Activation of Protein Kinase C α by Lipid Mixtures Containing Different Proportions of Diacylglycerols†

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Received July 23, 2001; Revised Manuscript Received September 17, 2001

ABSTRACT: Lipid activation of protein kinase C α (PKC α) was studied using a model mixture containing POPC/POPS (molar ratio 4:1) and different proportions of either DPG or POG. The lipid mixtures containing DPG were physically characterized by using different physical techniques, and a phase diagram was constructed by keeping a constant POPC/POPS molar ratio of 4:1 and changing the concentration of 1,2-DPG. The phase diagram displayed three regions delimited by two compounds: compound 1 (CO_1) with 35 mol % of 1,2-DPG and compound 2 (CO_2) with 65 mol % of 1,2-DPG. PKC α activity was assayed at increasing concentrations of 1,2-DPG, maximum activity being reached at 30 mol % 1,2-DPG, which decreased at higher concentrations. Maximum activity occurred, then, at concentrations of 1,2-DPG which corresponded to the transition from region 1 to region 2 of the phase diagram. It was interesting that this protein was maximally bound to the membrane at all DPG concentrations. Similar results were observed when the enzyme was activated by POG, when a maximum was reached at about 10 mol %. This remained practically constant up to 50 mol %, about which it decreased, the binding level remaining maximal and constant at all POG concentrations. The fact that in the assay conditions used maximal binding was already reached even in the absence of diacylglycerol was attributed to the interaction of the C2 domain with the POPS present in the membrane through the Ca^{2+} ions also present. To confirm this, the isolated C2 domain was used, and it was also found to be maximally bound at all DPG concentrations and even in its absence. Since the intriguing interaction patterns observed seemed to be due then to the C1 domain, the PKC α mutant D246/248N was used. This mutant has a decreased Ca^{2+}-binding capacity through the C2 domain and was not activated nor bound to membranes by increasing concentrations of DPG. However, POG was able to activate the mutant, which showed a similar dependence on POG concentration with respect to activity and binding to membranes. These data underline the importance of unsaturation in one of the fatty acyl chains of the diacylglycerol.

Protein kinase C (PKC)[1] isoenzymes are a large family of serine/threonine kinases involved in cellular signaling (*1*−*4*). Within this family, the so-called classic isoenzymes, such as the PKC α studied in this paper, are activated by Ca^{2+}, phosphatidylserine, and diacylglycerols. They are lipid second messengers that are produced in vivo by the hydrolysis of phosphatidylinositols and phosphatidylcholine, as a result of the action of phospholipase C. Diacylglycerols are considered to serve as hydrophobic anchors which may recruit protein kinase C to the membrane, leading to an increase in the enzyme's membrane affinity (*4*) and to activation of PKC (*5*−*7*).

There is a great deal of information that suggests that membrane structure is modulated by the presence of DAGs. For example, it has been shown that DAGs produce structural changes in membranes, including lateral phase separation (*8*−*13*), the formation of nonbilayer phases (*9*, *10*, *14*−*16*) and dehydration of the membrane interface (*16*, *17*). Significantly, the dehydration produced by DAG has a more drastic effect on phosphatidylserine than on phosphatidylcholine (*16*), which may be responsible for facilitating membrane fusion (*18*−*23*), and perhaps for the activation not only of PKC but also of other enzymes such as phospholipases (*23*−*26*), phosphocholine cytidyltransferase (CTP) (*27*), and tyrosine kinase (*28*).

On the other hand, several suggestions have been made concerning the modulation of PKC activity by DAGs. It has been suggested, for example, that PKC activity increases with the tendency of lipids to form nonbilayer phases, as could be the case in regions of high bilayer curvature produced by molecules, such as diacylglycerols or phosphatidylethanol-

† This work was supported by Grant PB98-0389 from Dirección General de Enseñanza Superior e Investigación Científica (Spain).

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[1] Abbreviations: DAGs, diacylglycerols; 1,2-DMG, 1,2-dimyristoyl-*sn*-glycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine; 1,2-DPG, 1,2-dipalmitoyl-*sn*-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; Δσ, chemical shift anisotropy; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; FTIR, Fourier transform infrared spectroscopy; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MLVs, multilayer vesicles; PMSF, phenylmethanesulphonyl fluoride; PKC, protein kinase C; ^{31}P NMR, ^{31}P-nuclear magnetic resonance; 1,2-POG, 1-palmitoyl-2-oleoyl-*sn*-glycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi015599+ CCC: $20.00
Published on Web 11/14/2001

amines, which have small polar headgroups (*12*, *14*, *29*, *30*). It has also been suggested that the presence of DAGs increase the spacing between phospholipid headgroups because of the interposition of the small groups of DAGs (*14*, *31*−*34*). It might also be possible that the effect of membrane surface dehydration induced by DAGs could facilitate immersion of the protein in the membrane (*17*). It has recently been suggested that membrane heterogeneity, involving coexistence of rich and poor phases in DAGs, may contribute to the activation of PKC (*13*, *35*) and similarly 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 (*36*).

There are a number of reports that indicate that PKC activity is modulated by other membrane structural properties (*33*, *37*, *38*). It was shown, for example, that PKC activity is sensitive to acyl chain unsaturation (*33*), the effects of unsaturation probably arising from alterations in headgroup spacing (*33*, *34*). PKC activity is also sensitive to phosphatidylethanolamine, and this has been attributed to its tendency to disrupt the bilayer structure of the membrane (*39*, *40*). It has been recently found that the physical state of the membrane affects the enzymatic activity and the energy of activation of PKC α (*41*).

