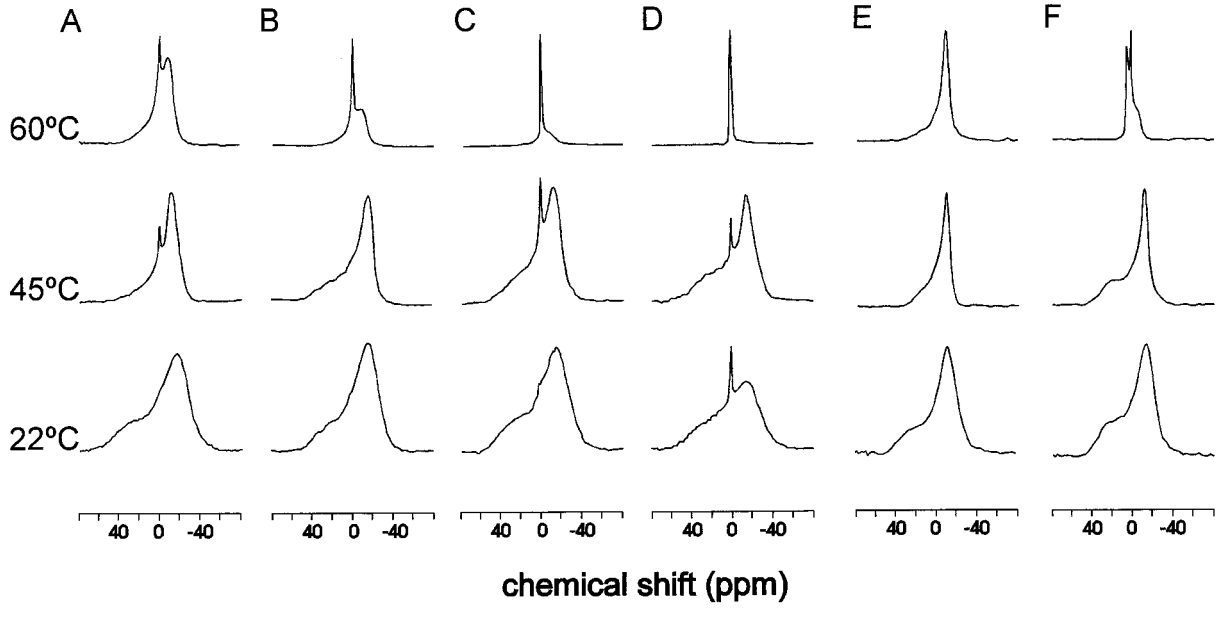

FIGURE 4 ^{31}P-NMR spectra of aqueous dispersions of mixtures of DMPC/DMPS (4:1 molar ratio) as a function of the temperature and 1,2-DMG content in the absence of cations. (*A*) 20 mol% of 1,2-DMG. (*B*) 45 mol% of 1,2-DMG. (*C*) 63 mol% of 1,2-DMG. (*D*) 88 mol% of 1,2-DMG. *E* and *F* correspond to the same samples as *A* and *B*, respectively, in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}. Temperatures are indicated in the figure.

of Ca^{2+} and Mg^{2+} and to establish any possible correlation between this activity and the structure of the lipids, two types of activity measurements were performed.

First, the effect of temperature on the activity of PKC was studied at different concentrations of 1,2-DMG (Fig. 5). A linear increase in activity with increasing temperatures was found for samples containing 5, 20, and 45 mol% diacylglycerol. In all three cases the activity began to fall at 38–39°C, probably as a result of protein denaturation. This was confirmed from changes in intrinsic tryptophan fluorescence by recording the maximum of the emission peak of protein kinase C α at 340 nm and its variation with temperature (not shown). The drastic change in the environment of the tryptophans that was detected at ~40°C would explain the decrease in the activity above 38–39°C. At 5 mol%, the level of activity was rather low at all temperatures compared to the activity recorded with other DAG concentrations. At 20 mol%, the activity sharply increased as the temperature increased, reaching a maximum at 38°C. It is worth noting that the maximum activity found at 45 mol% was lower than that at 20 mol% 1,2-DMG.

