A comparative study of the activation of protein kinase C α by different diacylglycerol isomers

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The lipid activation of protein kinase C α (PKC α) has been studied by comparing the activation capacity of different 1,2-diacylglycerols and 1,3-diacylglycerols incorporated into mixed micelles or vesicles. Unsaturated 1,2-diacylglycerols were, in general, more potent activators than saturated ones when 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS)/Triton X-100 mixed micelles and pure POPS vesicles were used. In contrast, these differences were not observed when 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/POPS (4:1, molar ratio) vesicles were used. Diacylglycerols bearing short fatty acyl chains showed a very high activation capacity, however, the capacity was less in mixed micelles. Furthermore, 1,2-diacylglycerols had a considerably higher activating capacity than 1,3-diacylglycerols in POPS/Triton X-100 mixed micelles and in POPC/POPS vesicles. However, the differences between the two types of diacylglycerols were smaller when pure POPS vesicles were used. Differential scanning calorimetry (DSC) showed that POPC/POPS membrane samples containing diacylglycerols had endothermic transitions in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}. Transitions were not detected when using pure POPS vesicles due to the formation of dehydrated phases as demonstrated by FTIR (Fourier-transform infrared) spectroscopy. PKC α binding studies, performed by differential centrifugation in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}, showed that 1,2-*sn*-dioleoylglycerol (1,2-DOG) was more effective than 1,3-dioleoylglycerol (1,3-DOG) in promoting binding to POPC/POPS vesicles. However, when pure POPS vesicles were used, PKC α was able to bind to membranes containing either 1,2-DOG or 1,3-DOG to the same extent.

Key words: differential scanning calorimetry; Fourier-transform infrared spectroscopy; lipid–protein interactions; phospholipid vesicles.

INTRODUCTION

Protein kinase C (PKC) isoenzymes are a large family of serine/threonine kinases involved in cellular signalling [1–4]. Within this family the so called classic isoenzymes, such as PKC α, are activated by Ca^{2+}, phosphatidylserine and diacylglycerols. Diacylglycerols are lipid second messengers which are produced *in vivo* by the hydrolysis of phosphatidylinositols, as a result of the action of phospholipase C. Diacylglycerols are considered to serve as hydrophobic anchors which may recruit PKC to the membrane, leading to an increase in the enzyme's membrane affinity [3] and to the activation of PKC [5–7].

With respect to the specificity for diacylglycerols, it has been shown that there is stereospecificity in the activation of PKC, thus 1,2-*sn*-diacylglycerols are more potent activators than 1,3-isomers and 2,3-*sn*-isomers when using the standard assay, i.e. with phospholipid vesicles [8–10]. The other factor which was claimed to be a determinant of the specificity of diacylglycerols was the nature of the fatty acyl chains. Isomers with long and saturated chains were less effective than those with unsaturated or saturated short chains [11–14].

On the other hand, the mixed micellar assay [15–16] offers new insight for a detailed molecular analysis of protein kinase C activation in order to elucidate a model for the activation of this enzyme. With the development of these assays, the specificity and stoichiometry of PKC–lipid interactions have been demonstrated [15], showing full activation of monomeric PKC when Ca^{2+}, phosphatidylserine and a single molecule of diacylglycerol were present. However, it should be noted that a phosphatidylserine trapping artifact has been described in the Triton X-100 system [17] and hence, this system may be more complex than was thought previously. In any case, studies performed using the micellar system concluded that there is a high degree of structural specificity within the head group, composed of two oxygen esters and the 3-hydroxyl group [16]. Furthermore, other studies concluded that at least three points of physical contact occurred between PKC and diacylglycerols, presumably through hydrogen bonds. Chain length of the diacylglycerol fatty acids did not appear to be a critical factor as long as the acyl chains were long enough to allow their association with the micelle; a minimum length of five carbons was required for activation in mixed micelles [18].