This paper describes our studies on the activation of PKC α by lipid systems, formed by POPC/POPS (4:1 molar ratio) with varying DPG or POG contents. We also show that when PKC α was activated by incorporation of DPG, the activity was maximal at 30 mol %, a concentration at which the pure CO_1 (phospholipid/diacylglycerol) complex was present (as defined by the phase diagram). However, the enzyme was maximally bound at all concentrations even in the absence of DPG. POG, on the other hand, activated the enzyme to a maximum degree at 20−50 mol % with the enzyme being maximally bound at all concentrations. Through the use of isolated C2 domain and double mutant D246/248N, we show that the enzyme may be activated simply by binding through C1 domain in the presence of POG but not in the presence of DPG, demonstrating the importance of an unsaturated fatty acyl chain in the DAG molecule.

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocoline (DPPC) were purchased from Avanti Polar Lipids (Alabaster, Alabama). 1,2-Dipalmitoyl-*sn*-glycerol (DPG) and 1-palmitoyl-2-oleoyl-*sn*-glycerol (POG) 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 the diacylglycerols was determined by thin-layer chromatography on plates of silica gel 60 (Macherey-Nagel, Dürren, Germany) using chloroform/acetone/methanol (94.5:5.0:0.5, v/v/v) as solvent. Anti-HA antibody (HA-probe, mouse monoclonal) was purchased from Santa Cruz Biotechnology (Madrid). Chemiluminiscence Western Blotting kit (mouse/rabbit) was purchased from Boehringer-Mannheim (Barcelona). Glassfiber prefilter (25 μm pore size) was purchased from Sartorius (Germany). Water was twice distilled and deionized in a Millipore system from Millipore Ibérica (Madrid).

DSC Measurements. Samples containing 3 μmol of phospholipid (POPC/POPS, 4:1, mol/mol), the appropriate amount of diacylglycerol (DPG or POG) and ionophore A23187, at a molar ratio phospholipid/ionophore of 1000:1, were dried under a stream of oxygen-free N_2, and the last traces of organic solvent were removed by keeping the samples under vacuum for at least 2 h. Multilamellar vesicles were formed by incubating the dried lipids in a buffer containing 20 mM Tris-HCl, pH 7.5, 200 μM $CaCl_2$, and 5 mM $MgCl_2$ (Ca^{2+}/POPS molar ratio of 2.1 and Mg^{2+}/POPS of 52.1) for 15 min at a temperature above the gel to fluid phase transition. After this period, samples were incubated at the same temperature for an additional period of 60 min, with occasional and vigorous vortexing, and left to cool slowly to 20 °C in a water bath at a cooling rate of approximately 0.3 °C/min. Then, the samples were centrifuged at 15000*g* for 45 min at room temperature, and the pellets were transferred to small aluminum pans.

Thermograms were recorded using a Perkin-Elmer (Norwalk, CT) DSC-4 calorimeter using a sample pan containing the same buffer without lipids as a reference. The DSC instrument was calibrated using indium as standard. The samples were scanned over a temperature range from −12 to 74 °C, at a heating rate of 4 °C/min and a sensitivity of 1 mcal/s. The samples were cooled at the same temperature rate and sensitivity. The scans were repeated until identical profiles were obtained. Normally, the fourth scan was used for transition calculations.

To determine the acyl migration of 1,2-diacylglycerol to 1,3-diacylglycerol after the experiments, we analyzed the different samples as previously described (*11*), leading to the formation of 1,3-diacylglycerol, which represented 5−8% of total diacylglycerol. Organic phosphorus was analyzed (*42*) to quantify the phospholipid present in each sample.

^{31}P NMR Spectroscopy. Samples for ^{31}P NMR were prepared by combining organic solutions containing 25 μmol of phospholipid (POPC/POPS, 4:1, mol/mol), ionophore A23187 (at a molar ratio phospholipid/ionophore of 1000:1), and the appropriate amount of 1,2-DPG. Lipids were placed in conventional 5-mm NMR tubes and dried under a stream of oxygen-free N_2. The last traces of organic solvent were removed by keeping the samples under vacuum for at least 2 h.

Multilamellar vesicles were formed incubating the dried lipids in 20 mM Tris-HCl, pH 7.5, 260 mM $CaCl_2$, and 6.5 M $MgCl_2$ (to ensure that conditions were exactly the same as for the calorimetric and enzymatic assays, with a Ca^{2+}/POPS molar ratio of 2.1 and Mg^{2+}/POPS molar ratio of 52.1) as described above.

^{31}P NMR spectra were obtained in the Fourier Transform mode in a Varian Unity 300 spectrometer. All chemical shift values are quoted in parts per million (ppm) with reference to pure lysophosphatidylcholine micelles (0 ppm), positive values referring to low-field shifts. All spectra were obtained in the presence of a gated broad band proton decoupling (5 W input power during acquisition time), and accumulated free inductive decays were obtained from up to 5000 scans. A spectral width of 25 000 Hz, a memory of 32 000 data points, a 2 s interpulse time, and a 80° radio frequency pulse

(11 μs) were used. Prior to Fourier transformation, an exponential multiplication was applied, resulting in a 60 Hz line broadening.

Fourier Transform Infrared Spectroscopy. Samples for Fourier transform infrared spectroscopy (FTIR) contained 5 μmol of total phospholipid (POPC/POPS, 4:1), ionophore A23187 (at a molar ratio phospholipid/ionophore of 1000:1), and the appropriate amount of 1,2-*sn*-DPG-d_{62}. Multilamellar vesicles were formed as described above.

Infrared spectra were obtained by using a Bruker Vector 22 spectrometer equipped with a deuterated triglycine sulfate detector. Samples were examined in a thermostated Specac 20710 cell (Specac, Kent, England) equipped with CaF_2 windows and 25 μm Teflon spacers. Samples were equilibrated for 15 min at −10 °C, the infrared cell before the spectra were recorded. Each spectrum was obtained by collecting 128 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization, using a sample shuttle accessory to obtain the 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 −10 to 74 °C at 2 °C intervals, with a 4-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).