Looking at the different levels of maximum activity obtained for the samples containing different amounts of 1,2-DMG and to better understand the dependence of PKC α activity on phase behavior, a different type of experiment was performed. In this case, the activity of the enzyme was measured by changing the 1,2-DMG concentration at two different temperatures, 15°C and 35°C (Fig. 6, *A* and *B*, respectively). It can be seen that although the activity levels were much higher at 35°C, the patterns of activation caused by the different concentrations of diacylglycerol were very similar at the two temperatures. There was a gradual increase in the activity recorded up to 30 mol%, when a maximum of activity was reached, and a decrease at higher concentrations. Again, different levels of maximum activity were demonstrated at 20 and 45 mol% 1,2-DMG, in agreement with the results of Fig. 5.

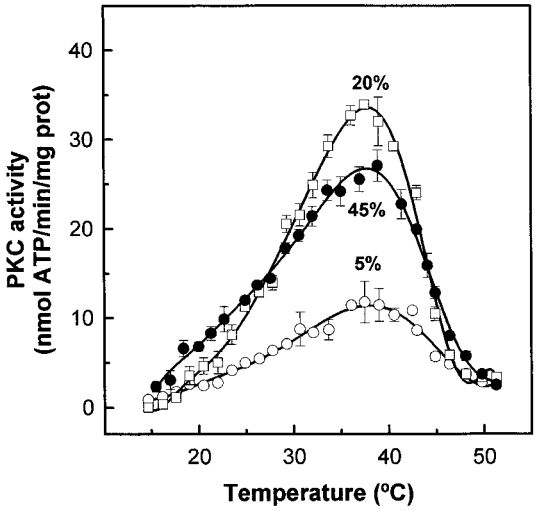

FIGURE 5 PKC α activity as a function of the temperature increase in mixtures of DMPC/DMPS (4:1 molar ratio) with different concentrations of 1,2-DMG. The 1,2-DMG concentrations were 5 (○), 20 (□), and 45 mol% (●) of 1,2-DMG. (*Inset*) Variation in the maximum of the emission peak of protein kinase C α located at 340 nm with the temperature (excitation at 295 nm). F_0 stands for fluorescence intensity at 20°C and F_I for fluorescence intensity at each temperature.

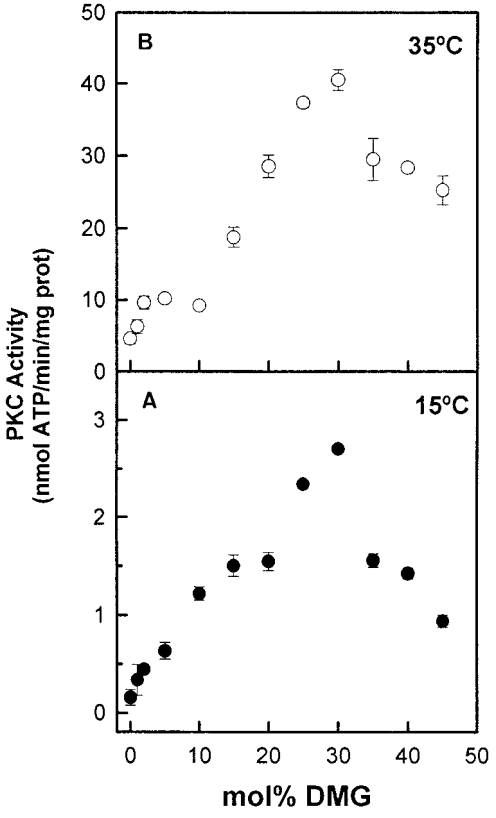

FIGURE 6 PKC α activity as a function of the increasing percentage of 1,2-*sn*-DMG in DMPC/DMPS mixtures (4:1 molar ratio), at (*A*) 15°C and (*B*) 35°C.

DISCUSSION

The behavior of the DMPC/DMPS/1,2-DMG system in the absence of Ca^{2+} and Mg^{2+} is very similar to to that of binary systems containing a phosphatidylcholine (PC) and a DAG that bear identical saturated acyl chains, such as DMPC/1,2-DMG (Heimburg et al., 1992) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/1,2-dipalmitoylglycerol (DPPC/1,2-DPG) (López-García et al., 1994b), and is certainly more similar to these phosphatidylcholine systems than to the 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine/dipalmitoylglycerol (DPPS/DPG) system (López-García et al., 1994a). The inclusion of DMPS at a 4:1 DMPC/DMPS molar ratio does not seem to alter the system much compared with the binary system DMPC/1,2-DMG system previously described (Heimburg et at., 1992).

