Influence of the Physical State of the Membrane on the Enzymatic Activity and Energy of Activation of Protein Kinase C α†

Antonia M. Jiménez-Monreal, Francisco J. Aranda, Vicente Micol,‡ Pilar Sánchez-Piñera, Ana de Godos, and Juan C. Gómez-Fernández*

Departamento de Bioquímica y Biología Molecular “A”, Facultad de Veterinaria, Universidad de Murcia, Apartado de Correos 4021, E–30080–Murcia, Spain

Received December 29, 1998; Revised Manuscript Received March 16, 1999

ABSTRACT: The activation of protein kinase C α was studied by using a lipid system consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) (molar ratio 4:1) and different proportions of 1-palmitoyl-2-oleoyl-sn-glycerol (POG). The phase behavior of the lipidic system was characterized by using differential scanning calorimetry and 31P NMR, and a phase diagram was elaborated. The results suggested the formation of two diacylglycerol/phospholipid complexes, one at 15 mol % of POG and the second at 30 mol % of POG. These two complexes would define the three regions of the phase diagram: in the first region (concentrations of POG lower than 15 mol %) there is gel–gel immiscibility at temperatures below that of the phase transition between C1 and pure phospholipid, and a fluid lamellar phase above of the phase transition. In the second region (between 15 and 30 mol % of POG), gel–gel immiscibility between C1 and C2 with fluid–fluid immiscibility was observed, while inverted hexagonal H2 and isotropic phases were detected by 31P NMR. In the third region (concentrations of POG higher than 30 mol %), gel–gel immiscibility seemed to occur between C2 and pure POG along with fluid–fluid immiscibility, while an isotropic phase was detected by 31P NMR. When PKC α activity was measured, as a function of POG concentration, maximum activity was found at POG concentrations as low as 5–10 mol %; the activity slightly decreased as POG concentration was increased to 45 mol % at 32 °C (above Tc) whereas activity did not change with increasing concentrations of POG at 5 °C (below Tc). When the activity was studied as a function of temperature, at different POG concentrations, and depicted as Arrhenius plots, it was found that the activity increased with increasing temperatures, showing a discontinuity at a temperature very close to the phase transition of the system and a lower activation energy at the upper slope of the graph indicating that the physical state of the membrane affected the interaction of PKC α with the membrane.

Protein kinases C (PKCs)† are a family of enzymes constituted by at least 12 isoenzymes which are involved in many cellular processes and which are major receptors of tumor promotors such as bryostatin and phorbol esters (see refs 1–3 for comprehensive reviews on PKCs). Of the different PKC isoenzymes, it is PKC α that will receive attention in this paper. This isoenzyme, which belongs to the classic type, is activated by the diacylglycerols generated by phospholipases after cell stimulation, and also by phosphatidylerine and Ca2+. It is thought that membrane structure may be important in determining the activation of PKC α (3, 4).

There is a great deal of information suggesting that membrane structure is modulated by the presence of DAGs. For example, it has been shown that DAGs may produce membrane structural changes in membranes such as lateral phase separations (5–10), nonbilayer phases (6, 7, 11–13), and dehydration of the membrane interface (13, 14). Significantly, the dehydration produced by DAG has more drastic effect on phosphatidylserine than on phosphatidylcholine (13). These effects may be responsible for the facilitating effect of membrane fusion (15–19) and perhaps for the activation not only of PKC but also of other enzymes such as phospholipases (20–22), CTP phosphocholine cytidylyltransferase (23), and tyrosine kinase (24).

On the other hand, several suggestions have been made concerning the modulation of PKC activity by DAGs. It has been suggested 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 phosphatidylethanolamines, which

† This work was supported by Grants PB95-1022 and PB96-1107 from Dirección General de Enseñanza Superior (Spain).
‡ To whom correspondence should be addressed. Tel: +34-968364766; Fax: +34-968364147. E-mail: jcgomez@fcu.um.es.
‡ Present address: Centro de Biología Molecular y Celular. Universidad Miguel Hernández. C/ Monóvar s/n. E-03206 Elche, Alicante, Spain.