Preparation of Protein Kinase C α *and Its Mutant*. PKC α wild type and mutants were generated as described by Conesa-Zamora et al. (*43*). HEK293 (human epithelial kidney) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Transfection was performed with the calcium phosphate method described by Wigler et al. (*44*). Briefly, DNA (10 μg) was diluted in Tris/EDTA buffer containing 125 mM $CaCl_2$. The mixture was then diluted in HEPES/Na_2HPO_4 buffer and incubated at room temperature for 15 min before being added to the culture medium. Twelve hours after transfection, media were aspirated and washed three times with 6 mL of PBS buffer. Cells were harvested at 48 h postransfection, pelleted, and resuspended in a volume of lysis buffer (5 mL of buffer/g of cells) containing 10 mM Tris, pH 7.5, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 200 μM PMSF, 10 μg/mL trypsin inhibitor, 1 mM Na_3VO_4, 10 mM benzamidine, 150 mM NaCl, and 50 mM NaF. The pellet was disrupted by sonication, and the resulting lysate was centrifuged at 15000*g* for 30 min at 4 °C. The level of protein expression in the supernatant was checked by immunoblot analysis of the epitope-tagged transiently expressed proteins and using anti-HA antibody, and developed with chemiluminescence Western blotting reagents.

Determination of Protein Kinase C Activity. Lipids (POPC/POPS, 4:1 mol/mol, and ionophore A23187 at a molar ratio phospholipid/ionophore of 1000:1) were added to the reaction to give a final total phospholipid concentration of 480 μM, and the diacylglycerol was varied from 0 to 80% molar ratio. A 25-μL sample of the dispersed lipids, prepared as described above, was added to the reaction vial (final volume 250 μL), containing 20 mM Tris-HCl, pH 7.5, 200 μM $CaCl_2$, 5 mM $MgCl_2$, 0.2 mg/mL histone III-S, and 20 μM [γ-^{32}P] ATP (approximately 300 000 cpm/nmol). The reaction mixture was incubated for 5 min at the desired temperature and started by adding 10 μL of the cell lysate (approximately 0.1 μg of protein) to the same 240 μL reaction mixture. Additional control experiments were performed with mock cells lysates to discard the endogenous PKC α activity, and they represent less than 1% of the total enzyme activity measured.

Reactions were stopped after 20 min by adding 1 mL of ice-cold 25% trichloroacetic acid and 1 mL of ice-cold 0.05% bovine serum albumin, in that order. After precipitation of the sample on ice for 30 min, the protein was collected on a 2.5-cm glass fiber filter and washed three times with 3 mL of ice-cold 10% trichloroacetic acid. The incorporation of ^{32}P into histone was measured by liquid scintillation counting. Basal activity was measured in the presence of 1 mM EGTA instead of POPC/POPS, diacylglycerol, and Ca^{2+}, and was subtracted from the sample results. Data are the means of triplicate determinations (±SD). The linearity of the assay was confirmed by plotting the nmoles of phosphorylated histone versus the amount of protein and also versus the time course up to 45 min.

PKC α *Membrane Binding Assay*. Multilamellar vesicles were prepared as described in the activity assay. A 25-μL sample of the dispersed lipids was added to the reaction vial (final volume 250 μL), containing 20 mM Tris-HCl, pH 7.5, 200 μM $CaCl_2$, and 5 mM $MgCl_2$. The binding mixture was incubated for 10 min at the desired temperature before adding the protein. In the case of PKC α, 0.1 μg of protein was used, and for the PKC α-C2 domain, 0.5 μg of protein was added. The binding assay ended after 20 min at the desired temperature, and the vesicle-bound protein was separated from the free protein by centrifuging the mixture at 15000*g* for 45 min at the binding temperature. Control experiments have been performed to discard protein interferences from the cell lysate by using wild type-PKC α expressed by recombinant baculovirus and highly purified from insect cells. The PKC α bound to lipids was detected by Western blotting as previously described (*43*). The amount of sample loaded into the SDS−polyacrylamide gel was in the linear range of detection of the chemiluminescence Western blotting kit.

PKC α-C2 domain bound to lipids was detected by checking aliquots from the supernatants and pellets by silver staining of a 15% SDS−PAGE. Quantification was carried out by densitometry.

Construction of Expression Plasmids. Clonation of the DNA fragment corresponding to the C2 domain of PKC α (residues 158−258) and expression and purification of this 6His-PKC α-C2 domain was performed as described by García-García et al. (*45*).

RESULTS

Thermal Studies of the Lipid Mixtures. Differential scanning calorimetry was used to study the physical state of the lipid mixture used to activate PKC α, which was POPC/POPS at 4:1 molar ratio with increasing proportions of 1,2-DPG and in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}, i.e., the same concentrations of cations used in the assay of enzymatic activity of PKC α. Figure 1 shows that the POPC/POPS sample in the absence of 1,2-DPG had a transition peak with an onset at −3 °C. This peak was not very cooperative, as was to be expected given the effect of Ca^{2+}