According to the FTIR results, DMPC and DMPS mix well in the absence of Ca^{2+} and Mg^{2+} and could not be separated, whereas 1,2-DMG does not seem to interact preferentially with either of these phospholipids. However,

in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}, which were the concentrations used for the enzyme activity assays, at least some of the DMPS molecules appeared to show an increased melting temperature (see Fig. 3).

1,2-DMG, on the other hand, was found to be heterogeneously distributed, so that, judging from the increases in frequencies of the two components of the transition, as seen through FTIR, a vast majority of it was concentrated in domains, even at very low concentrations. It should be stressed once again that these domains, in which most of the diacylglycerol molecules are concentrated, also contain phospholipid molecules. This description essentially coincides with other previously published results (Ortiz et al., 1988; Cunningham et al., 1989; Heimburg et al., 1992; López-García et al., 1994b; Quinn et al., 1995). The formation of these domains, in which most of the DAGs are concentrated (not in a pure state, but together with phospholipids), may explain why diacylglycerols are present in relatively low concentrations in biological membranes, and only in exceptional circumstances reach 2–10 mol% (Preiss et al., 1986; Basu, 1993).

Using the data obtained through DSC, FTIR, and ^{31}P-NMR, phase diagrams were constructed for samples both in the absence and in the presence of Ca^{2+} and Mg^{2+} (Fig. 7). Pretransition data were ignored for the sake of simplicity. The phase boundaries of the solidus and fluidus lines were established from the respective onset and completion temperatures of the scans of DMPC/DMPS (4:1 molar ratio) mixtures containing variable percentages of 1,2-DMG. FTIR data were used to help in the identification of the origin of some thermal transitions, and ^{31}P-NMR was used to characterize the phase organization of samples at the different temperatures.

In the absence of Ca^{2+} and Mg^{2+} (Fig. 7 *A*), the resulting phase diagram was similar to those previously published for DMPC/1,2-DMG (Heimburg et al., 1992) and for DPPC/1,2-DPG (López-García et al., 1994b). This is not surprising, because the phospholipid mixture used in this study mainly contained DMPC, and the diacylglycerol used had the same fatty acyl chains as the phospholipid, which was also true for the two previously described cases. The diagram has three well-differentiated regions. The first one corresponds to concentrations of 1,2-DMG lower than 45 mol%. This region can be approached by an eutectic model, with the eutectic point located at a very low concentration of 1,2-DMG. The immiscibility in the gel phase is presumed to occur between the phospholipid molecules without diacylglycerol (DMPC/DMPS) and a compound (that can be called C_1) with a phospholipid/1,2-DMG molar ratio of ~55:45. There are, nevertheless, some deviations from total immiscibility in the gel phase, which probably occur because of the limited solubility of the phospholipid in the compound C_1. Similar situations have been observed in other systems (Van Dijck et al., 1977; López-García et al., 1994b). This very limited solubility is probably the reason why FTIR spectroscopy showed a small proportion of 1,2-DMG with a lower temperature transition than the bulk of the diacylglycerol. Hence, the gel phase immiscibility, leading to a phase containing almost pure phospholipid and another formed by phospholipid and diacylglycerol, is very much supported by the FTIR experiments described above (Fig. 3 *A–C*). Above the phase transition temperature, there seems to be good miscibility between phospholipids and diacylglycerols. ^{31}P-NMR experiments corresponding to this region of the phase diagram show that at temperatures below those of the phase transition, the mixture adopts a lamellar structure.

In the second region of the phase diagram (between 45 and 60% 1,2-DMG), immiscibility occurs both above and below the phase transition, at which point it is postulated that two compounds, C_1 and C_2, will coexist. The existence of these two complexes is deduced from the fact that a pure compound was reached at 45 mol%, and the addition of more 1,2-DMG did not produce any noticeable change; however, as the 1,2-DMG concentration reached 60 mol%, pure 1,2-DMG was separated, indicating that the phospholipid present was not able to complex more diacylglycerol. C_2 probably has a composition of ~60 mol% 1,2-DMG and 40 mol% phospholipid. A second eutectic point is probably present at a composition very close to that of compound C_2. It should also be pointed out that whereas in the heating experiments only a narrow transition peak was observed