1 Abbreviations: DAGs, diacylglycerols; 1,2-DAG, 1,2-dimyristoyl-sn-glycerol; DMP, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-sn-glycero-3-phosphoserine; 1,2-DPG, 1,2-dipalmitoyl-sn-glycerol; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSC, differential scanning calorimetry; Δν, chemical shift anisotropy; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PC, phosphatidylcholine; PKC, protein kinase C; 31P NMR, 31P-nuclear magnetic resonance; POG, 1-palmitoyl-2-oleoyl-sn-glycerol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine.
have small polar headgroups (9, 11, 25, 26). It has also been suggested that the presence of DAGs increases the spacing between phospholipid headgroups because of the interposition of the small groups of DAGs (11, 27–30). It might also be possible that the effect of membrane surface dehydration induced by DAGs could facilitate immersion of the protein in the membrane (14). Finally, it has recently been suggested that membrane heterogeneity, involving the coexistence of rich and poor phases in DAGs, may contribute to the activation of PKC (10, 31).

There are a number of reports that indicate that PKC activity is modulated by other membrane structural properties (29, 32, 33). It was shown, for example, that PKC activity is sensitive to acyl chain unsaturation (29), the effects of unsaturation probably arising from alterations in headgroup spacing (29, 30). PKC activity is also sensitive to phosphatidylethanolamine, and this has been attributed to its tendency to disrupt the bilayer structure of the membrane (34, 35).

We will present in this paper our studies on the activation of PKCα by an unsaturated lipid system, formed by POPC/POPS (4:1 molar ratio) and 1,2-POG.

**MATERIALS AND METHODS**

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and 1-oleoyl-2-palmitoyl-sn-glycerol (POG) were purchased from Avanti Polar Lipids (Alabaster, Alabama). POG was also prepared from POPC 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 diacylglycerol was determined by thin-layer chromatography on plates of silica gel 60 (Macherey-Nagel, Düren, Germany) using chloroform/methanol (94.5:5.0:0.5, v/v/v) as a solvent.

**DSC Measurements.** Samples containing 3 μmol of phospholipid, the appropriate amount of POG, and ionophore A23187, at a molar ratio phospholipid/ionophore of 1000:1, were dried under a stream of N2, 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 6.25 mL of 20 mM Tris-HCl, pH 7.5, for 15 min at a temperature above transition with occasional and vigorous vortexing. After this period, CaCl2 and MgCl2 were added to give final concentrations of 200 μM Ca2+ and 5 mM Mg2+, that is, the same concentrations of these cations used for the enzymatic assays, so that the Ca2+/POPS molar ratio was 2.1 and Mg2+/POPS was 52. Samples were incubated at the mentioned temperature for an additional period of 60 min and left to cool slowly to 20 °C in a water bath at a cooling rate of approximately 0.3 °C/min. Samples were centrifuged at 16000g for 30 min, 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 as a reference. The DSC instrument was calibrated using indium as standard. The samples were scanned over a temperature range from −10 to 40 °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, but it provided no better resolution of the thermograms). The scans were repeated until identical profiles were obtained. Normally, the third scan was used for transition calculations. To determine the acyl migration of 1,2-POG to 1,3-POG after the experiments, we analyzed the different samples as previously described (8) leading to the formation of no more than 7% 1,3-POG of total diacylglycerol. Organic phosphorus was analyzed (36) in order to quantify the phospholipid present in each sample.

**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 from 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 (37) with slight modifications. A 2 L culture of SF9 insect cells at 3 × 106 cells/mL was infected with the recombinant baculovirus. Cells were harvested 60 h postinfection (cell viability of 70%), pelleted at 1500g 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 Na3VO4, and 50 mM NaF). The pellet was disrupted by sonication (6 × 10 s), and the resulting lysate was centrifuged at 10000g for 60 min. The supernatant was applied in a batch mode to a 100 mL diethylaminoethyl-Sepharose 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 application of a linear gradient (0–0.5 M NaCl) at a flow rate of 1 mL/min. The PKC-containing fractions were then applied to a 10 mL protamine agarose 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 application of a linear gradient (0–1.5 M NaCl). Fractions containing PKCα activity were pooled and loaded onto a 10 mL phenyl Sepharose column, and PKC was then eluted in the same buffer with a linearly descending salt gradient (1–0 M NaCl). The pure PKCα, as seen by a silver-stained SDS–polyacrylamide gel, was stored at −80 °C in the presence of 10% glycerol and 0.05% Triton X-100.