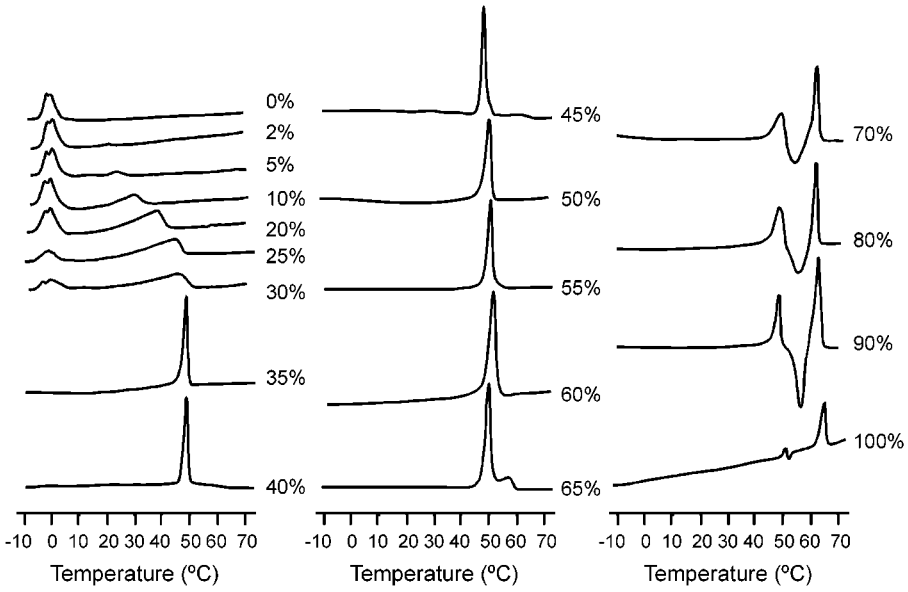

FIGURE 1: DSC heating thermograms of aqueous dispersions of mixtures of POPC/POPS at a 4:1 molar ratio containing DPG in the presence of 200 μM $CaCl_2$ and 5 mM$MgCl_2$. The mol % of DPG in the mixture is indicated on the thermograms. The fourth scan is shown for each sample.

and Mg^{2+} on POPS (*16*, *36*), and had a peak split into two, indicating phase separation of both phospholipids in the presence of the cations. The inclusion of only 2 mol % of 1,2-DPG produced a very clear effect, giving rise to the appearance of a new small transition centered at 21 °C together with a transition at the same temperature as the initial phospholipid mixture. At 5 mol %, a pattern very similar to that of 2 mol % was found, with two peaks, one at 24 °C and the other the transition appearing at the same temperature as the pure phospholipid mixture. At 10, 20, 25, and 30 mol %, the pattern was similar to those previously described, although it was noticed that the peak appearing at the same temperature as pure phospholipid (−3 °C) decreased in size as the DPG content was increased, and the second peak shifted to higher temperatures as the concentration of DPG was increased. In this way, the second peak was observed to be centered at 30, 37, 42, and 45 °C for 10, 20, 25, and 30 mol % DPG, respectively. At 35 mol % DPG, the peak appearing at the same temperature as pure phospholipid was no longer observed, and the only one remaining was centered at 49 °C. Very similar transitions were observed between 40 and 60 mol %. Things changed at 65 mol % with a small transition appearing at a temperature slightly higher than that of the main transition peak centered at 49 °C. At 70 mol %, the pattern was totally different with the appearance of a second peak located at a higher temperature, which must correspond to the separation of free DPG. Therefore, an endothermic transition at 49 °C preceded an exothermic transition and another endothermic one centered at 66 °C. Phase separation of excess DPG started at 65 mol %, as indicated by the small additional peak observed. The same pattern was observed for the samples containing 80 and 90 mol % DPG, indicating the presence of pure DPG. Pure diacylglycerols have been described as producing this type of behavior, even when mixed with phospholipids (*10*). The first endothermic transition corresponds to the melting of the metastable α-form centered at about 50 °C, and the exothermic transition corresponds to the transformation of the stable crystalline β′-form that melts endothermically at 66 °C (*11*, *46*, *47*). The problem arising, in this case, is that there is an overlapping of the peak corresponding to the phospholipid−diacylglycerol complex and the peak of the transition of the α-form.

Infrared Spectroscopy Measurement of the Phase Transition. To further clarify the origin of the different transitions observed in the thermograms obtained by DSC, infrared spectroscopy was used to monitor the phase transitions of mixtures of POPC/POPS (4:1 molar ratio) containing different proportions of 1,2-DPG-d_{62}. The frequencies of the maxima of the CH_2 and C^2H_2 symmetric stretching vibration of the lipid acyl chains were plotted as a function of temperature. It is well established that a shift in the frequency of this vibration is a reliable index of the gel to liquid-crystalline phase transition in phospholipid aqueous dispersions (*48*, *49*). The pure phospholipid mixture (monitoring the CH_2 band) had a transition with an onset at about −3 °C (Figure 2A). When the sample that contained 20 mol % of 1,2-DPG-d_{62} was studied, the maximum of the phospholipid CH_2 symmetric stretching vibration changed with the temperature and showed a broad transition, with a profile that suggested the existence of two different components in this transition that started at about 9 °C and ended at about 36 °C (Figure 2A). This range of temperatures was quite similar to that found in DSC for the sample containing the same lipid composition (Figure 1). When the behavior of the 1,2-DPG-d_{62} was followed through its C^2H_2 stretching vibration, it was noted that this transition was narrower than that of the CH_2 since it started at 29 °C and ended at about 38 °C (Figure 2B), which seemed to account for the second component observed in the broad transition of the CH_2 stretching vibration. It seems, then, that there may be a phase separation between a phase that melts at a lower temperature and that is composed mainly of phospholipid with a very small amount of 1,2-DPG, if any, and another phase containing most of the 1,2-DPG and a fraction of the phospholipids. The broad transition of the phospholipid probably corresponds to the broad peak observed in DSC