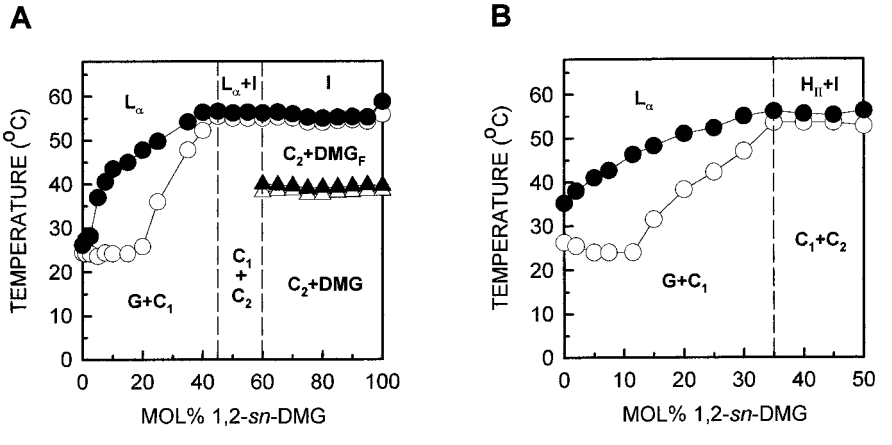

FIGURE 7 Phase diagrams for aqueous dispersions of DMPC/DMPS (4:1 molar ratio) containing 1,2-DMG constructed from data derived from differential scanning calorimetry, FTIR, and ^{31}P-NMR. The open and closed circles were obtained from T_c of the onset and completion temperatures of the heating scans respectively. (*A*) Phase diagram in the absence of Ca^{2+} and Mg^{2+}. (*B*) Phase diagram in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}.

through DSC, in the cooling thermograms (not shown for brevity) two components could be discerned. This was also the case in previous similar studies (Heimburg et al., 1992; López-García et al., 1994b). Finally, ^{31}P-NMR results indicate that below the phase transition, only the lamellar phase can be observed, whereas above the phase transition, a fluid lamellar phase (L_α) and an isotropic phase (I) coexist.

In the third region of the diagram, phase-separated diacylglycerol coexists with C_2 compound. Below the phase transition, the C_2 compound adopts a lamellar structure, although above it, it has an isotropic structure, according to the ^{31}P-NMR results. The isotropic structure adopted by C_2 in both regions 2 and 3 of the diagram is presumably cubic, according to several detailed studies that have characterized different phospholipid/diacylglycerol mixtures with a high diacylglycerol content (Das and Rand, 1986; Seddon, 1990; Luzzati et al., 1992; Quinn et al., 1995).

In the presence of 200 μM $CaCl_2$ and 5 mM $MgCl_2$ (Fig. 7 *B*), the phase diagram was slightly different from that observed in the absence of the ions, reflecting the peculiarities observed in the DSC and FTIR studies. First of all, the thermograms were quite different in the presence of the cations and in their absence, and, in addition, the results obtained from FTIR spectroscopy revealed that the cation concentrations used in this work affected the phospholipid organization and hence their thermotropic properties. In this case, the first region of the phase diagram ends at 35 mol%, instead of at the 45 mol% observed in the absence of Ca^{2+} and Mg^{2+}.

The phase diagram in the presence of Ca^{2+} and Mg^{2+} (Fig. 7 *B*) indicates that at 15°C the DMPC/DMPS/1,2-DMG vesicles were in a gel state throughout the range of concentrations of 1,2-DMG studied. In contrast, at 35°C this system showed different phases as the 1,2-DMG concentration was increased. From 0 to 17.5 mol% 1,2-DMG, this lipidic system was in the interphase between the solidus and fluidus lines, whereas at concentrations of 1,2-DMG higher than 17.5 mol%, it was in the gel phase. As deduced from FTIR spectroscopy (Fig. 3), a substantial percentage of the DMPS molecules were affected by the presence of the cations, so that the phase transition of this phospholipid was shifted slightly to a higher temperature (see Fig. 3 *E*).

The data obtained with DSC and FTIR (Figs. 2 and 3) suggest that at a concentration such as 15 mol% 1,2-DMG and in the presence of Ca^{2+} and Mg^{2+}, there was a phase separation, just as there was in their absence. However, it should be stressed that the presence of the cations determined a change in the composition of the separated phases.