**Determination of Protein Kinase C Activity.** Lipids for use in the reaction were dried under a stream of N2, and the last traces of organic solvent were removed by keeping the samples under vacuum for 2 h. The lipids were then 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 than unilamellar extruded vesicles and were used for all of the assays described. MLVs also have the advantage of being more informative for the type of DSC and 31P NMR studies that we have carried out here. Liposomes were added to the reaction to a final total phospholipid concentration of 480 μM, and the diacylglycerol was varied from 0 to 45 mol % of the total lipid. The reaction medium (final volume 250 μL) contained 20 mM Tris-HCl, pH 7.5, 0.2 mg/mL histone III-S, 20 μM γ-[32P] ATP (300000 cpm/nmol), 5 mM MgCl2, and 200 μM CaCl2. Therefore Ca2+/POPS molar ratio was 2.1, and Mg2+/POPS was 52. The reaction mixture was incubated for 2 min at the desired temperature, and then the reaction was started by the addition of 25 μL (0.5 μg) of the diluted enzyme. The reaction was terminated after 30 min for those reactions performed between 15 and 20 °C, or after 10 min for those at temperatures above 20 °C, by the addition of 1 mL of ice-cold trichloroacetic acid and 1 mL of ice-
Influence of Membrane on PKC α Activity

Biochemistry, Vol. 38, No. 24, 1999 7749

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 glassfiber 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. Activity in the absence of phospholipids was subtracted for each experiment. The data presented refer to the means of triplicate determinations (±sd). The linearity of the assay was confirmed by the time course of histone phosphorylation up to 30 min.

Assays were also made by using Triton X-100 mixed micelles, containing 3% of Triton X-100 (v/v). Lipids were solubilized by vortexing for 1–2 min and then incubating at 37 °C for 5 min. POPS was added to a final concentration of 0.5 mM, and POG was 0.24 mM.

$^{31}$P NMR Spectroscopy. The samples for $^{31}$P NMR were prepared by combining organic solutions containing 20 mg (26.7 μmol) of phospholipid with the appropriate amount of 1,2-POG, evaporating the solvents, forming multilamellar vesicles by adding 56 mL of 20 mM Tris-HCl buffer, pH 7.5, containing 200 μM CaCl$_2$ and 5 mM MgCl$_2$ to ensure that conditions were exactly the same as with the assays of enzymatic activity. The suspensions were centrifuged and $^{31}$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.

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-POG and in the presence of 200 μM Ca$^{2+}$ and 5 mM Mg$^{2+}$, that is, the same concentrations of cations used for the assay of enzymatic activity of PKC α. Figure 1 shows that the POPC/POPS sample with no 1,2-POG had a transition peak with an onset at −3 °C. This peak was not very cooperative, as it reached a value of about 15 kcal mol$^{-1}$.

FIGURE 1: DSC heating thermograms of aqueous dispersions of mixtures of POPC/POPS at 4:1 molar ratio containing POG in the presence of 200 μM Ca$^{2+}$ and 5 mM Mg$^{2+}$. The mol % of POG in the mixture is indicated on the thermograms. The third scan is shown for each sample.
depicted in Figure 3. In the absence of POG, at both 1 and after the conclusion of the phase transition (Figure 1), the bilayer gel phase with a shows a broad asymmetric pattern, which is indicative of a bilayer structure, with a chemical shift anisotropy ($\Delta \sigma$) of 65 ppm. At 35 °C, a composite spectrum was seen with an anisotropic 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 54 ppm at 1 °C and 42 ppm at 35 °C. According to the DSC results shown above, at 1 °C the system is undergoing a phase transition which begins at −7 °C and ends at 3 °C, whereas at 35 °C it is already in the fluid state.

At 5 mol % (Figure 3B) the line shapes are similar to those seen at the same temperatures in pure phospholipids, with a $\Delta \sigma$ at 1 °C of 55 ppm, and 42 ppm at 35 °C. According to DSC the sample undergoes the phase transition at 1 °C and is already in the fluid condition at 35 °C.