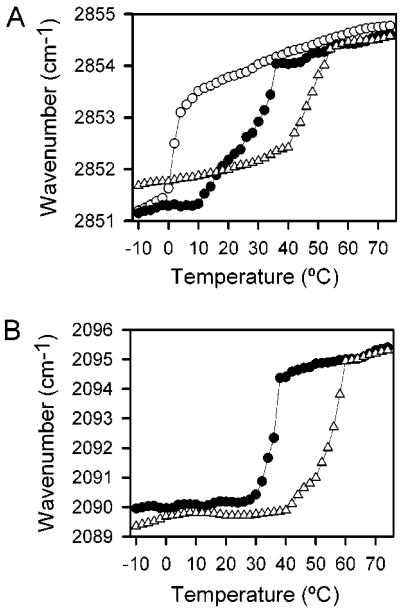

FIGURE 2: Temperature dependence of the frequency of the symmetrical CH_2 (A) and symmetrical C^2H_2 (B) stretching of mixtures containing POPC/POPS (4:1 molar ratio) and 0 mol % 1,2-DPG-d_{62} (○), 20 mol % 1,2-DPG-d_{62} (●), and 50 mol % 1,2-DPG-d_{62} (△), in the presence of $CaCl_2$ 200 μM and $MgCl_2$ 5 mM.

for the sample containing 20 mol % of 1,2-DPG in POPC/POPS (Figure 1).

The sample containing 50 mol % 1,2-DPG-d_{62} has a transition, as observed through its CH_2 stretching vibration, at about 41 °C (Figure 2A), which coincided with the transition temperature observed though the C^2H_2 stretching vibration (Figure 2B). Therefore, both the phospholipids (CH_2) and DPG (C^2H_2) underwent the transition at the same temperature, and indicating that both lipids are well mixed in this sample.

^{31}P NMR Spectroscopy. The effect of DPG on the phase polymorphism of the POPC/POPS mixture (4:1 molar ratio) was investigated by ^{31}P NMR spectroscopy. The spectra of aqueous dispersions of lipid mixtures at various temperatures and in the presence of Ca^{2+} and Mg^{2+} are depicted in Figure 3. In the absence of DPG, at all the temperatures studied, the phospholipid mixture gave rise to an asymmetric line shape with a high-field peak and a low-field shoulder (Figure 3A), characteristic of an axially symmetrical shift tensor and consistent with the arrangement of the phospholipids in a bilayer configuration. The line shapes are broad with a chemical shift anisotropy ($\Delta\sigma$) of 43 ppm at 25 °C, 42 ppm at 35 °C, and 41 at 70 °C. According to the DSC results shown above, it is already in the fluid state at all the temperatures studied.

At 20 mol % (Figure 3B), the line shapes are similar to those seen at the same temperatures in pure phospholipids, with a $\Delta\sigma$ at 25 °C of 56 ppm, 42 ppm at 35 °C, and 41 ppm at 70 °C. According to DSC, the sample undergoes the second phase transition ending at about 41 °C, and therefore the sample at 25 °C is partially in the gel state, and the sample at 35 °C may also have a small percentage of phospholipids in the gel phase. In constrast, the sample at 70 °C is already in the fluid condition.

At 40 mol % of DPG (Figure 3C), the system at 25 and 35 °C is in the gel state, as shown by DSC (see Figure 1).

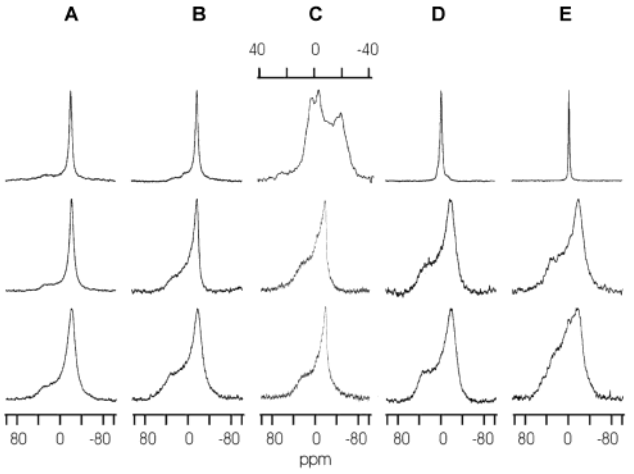

FIGURE 3: ^{31}P NMR spectra of aqueous dispersions of mixtures of POPC/POPS at 4:1 molar ratio as a function of the temperature and DPG content in the presence of $CaCl_2$ and $MgCl_2$: (A) 0 mol % of DPG, (B) 20 mol % of DPG, (C) 40 mol % of DPG, (D) 60 mol % DPG, and (E) 80 mol % DPG. Temperatures are shown for each case. Note that the parts per million scale of the 40 mol % sample at 70 °C has been amplified to allow a better observation of this complex spectrum.

The line shape of the corresponding ^{31}P NMR spectra (Figure 3C) shows broad asymmetric patterns, which are indicative of a bilayer gel phase with a $\Delta\sigma$ of 65 ppm at 25 °C and 64 ppm at 35 °C. At 70 °C, the system is above the phase transition according to DSC (Figure 1) and the line shape of the corresponding spectrum is different, with an isotropic component superimposed on other components that present axially anisotropic spectra. The spectrum showed a chemical shift anisotropy of an opposite sign to that of the lamellar phase, which is characteristic of a phase of cylindrical symmetry and corresponds to an inverted hexagonal H_{II} phase, although a lamellar component also seems to be present. The heterogeneous phase composition of this sample explains the observed $\Delta\sigma$ value of 35 ppm, which is too high for a pure H_{II} phase spectrum, which has been described to have a $\Delta\sigma$ of only 20 ppm (*50*, *51*).

At 60 mol % of DPG (Figure 3D) at 25 and 35 °C, i.e., below the phase transition temperature as indicated by DSC (see Figure 1), the spectra are broad and anisotropic, with $\Delta\sigma$ of 64 ppm both at 25 °C and at 35 °C, as is to be expected from a bilayer in the gel state. Above the phase transition temperature, as for example at 70 °C, a predominantly isotropic spectrum was found, although very small anisotropic components could be detected.