In the presence of the cations, as suggested by the DSC and FTIR data, a substantial part of DMPS was separated from DMPC. It is difficult to tell from the available data whether 1,2-DMG was uniformly distributed with respect to both phospholipids or whether it had a preference for one of them. It should be stressed, however, that the DSC results showed that the phase separation existing at low 1,2-DMG concentrations totally disappeared when sufficient diacylglycerol was added to the system, so that at 35 mol% 1,2-DMG only one phase, the pure complex, was present. The structure of this complex was nevertheless different from that of its analog observed in the absence of cations, which contained 45 mol% 1,2-DMG.

The ^{31}P-NMR results obtained in the presence of Ca^{2+} and Mg^{2+} deserve some comment, especially in the case of the 45 mol% 1,2-DMG sample, where an H_{II} inverted hexagonal phase was detected at temperatures above those of the phase transition, together with an isotropic phase. H_{II} inverted hexagonal phases have been described for other diacylglycerol-containing systems, at compositions similar to that of the sample mentioned (Heimburg et al., 1992; López-García et al., 1994). However, in the samples without Ca^{2+} and Mg^{2+}, described above, hexagonal H_{II} inverted phases were not observed. The reason for this may be that the composition range at which this phase appears seems to be very narrow (Heimburg et al., 1992; López-García et al., 1994), and therefore it does not appear at 45 mol% without Ca^{2+} and Mg^{2+}, whereas it does so at slightly higher concentrations of 1,2-DMG. Note also that there is no exact correspondence between concentrations and the boundaries of the phase diagrams when samples with and without Ca^{2+} and Mg^{2+} are compared, so that samples with Ca^{2+} and Mg^{2+} may show phase properties expected in samples containing higher concentrations of 1,2-DMG but that do not contain Ca^{2+} and Mg^{2+}.

When we look at the activation of protein kinase C α by these lipid mixtures, it should be remembered that in the cases examined here, the system remained in the gel state. It is worth noting that maximum activity is reached at a high concentration of 1,2-DMG (30 mol%), at both 15°C and 35°C. In the presence of calcium, region 2 of the phase diagram is reached at higher concentrations of 1,2-DMG, at which point free phospholipid disappears.

The phase diagrams reveal that the decrease in maximum activity observed in the sample of 45 mol% 1,2-DMG (Fig. 5) contains no free phospholipid, because at this concentration, in the gel phase, all of the molecules in the sample exist as complexes of 1,2-DMG and phospholipid. It is interesting to note that the sample containing 5 mol% 1,2-DMG reached the melting interval at 25°C, i.e., within the range of temperatures at which enzyme activity was studied here. However, at 20 mol% 1,2-DMG the melting range is reached at a temperature higher than that at which protein denaturation occurs.

PKC α activity began to decline at concentrations above 35 mol% 1,2-DMG (Fig. 6). We suggest that the structure of the membrane may be less suitable for supporting enzyme activity when the lipid composition corresponds to region 2 of the phase diagram than when it corresponds to region 1. It should be remembered here that the gel phase of the diacylglycerol/phospholipid stoichiometric complexes, like those appearing at compositions corresponding to region 2, has a structure that differs from that of the pure phospholipid. It has been described that the lipid bilayer and the water layer of the gel phase are thicker in these complexes than in the pure phospholipid (López-García et al., 1994b;

Quinn et al., 1995). This may hinder the enzyme when it penetrates the membrane. If the membrane is relatively dehydrated, it would be easier for the protein to become inserted in the hydrophobic domain of the membrane than if the tightly bound layer were thicker. It may be mentioned in this respect that a certain degree of enzyme penetration of the membrane is necessary for optimum activity (see Zidovetzki and Lester, 1992, and Newton, 1993, for reviews).