At 20 mol % of 1,2-POG (Figure 3C), the system at 1 °C is in the gel state, as shown by DSC (see Figure 1). The line shape of the corresponding $^{31}$P NMR spectrum (Figure 3C) shows a broad asymmetric pattern, which is indicative of a bilayer gel phase with a $\Delta \sigma$ of 65 ppm. At 35 °C, that is, after the conclusion of the phase transition (Figure 1), the line shape of the corresponding spectrum is different, with an isotropic component superimposed on another component which presents an axially anisotropic spectrum. The spectrum shows a chemical shift anisotropy of a sign opposite of that of the lamellar phase which is characteristic of a phase of cylindrical symmetry and corresponds to an inverted hexagonal HII phase, although a minor lamellar component seems to be also present. The heterogeneous phase composition of this sample explains the observed $\Delta \sigma$ value of 32 ppm which is too high for a pure HII phase spectrum, which has been described to have a $\Delta \sigma$ of only 20 ppm (39, 40).

At 40 mol % of POG (Figure 3D) at 1 °C, which is below the phase transition temperature as indicated by DSC (see Figure 1), the spectrum is broad and anisotropic, with a $\Delta \sigma$ of 64 ppm, as it is expected from a bilayer in the gel state. Above the phase transition temperature, as it is the case at 35 °C, a composite spectrum was seen with an anisotropic component, which is indicative of a bilayer structure, superimposed to an isotropic one.

Finally, at a concentration of 60 mol % of 1,2-POG (Figure 3E), at 1 °C the sample was again below the phase transition temperature, according to DSC (Figure 1), and the line shape of the $^{31}$P NMR spectrum (Figure 3C) showed a broad anisotropic spectrum, characteristic of a lamellar gel phase, with a $\Delta \sigma$ of 65 ppm. However at 35 °C, that is, above the phase transition, only an isotropic spectrum was detected.

$^{31}$P NMR Spectroscopy. The effect of POG 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 200 μM Ca$^{2+}$ and 5 mM Mg$^{2+}$ are depicted in Figure 3. In the absence of POG, at both 1 and 35 °C, 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 54 ppm at 1 °C and 42 ppm at 35 °C. According to the DSC results shown above, at 1 °C the system is undergoing a phase transition which begins at −7 °C and ends at 3 °C, whereas at 35 °C it is already in the fluid state.

At 5 mol % (Figure 3B) the line shapes are similar to those seen at the same temperatures in pure phospholipids, with a $\Delta \sigma$ at 1 °C of 55 ppm, and 42 ppm at 35 °C. According to DSC the sample undergoes the phase transition at 1 °C and is already in the fluid condition at 35 °C.

At 20 mol % of 1,2-POG (Figure 3C), the system at 1 °C is in the gel state, as shown by DSC (see Figure 1). The line shape of the corresponding $^{31}$P NMR spectrum (Figure 3C) shows a broad asymmetric pattern, which is indicative of a bilayer gel phase with a $\Delta \sigma$ of 65 ppm. At 35 °C, that is, after the conclusion of the phase transition (Figure 1), the line shape of the corresponding spectrum is different, with an isotropic component superimposed on another component which presents an axially anisotropic spectrum. The spectrum shows a chemical shift anisotropy of a sign opposite of that of the lamellar phase which is characteristic of a phase of cylindrical symmetry and corresponds to an inverted hexagonal HII phase, although a minor lamellar component seems to be also present. The heterogeneous phase composition of this sample explains the observed $\Delta \sigma$ value of 32 ppm which is too high for a pure HII phase spectrum, which has been described to have a $\Delta \sigma$ of only 20 ppm (39, 40).

At 40 mol % of POG (Figure 3D) at 1 °C, which is below the phase transition temperature as indicated by DSC (see Figure 1), the spectrum is broad and anisotropic, with a $\Delta \sigma$ of 64 ppm, as it is expected from a bilayer in the gel state. Above the phase transition temperature, as it is the case at 35 °C, a composite spectrum was seen with an anisotropic component, which is indicative of a bilayer structure, superimposed to an isotropic one.

Finally, at a concentration of 60 mol % of 1,2-POG (Figure 3E), at 1 °C the sample was again below the phase transition temperature, according to DSC (Figure 1), and the line shape of the $^{31}$P NMR spectrum (Figure 3C) showed a broad anisotropic spectrum, characteristic of a lamellar gel phase, with a $\Delta \sigma$ of 65 ppm. However at 35 °C, that is, above the phase transition, only an isotropic spectrum was detected.