Finally, at a concentration of 80 mol % of DPG (Figure 3E) and at 25 and 35 °C, the sample was again below the phase transition temperature, according to DSC (Figure 1), and the ^{31}P NMR spectra showed broad anisotropic line shapes, characteristic of a lamellar gel phase, with a $\Delta\sigma$ of 67 ppm for 25 °C and 64 ppm for 35 °C. However at 70 °C, i.e., above of the phase transition, the spectrum was clearly isotropic.

POPC/POPS/DPG Phase Diagram. Using the data obtained through DSC and ^{31}P NMR, a phase diagram was constructed for samples in the presence of Ca^{2+} and Mg^{2+} (Figure 4). The phase boundaries of the solidus and fluidus lines were established from the respective onset and completion temperatures of the scans of mixtures of POPC/POPS

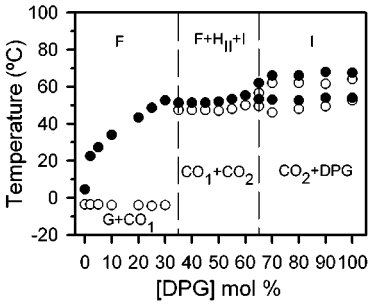

FIGURE 4: Phase diagram for aqueous dispersions of POPC/POPS (4:1 molar ratio) containing DPG constructed from data derived from differential scanning calorimetry and ^{31}P NMR. The open and closed circles were obtained from the temperature of the onset and completion temperature, respectively, of the heating scans, in the presence of Ca^{2+} and Mg^{2+}. G and F denote phospholipid in gel and fluid phases, respectively. H_{II} and I are inverted hexagonal and isotropic phases, respectively. CO_1 and CO_2 denote phospholipid/diacylglycerol complexes.

(4:1 molar ratio) with variable percentages of DPG. ^{31}P NMR was used to characterize the phase organization of samples at the different temperatures. The phase diagram seems to be characterized by the presence of two complexes, and therefore three regions, as can also be seen in other diacylglycerol/phospholipid mixtures of both saturated DMPC and DPPC (*10*, *11*, *35*) and unsaturated POPC and POPS (*40*). Two eutectic points are proposed, one at very low DPG concentration and the other at a DPG concentration close to 65 mol % of DPG. A first complex seems to be formed at a concentration of about 35 mol % (CO_1), and this is proposed on the basis of the heating and the cooling scans (not shown for the sake of brevity) in which a very narrow peak centered at 48 °C appeared at 35 mol %. A second pure complex was formed at about 65 mol % (CO_2), as suggested by the fact that pure DPG appeared phase-separated at higher concentrations. Solid−solid immiscibilities are suggested by the solidus lines of the phase diagram. In region I of the phase diagram, i.e., from 0 to 35 mol % of DPG and below the phase transition, the immiscibility was between the first complex formed and the free phospholipid. However, above the phase transition there was good miscibility. In region II, between 35 and about 65 mol % of DPG, there was solid−solid immiscibility in the gel phase between the first and the second complexes, i.e., the one with 35 mol % of DPG and the other with 65 mol % of DPG. In the fluid phase, on the other hand, there was coexistence of a lamellar phase, an inverted hexagonal H_{II} phase and an isotropic phase. Free DPG began to separate at concentrations higher than 65 mol %. In region III, there was phase separation in the gel phase between the gel form of the second complex and free DPG. In the fluid phase, only an isotropic phase was detected by ^{31}P NMR, corresponding most probably to a cubic phase, according to detailed studies involving other diacylglycerol/phospholipid systems (*11*, *52*−*54*).

PKC α Activity and Binding to Membranes as a Function of the Concentration of Diacylglycerol. When DPG was used, measurements were performed at 27 °C, so that the system was below the phase transition at all the concentrations of DPG tested above 10 mol %, and it was only above the phase transition at concentrations lower than 10 mol %. It was not possible to study the activity as a function of DPG

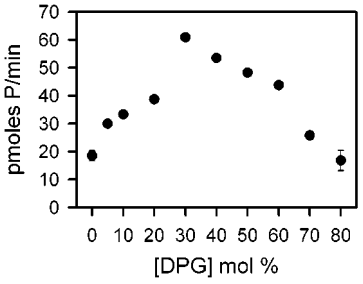

FIGURE 5: PKC α-WT activity as a function of the increasing percentage of DPG in aqueous dispersions of POPC/POPS (4:1 molar ratio) at 27 °C in the presence of 200 μM $CaCl_2$ and 5 mM $MgCl_2$. Mean values and error bars obtained from three experiments are shown.

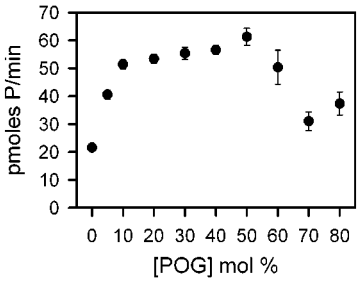

FIGURE 6: PKC α-WT activity as a function of the increasing percentage of POG in aqueous dispersions of POPC/POPS (4:1 molar ratio) at 32 °C in the presence of 200 μM $CaCl_2$ and 5 mM $MgCl_2$. Mean values and error bars obtained from three experiments are shown.

concentration in the fluid state since this system undergoes phase transition over than 40 °C at concentrations higher than 20 mol % DPG. Figure 5 shows that the activity of PKC α increased considerably with low concentrations of DPG (5 mol %), reaching a maximal at 30 mol %. The activity declined from 30 mol % onward, reaching very low values at 70 and 80 mol %.