Another way in which structural alteration of the membrane may lead to a loss of enzyme activation may be that the tilted structure of the phospholipid in the complex (Quinn et al., 1995) prevents it from binding to the enzyme in a suitable way for it to be an optimum activator. It is known, for example, that the enzyme needs the L stereoisomer of phosphatidylserine for the maximum activation of PKC to occur (Lee and Bell, 1989; Newton and Keranen, 1994), so that a very specific PKC-phosphatidylserine interaction must be established for activation. It should also be remembered that PKC seems to establish two types of interaction with anionic phospholipids. One of them leads to the binding of PKC to the membrane, with phosphatidylserine perhaps being replaced by other anionic phospholipid without impairing this binding process. The other interaction allows activation of the enzyme, which seems to be highly specific for phosphatidylserine (Orr and Newton, 1992; Mosior and Epand, 1993).

According to these arguments, in region 1 of the phase diagram, the enzymatic activity should increase gradually when the concentration of 1,2-DMG is increased. However, when the concentration of 1,2-DMG increases to the point at which the stoichiometric complex phospholipid/diacylglycerol is the predominant structure, the membrane structure will not be the most suitable for stimulating enzymatic activity, which would decline as a consequence.

In a recent paper, in which systems similar to those examined here were studied (Hinderliter et al., 1997), a slightly different structural explanation was suggested to explain the activation of PKC, i.e., the coexistence of diacylglycerol-rich and -poor phases. In samples containing DMPC and DMPS at a 1:1 molar ratio and in the gel state, the maximum activity of PKC α was observed at 36 mol% 1,2-DMG, although these authors did not show the phase diagram in the presence of the concentrations of Ca^{2+} and Mg^{2+} used for the enzyme assay, which were 5 μM Ca^{2+} and 5 mM Mg^{2+}, i.e., very low Ca^{2+} but relatively high Mg^{2+}. Note that we used 200 μM Ca^{2+} because in our experimental conditions this concentration produced activities close to the maximum. However, in the absence of these ions, and judging from the thermograms shown, a pure compound was reached at 40–50 mol% 1,2-diacylglycerol. As demosntrated in the present work, and in the presence of Ca^{2+} and Mg^{2+}, the pure compound C_1 appears at concentrations lower than those found in the absence of these cations. It should be expected, therefore, that in the abovementioned study, and in the presence of Ca^{2+} and Mg^{2+}, a pure compound similar to C_1 would appear at concentrations slightly higher than 40–50 mol% diacylglycerol. If this is the case, the results of Hinderliter et al. (1997) could also be explained by the interpretation that we give in our work, because these authors observed a decrease in activity for samples containing concentrations of diacylglycerol higher than ~35 mol%, i.e., as in our case, at a concentration of diacylglycerol at which the pure compound appeared. It should be said, nevertheless, that their system and ours are similar but not identical, because our study was carried out with a DMPC/DMPS molar ratio of 4:1, whereas Hinderliter et al. (1997) used a 1:1 DMPC/DMPS molar ratio.

In the same paper (Hinderliter et al., 1997) and in a previous paper by the same group (Dibble et al., 1996), similar studies on the effect of phospholipid/diacylglycerol systems were carried out, but using unsaturated diacylglycerols such as diolein mixed with DMPC/DMPS in a 1:1 molar ratio. The phase behavior of these mixtures of saturated phospholipids and unsaturated diacylglycerols has been studied less than the corresponding behavior of saturated phospholipid mixed with saturated diacylglycerols.

Nevertheless, the general explanation given in the abovementioned papers for the several systems studied was that the enzyme was maximally activated by membrane compositions in which separated 1,2-DMG-rich and -poor phases coexisted. Certainly this suggestion may also be valid for our results, because in region 1 pure phospholipid and complex C_1 coexisted, so that one phase was poorer and the other richer in diacylglycerol. As soon as the pure phospholipid disappeared, upon entering region 2, the diacylglycerol-poorer phase disappeared.

In summary, our results indicate that PKC activity increased as 1,2-DMG was increased, whereas the activity decreased when the membrane was formed by a pure stoichiometric compound. We suggest, therefore, that for PKC α to be maximally activated, the structure of the membrane must be adequate. Hence, whereas membrane compositions corresponding to region 1 of the phase diagram will be perfectly suitable to encourage PKC α activity, those compositions corresponding to region 2 will not provide an adequate environment for the expression of maximum enzymatic activity.

We are very grateful to Dr. Robert M. Bell for providing us with the recombinant baculovirus of protein kinase C α. We also thank Dr. Elaine Bardes for technical advice on the PKC purification and insect cell culture work.

This work was supported by grant PB95-1022 from Dirección General de Enseñanza Superior (Spain).

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