$POPC/POPS/POG$ Phase Diagram. By using the data obtained through DSC and $^{31}$P NMR, we constructed a phase diagram 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 (4:1 molar ratio) with variable percentages of POG. $^{31}$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 diacylglycerols/phospholipid mixtures of both saturated DMPC and DPPC (7, 8) and unsaturated POPC and POPS (41). Two eutectic points are proposed, one at a very low POG concentration and the other at a POG concentration close to 30 mol % of POG. Apparently, POG in POPC/POPS (4:1 molar ratio) shows limited solubility in the presence of Ca$^{2+}$ and Mg$^{2+}$ so that a first complex seems to be formed...
at a concentration of about 15 mol % (C1). This is proposed on the basis of the heating and cooling scans (not shown for the sake of brevity) in which a very narrow peak centered at 27 °C appeared at 15 mol %, and from the ΔH measurements, which reached a value which remained constant, within error, up to 60 mol %. A second pure complex was formed at about 30 mol % (C2), as suggested by the fact that pure POG appeared phase separated at higher concentrations. Solid–solid and fluid–fluid immiscibilities were suggested by the solidus and fluidus lines of the phase diagram. In region I of the phase diagram, that is, from 0 to 15 mol % of POG and below the phase transition, the immiscibility was between the first complex formed and free phospholipid. However, limited solubility of the phospholipid in the first complex was observed similarly to that observed in previous works (8, 41, 42). However above the phase transition there was good miscibility. In region II, between 15 and about 30 mol % of POG, there was solid–solid immiscibility, in the gel phase between the first and the second complexes, that is, the one with 15 mol % of POG and the other with 30 mol % of POG. In the fluid phase, on the other hand, there was phase separation between a lamellar phase, an inverted hexagonal HII phase and an isotropic phase. This isotropic phase corresponds most probably to a cubic phase, according to detailed studies involving other diacylglycerol/phospholipid systems (11, 43–45). Free POG began to separate at concentrations higher than 30 mol %.

In region III, there was phase separation in the gel phase between the gel form of the second complex and free POG, although free POG showed a limited solubility in the phospholipid/POG complexes, as indicated by the low-temperature broad peak appearing at concentrations of POG of 30 mol % and higher. In the fluid phase, only, an isotropic phase was detected by 31P NMR, although the fluidus line of the phase diagram indicated phase separation.

PKC α Activity as a Function of POG Concentration.

Figure 5 shows that the activity of PKC α increased considerably with low concentrations of POG (5 mol %), compared with the control system which did not contain any POG. This was true at both 5 and 32 °C, that is below and above of the phase transition, although the 5 mol % sample at 5 °C was still undergoing phase transition. Maximal activity was obtained at 10 mol %. However, further additions of POG did not increase the activity at either temperature. On the contrary, at 32 °C there was a slight fall in activity as the concentration of POG was increased, so that at 5 and 10 mol % the specific activity was 118 nmol min⁻¹ mg⁻¹ protein, falling to 95 nmol min⁻¹ mg⁻¹ at 45 mol % (the maximum concentration of POG studied in this experiment).

Activity of PKC α Versus Temperature.

The activity of PKC α was measured versus temperature at different POG concentrations, and the results of representative experiments are presented in the form of Arrhenius plots in Figure 6. At all of the concentrations studied (10, 20, 25, and 40 mol % of POG), the plots presented a biphasic pattern with a break which defined a change in Eα. At 10 mol % the break was located at 25.0 ± 1.3 °C, and Eα leveled off at this temperature. At 20 mol % of POG the break was at 27.7 ± 1.4 °C. At 25 mol % of POG the break occurred at 25.9 ± 1.8 °C, and finally, at 40 mol %, it was found at 26.9 ± 1.4 °C that these are mean values and standard deviation obtained from 3 different experiments. As a control, assays were carried out with a Triton X-100 mixed micelle system, and the result in the range of temperatures studied was a linear plot, demonstrating that PKC α does not present a discontinuity in its Eα in the absence of a membrane undergoing a phase transition.