The influence of DPG concentration on the binding of the enzyme showed that PKC α was bound to practically the same extent at all the concentrations tested (between 0 and 80 mol % of DPG) up to about 80% of the total protein (data not shown).

To ascertain whether the behavior of the enzyme was similar for other isomers of DAG, enzyme activity and binding were also examined in the presence of the unsaturated diacylglycerol POG. When the dependence of PKC α activity on POG concentration was studied at 32 °C, which is above the phase transition (*41*), it increased substantially at low concentrations, e.g., as 5 and 10 mol %, remained practically stable up to 40 mol % [similar to that studied in a previous report (*41*)], and reached maximal values at 50 mol %, after which it decreased (Figure 6). Very little activity however was observed at 12 °C, which is below of the phase transition (*41*) (data not shown).

When the binding to membranes containing POG was studied at the same temperature of 32 °C, it was observed that it did not change appreciably at different POG concentrations, being about 70−80% at all the concentrations studied (data not shown).

These results indicated that in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+} and with POPS present in the membranes, the enzyme is able to bind maximally even in the total absence of diacylglycerol, probably through the C2

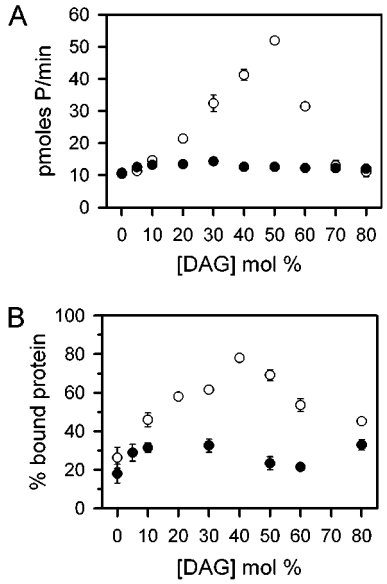

FIGURE 7: PKC α mutant (D246/248N) activity (A) and binding to membrane (B) as a function of the increasing percentage of DPG (●) or POG (○) in aqueous dispersions of POPC/POPS (4:1 molar ratio) at 27 or 32 °C, respectively, in the presence of 200 μM $CaCl_2$ and 5 mM$MgCl_2$. Mean values and error bars obtained from three experiments are shown.

domain, which is known to interact with Ca^{2+} and phosphatidylserine (*55*). Note that binding was not accompanied by maximal activation, which was only achieved in the presence of a sufficiently high concentration of DPG or POG, suggesting the involvement of the binding of the C1 domain.

The above interpretation was further confirmed by a study of the binding of the isolated C2 domain to membranes containing increasing proportions of DPG, showing that the domain was maximally bound (at about 75−85% of the total protein) at all the concentrations of DPG and even in its total absence (data not shown).

Since the above results suggested that the patterns of activation by different DAGs shown in Figures 5 and 6 seem to depend then on the interaction of diacylglycerols with the C1 domain, we used a double mutant of the enzyme, namely, D246/248N, in which the C2 domain is rendered unable to bind Ca^{2+}, and so any binding and activity observed will be due to the C1 domain. Residues D246 and D248 are known to be involved in the binding of Ca^{2+} in the C2 domain of PKC α (*55*), and these two mutations inhibit the binding of the C2 domain to membranes, both in vitro and in vivo (*56*). It was interesting in this context that this mutant was not activated by the addition of DPG at any concentration but was increasingly activated by POG, as its concentration increased, reaching a maximum at about 50 mol %, and showing a steep decrease at higher concentrations, which is reminiscent of the pattern shown by the wild type (Figure 7A). It was also of note that the observed pattern of activity was well correlated with the pattern of binding (Figure 7B), so that in the presence of POG, binding reached a maximum at 40 mol % but decreased at higher concentrations, showing therefore a similar dependence on POG concentration as the enzyme activity. On the other hand, the binding was much reduced in the presence of DPG and was not appreciably changed by increased concentrations of DPG. Hence, it was clear that the introduction of unsaturation in one of the acyl chains of the diacylglycerol greatly modified its capacity to activate the enzyme.

DISCUSSION

In this paper, we have tried to correlate the physical properties of a lipid mixture with its influence on the binding of PKC α to membranes and its activity parameters. The lipid mixture selected was formed of unsaturated phospholipids resembling those of biological membranes and either saturated (DPG) or unsaturated (POG) diacylglycerols. It has been described, in fact, that both types of diacylglycerol may be present during cell stimulation, which is often biphasic: during the early stages, the accumulated DAGs are mainly monounsaturated or saturated in the acyl *sn*-1 position and polyunsaturated in the acyl *sn*-2 position, whereas during the sustained phase the predominant DAG types bear saturated acyl chains in both positions or a saturated chain in position *sn*-1 and a monounsaturated chain in position *sn*-2 (*57*). Note that according to the results described above the C2 domain is very important for activation during the sustained phase, when saturated diacylglycerols, such as DPG, are abundant and when they cannot activate an enzyme form with a disabled C2 domain.

While the physical characteristics of the system containing POG has already been described in a previous paper from this laboratory (*41*), we now carry out the physical characterization of the DPG containing system. The lipid mixtures were characterized by including the same concentrations of Ca^{2+} and Mg^{2+} as were used in the enzymatic assays, and lamellar structures were maintained, at least at low DAG concentrations, because POPC/POPS mixtures with a 4:1 molar ratio were used. It is known that using pure POPS will lead to a cochleate phase in the presence of Ca^{2+} and Mg^{2+} (*58*−*60*) and that this type of phase lacks a phase transition in the physiological range of temperatures used in this work (*61*). However, we showed previously by FT-IR that a POPC/POPS mixture at a 4:1 molar ratio was not dehydrated by Ca^{2+} and Mg^{2+}, when the Ca^{2+}/POPS molar ratio was 2.6 and Mg^{2+}/POPS was 66, i.e., very similar values to those used in this paper (*62*). When POPC amounted to 80 mol % of the total phospholipid, this protects POPS from the formation of a cochleate phase, even in the presence of 33 mol % of 1,2-dioleoylglycerol (*62*).

We have tried to correlate the activation of PKC α at different concentrations of diacylglycerol with the physical state of the membrane, since we have shown that changing DAG concentration has a great effect on membrane organization.

In the case of the system containing DPG, maximum activity was reached at the DPG concentration at which the pure compound CO_1 was obtained (30 mol %), which may be because the coexistence of separate domains (one of them rich in DAG) stimulates the activity, as has been previously suggested (*35*). Another possibility may be that the increase in CO_1 complex may contribute to increasing the activity, while the CO_2 complex, which begins to appear at concentrations of DPG higher than 30 mol %, would be less likely to be adequate to promote PKC activity. In the case of POG-induced activation, activity increased with increasing POG concentrations, up to 10 mol %, when it reached a plateau. Note that in this case the activity is measured above of the

phase transition and therefore no phase separation seems to occur.

It is nevertheless clear that the wild-type enzyme is bound to the membrane to the same extent in the whole range of DPG concentrations studied perhaps due to lipid−protein interactions established either through the C1 or the C2 domain. However, since we have observed that the isolated C2 domain binds maximally at all the concentrations of DPG and even in its absence, it is clear that DPG must affect only the activation of the enzyme through the C1 domain and not through the C2 domain, despite the fact that at very high concentrations of DPG a substantial dilution of POPS may take place. This dilution probably has no effect on the activity since the excess of DPG accumulating in region III of the phase diagram will be phase-separated from compound CO_2, where the POPS is located.

To further clarify the role of the C1 domain, the double mutant D246/248N was used. This mutant was previously characterized in our laboratory and was shown to have a very limited capacity to be activated by phospholipids and Ca^{2+} (*56*) because the mutated residues are involved in the binding of Ca^{2+} to the C2 domain (*55*). According to our results, the D246/248N mutant was not activated by DPG and was not able to bind to the membrane to any significant extent in the presence of this DAG. This showed that DPG was not able to compensate for the decrease in binding capacity brought about by the mutation in the C2 domain responsible for the binding through Ca^{2+} and phosphatidylserine. However, in the presence of POG, the enzyme could be activated to the same extent as the wild type at a sufficiently high concentration (50 mol %), which correlates quite well with the extent binding. The question is why POG is a better activator than DPG. The answer is a higher capacity to induce binding of the mutant enzyme to the membrane. An explanation for this behavior has been suggested previously (*33*); unsaturated diacylglycerols produce an increase in the hydrocarbon volume and also an increase in the headgroup spacing, which may facilitate the insertion of the enzyme in the membrane. Alternatively, since the system containing POG was in a fluid condition, whereas that containing DPG was below the phase transition, another possibility is that it is more difficult for the enzyme to insert its C1 domain in a rigid than in a fluid membrane. This could not be tested by assaying PKC below the phase transition in the presence of POG, since the activity of the enzyme was very low even at temperatures of around 12 °C. It should be remembered here that we have previously shown that in Triton X-100 mixed micelles both DPG and POG showed a similar capacity to activate PKC (*62*), and hence their different abilities seen here are likely to be due to effects on membrane structure rather than to differential recognition on the part of the C1 domain.

The other question is why at very high POG concentrations activity decreases with decreasing membrane binding, as observed at temperatures higher than the phase transition. One possible explanation may be the formation of cubic phases which are known to be adopted by mixtures of phospholipids and diacylglycerols with high concentrations of the latter (*52*, *53*) and which may be less suited to supporting PKC activity.

In the case of PKC, there is abundant evidence to support the interaction between PKC α and membranes. It was concluded from monolayer experiments (see for example refs *63*−*65*) that PKC α penetrates the membrane at certain lipid concentrations. Also, Souvignet et al. (*64*) deduced from monolayer experiments that those hydrophobic interactions between PKC and lipid acyl chains are essential for PKC activity. Medkova and Cho (*65*) concluded recently that PKC α partially penetrates the membrane during its activation by phosphatidylserine. There are also a number of studies suggesting that the activation of PKC by diacylglycerols may involve a specific diacylglycerol-induced perturbation of the structure of the membrane phospholipid bilayer. For example, it was demonstrated that changes in the acyl-chains of the membrane lipids affect both the lipid-dependent activation of PKC and the action of diacylglycerols (*33*, *66*). It is clear, on the other hand, that diacylglycerols strongly affect membrane structure (see references given in the introduction section, and for a recent review ref *67*). All these arguments thus support the possibility that a diacylglycerol with an unsaturated chain, such as POG, may be a better promoter of PKC membrane-binding than a totally saturated molecule like DPG.

In summary, we have characterized the physical properties of lipid mixtures formed by a phospholipid mixture consisting of molecules bearing one saturated and one unsaturated acyl chain, so that they resemble to a certain extent the molecules present in animal membranes, with two different types of diacylglycerols: DPG and POG. Although both diacylglycerols were able to activate PKC α, a double mutant rendered unable to bind Ca^{2+} and phospholipid through its C2 binding domain was activated by POG but not by DPG. This was matched by a capacity to promote POG binding, whereas DPG was unable to promote the binding of this double-mutant of PKC α activity.

ACKNOWLEDGMENT

We are very grateful to Dr. Ono and Dr. Nishizuka for providing us with the recombinant cDNA coding for protein kinase C α. A.T. is a recipient of a fellowship from Fundación Séneca (Murcia, Spain).

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BI015599+