DISCUSSION

In this paper we have tried to correlate the physical properties of a lipid mixture with its influence on the activity parameters of PKC α. The lipid mixture selected was formed of unsaturated phospholipids, which resemble those of biological membranes more than the fully saturated phospholipids used in previous studies. Furthermore, the use of unsaturated lipids enabled us to study the effect of the phase transition temperature on enzymatic activity, since this transition takes place at relatively low temperatures. In other previous studies using saturated phospholipids from this (8) and other laboratories (10, 31), this was not possible because the transition took place at relatively high temperatures, at which the protein would be denatured. Physical characterization of the lipid mixtures was made by including the same concentrations of Ca²⁺ and Mg²⁺ as were used in the enzymatic assays, although lamellar structures were kept, at least at low POG 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 Ca²⁺ and Mg²⁺ (46–48), and this type of phase lacks a phase transition in the physiological range of temperatures used in this work (49). However we have shown previously, by using FT-IR, that a POPC/POPS mixture at a 4:1 molar ratio was not dehydrated by Ca²⁺ and Mg²⁺, when Ca²⁺/POPS molar ratio was 2.6 and Mg²⁺/POPS was 66, that is, very similar to those used in this paper (50). The presence of 80% of POPC over total phospholipid protects POPS from the formation of a cochleate phase, even in the presence of 33 mol % of 1,2-dioleoylglycerol.

The phase diagram proposed above from data obtained using DSC and 31P NMR suggests the existence of gel–gel and fluid–fluid immiscibilities, with three regions delimited by the existence of two complexes.

Changes in a number of physicochemical properties of the membrane have been related to PKC activity, as is the case for bilayer unsaturation (29), the tendency to form nonbilayer phases (9, 28, 32, 35, 51, 52), and lipid stress or frustration (33). Spacing and conformation of the lipid headgroups and the membrane interface have also been suggested to affect PKC activity (11, 13, 29, 33). Dibble et al. (10) and Hinderliter et al. (31) suggested that the lipid lateral
heterogeneity produced by coexisting diacylglycerol-enriched and diacylglycerol-poor lipid domains was responsible for the activation of PKC. Also, very recently, Goldberg and Zidovetki (53) showed that the alteration of curvature stress by compounds such as free fatty acids and diacylglycerols may modulate the activity of PKC.

In a previous paper from this laboratory describing the activation of PKC α by DMPC/DMPS (4:1, molar ratio), it was concluded that PKC α was increasingly activated as the concentration of DMG was increased, reaching a maximum at a concentration close to the appearance of a pure phospholipid/diacylglycerol complex, and the activity decreased at higher concentrations of diacylglycerol (38). In the present paper, where unsaturated lipids were used, maximal activity was also observed at a concentration of POG close to the formation of a pure complex, at least at 35 °C (above $T_c$) (Figure 5). Nevertheless, the decrease in activity observed at this temperature, at concentrations of POG where the complex is present, was only slight. Furthermore, no decrease was observed at 5 °C (below $T_c$). Although the reason for this difference in behavior between the saturated and the unsaturated systems is not clear, a possibility is that there might exist a higher degree of similarity between the structure of the membrane formed by pure phospholipid and that containing phospholipid/diacylglycerol complexes, when the lipid acyl chains are unsaturated than when they are saturated.

It should be discussed here that MLVs containing POPC/POPS were used to activate PKC α. It is possible that POG might affect the effective lipid concentration available for PKC α. This can be induced by the appearance of nonbilayer phases, as it happens in fact at temperatures above phase transition in some regions. The number of layers of the MLVs may be also affected by increasing concentrations of POG, even in those regions of the phase diagram where lamellar phases are predominant. Additionally, the presence of Ca$^{2+}$ and Mg$^{2+}$ may induce aggregation or fusion of the vesicles, and diacylglycerol may be an activator of this effect (16), at least at the higher concentrations studied, and this effect may produce a decrease in the availability of phospholipids to PKC α.

As a consequence of the circumstances described above, conflicting effects may be produced by the increase in POG concentrations. These increasing concentrations of POG may activate PKC α by direct allosteric effect on the enzyme, and by increasing the POPS available to the enzyme through the change in the structure of the MLVs vesicles. On the other hand, aggregation or fusion of MLVs vesicles may reduce the activation as less POPS would be available for the enzyme. The result of the confrontation of all these effects is that the activity is not changed as POG increased below $T_c$, and that only a slight decrease occurred, above $T_c$, as the concentration was increased from the maximum at 10 mol % of POG to 45 mol %.

Another interesting observation with respect to the influence of the membrane physical state on PKC α was obtained from the study of the enzymatic activities measured as a function of temperature. The activity changed gradually and linearly with temperature when the system was heated but kept in the gel state or in the mixed phase region where gel and liquid crystalline domains would coexist (not shown). The activity also increased linearly, as the temperature was increased, when the system was in a fully fluid condition, and the activity was not suddenly increased by the end of the membrane transition (not shown), all of that being in accord with a previous study (35). However, the activity was less sensitive to temperature when the membrane was fluid than when it was in gel state or in mixed state, and this was reflected by Arrhenius plots of these data which were biphasic, with breaks that indicate a decrease in $E_a$. Note that $E_a$ of the enzyme activated by micelles was more similar to those induced by membranes in the fluid condition than to those of the enzyme activated by membranes in the gel phase or in mixed gel and fluid conditions. The breaks observed coincided with melting of the lipid mixtures, as indicated by the DSC results: at 10 mol % of POG, the break in the activity occurred at 25 ± 1.3 °C and the end of the transition at 27 °C; at 20 mol % the corresponding temperatures were 27.7 ± 1.4 and 28 °C; at 25 mol %, 25.9 ± 1.8 and 28 °C; and at 40 mol %, 26.9 ± 1.4 and 28 °C. It is clear, then, that the phase transition of the membrane has a very important influence on the $E_a$ of PKC α.

Arrhenius plots have been widely used for many years to study the influence of the physical properties of membrane on membrane-related enzymes (54). For example, a close correlation between the break temperature of the Arrhenius plots and the $T_c$ values of the membrane lipid phase was established for the lactose permease system of Escherichia coli (55, 56). Another illustrative example is that of NADH-cytochrome b$_5$ reductase, for which breaks in the Arrhenius plots were interpreted in terms of phase changes in membrane lipids (57). More recently (38) biphasic Arrhenius plots were observed for the $(Na^+ + Mg^{2+})$-ATPase purified from Achloleplasma laidlawii B membranes, reconstituted into large, unilamellar vesicles formed from DMPC and variable amounts of cholesterol, and the results were interpreted in terms of the activity being determined by the physical state of the membrane. Nevertheless, the reasons for the correlation between the discontinuities in the Arrhenius plots and changes in the physical properties of the membrane are not always clear when attempting to describe a detailed molecular mechanism, but it was suggested that these discontinuities may be related with modifications in protein solubility in the lipid phase (55).

In the case of PKC, there is abundant evidence to support the interaction between PKC α and membranes. It was concluded, for example (59), from monolayer experiments that PKC penetrates the membrane at certain lipid concentra-
tions. Also, Souvignet et al. (60) deduced from monolayer experiments that those hydrophobic interactions between PKC and lipid acyl chains are essential for PKC activity. Medkova and Cho (61) 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 (29, 62). 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 63).

In the case examined here, the change in the membrane which takes place with the phase transition, detected through DSC, leads to a fluid bilayer membrane in region I of the phase diagram and to nonbilayer phases in regions II and III. It is obvious that penetration of PKC would be facilitated by the appearance of fluid $L_\alpha$ phase or nonbilayer phases such as inverted hexagonal $H_\text{II}$ or isotropic phases (most probably cubic). The packing of the phospholipid headgroups would be less dense, and hence, the enzyme would have a greater facility to penetrate the membrane. Therefore the energy of activation would decrease (as was observed in our measurements).

In summary, we have characterized the physical properties of a lipid mixture which consists of molecules bearing one saturated and one unsaturated acyl chain, meaning that they resemble to a certain extent the molecules present in animal membranes. Although this system was able to activate PKC $\alpha$, $E_a$ decreased after the phase transition. Therefore, some properties of the membrane affected by the phase transition, most probably the density of packing of the polar headgroups or the solubility of the protein in the lipid membrane, are important in determining PKC $\alpha$ activity.

ACKNOWLEDGMENT

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

REFERENCES