In addition to the cooperative effect of diacylglycerols on PKC binding and activity, it has been shown recently that PKC activation is also modulated by the structure of the lipid bilayer. For example, recent studies by Giorgione et al. [19] have shown

Abbreviations used: 1,2-DCG, 1,2-*sn*-dicapriloylglycerol; 1,3-DCG, 1,3-dicapriloylglycerol; 1,2-DMG, 1,2-*sn*-dimyristoylglycerol; 1,3-DMG, 1,3-dimyristoylglycerol; 1,2-DOG, 1,2-*sn*-dioleoylglycerol; 1,3-DOG, 1,3-dioleoylglycerol; 1,2-DPG, 1,2-dipalmitoylglycerol; 1,3-DPG, 1,3-dipalmitoylglycerol; DSC, differential scanning calorimetry; 1,3-DSG, 1,3-distearoylglycerol; FTIR, Fourier-transform infrared; PKC, protein kinase C; 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-phosphoserine; 1,2-SAG, 1-stearoyl-2-arachidonyl-*sn*-glycerol.

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that the specific activity of the enzyme bound to cubic phase membranes is much greater than that bound to phospholipid in the lamellar phase. Furthermore, other authors [20,21] have shown that it is possible to totally bypass the requirement of PKC for diacylglycerols by using short-chain phosphatidylcholine and/or phosphatidylserine. On the other hand, studies by other laboratories support the idea that diacylglycerols are necessary for PKC activation, and lipid mixtures that form local concentrations of diacylglycerols lead to an increase in PKC activity [22,23].

In the present work we have carried out an extensive and systematic study in order to compare the potency, as activators, of the different chain lengths, saturation and positional isomers of diacylglycerols. At the same time we have evaluated the influence of vesicles, formed with either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPC/POPS) or pure POPS, and POPS/Triton X-100 mixed micelles, on the activator potency. In this way, the activating capacity of diacylglycerols bearing long saturated chains, such as dimyristoylglycerol (DMG), dipalmitoylglycerol (DPG) and distearoylglycerol (DSG), were compared with those consisting of short saturated chains, such as dicapriloylglycerol (DCG), and unsaturated chains, such as dioleoylglycerol (DOG), 1-palmitoyl-2-oleoyl-*sn*-glycerol (1,2-POG) and 1-stearoyl-2-arachidonyl-*sn*-glycerol (1,2-SAG). In addition 1,2-isomers were compared with 1,3-isomers. We have also tested the capacity of 1,2-DOG and 1,3-DOG to induce the binding of PKC α to different types of vesicles and found that the activating capacity of the different isomers of diacylglycerols may be modulated by the organization of the surrounding lipids.

MATERIALS AND METHODS

Materials

POPS, POPC, 1,2-DOG and 1,2-DCG were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). 1,3-DMG, 1,3-DPG, 1,3-DSG, 1,2-SAG, 1,3-DOG and 1,3-DCG were purchased from Sigma Chemical Co. (Madrid, Spain). 1,2-DSG, 1,2-DMG, 1,2-DPG and 1,2-POG were prepared from their respective phosphatidylcholine by the action of phospholipase C (from *Bacillus cereus*; Boehringer-Mannhein, Barcelona, Spain) in diethyl ether/water (4:1, v/v) at 4 °C for 5 h and were then extracted from the ether phase. The purity of each diacylglycerol was determined by TLC on silica gel 60 plates (Macherey-Nagel, Düren, Germany) using chloroform/acetone/methanol (94.5:5.0:0.5, by vol.) as a solvent. All the diacylglycerols used in this work showed a single spot when the chromatograms were stained using iodine vapour.

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 (Duke University Medical Center, Durham, NC, U.S.A.). Porcine PKC α was expressed in Sf9 insect cells by infection with a high-titre recombinant baculovirus and purified to homogeneity from the cytosolic fraction. Purification was performed as described previously [24] with slight modifications. A 2-litre scale 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 70 %), pelleted at 1500 ***g*** for 10 min, and resuspended in buffer H containing 20 mM Tris/HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM PMSF, 0.01 % leupeptin, 100 μM $NaVO_3$ and 50 mM NaF. The pellet was disrupted by sonication (6×10 s) and the resulting lysate was centrifuged at 100000 ***g*** for 60 min. The supernatant was applied in batch mode to a 100 ml DEAE–Sephacel column and equilibrated with buffer E [20 mM Tris/HCl (pH 7.5)/0.5 mM EGTA/0.5 mM EDTA/10 mM β-mercaptoethanol]. The bound proteins were eluted by the application of a linear salt gradient (0–0.5 M NaCl) at a flow rate of 1 ml/min. The PKC α containing fractions were then applied to a 10 ml protamine–agarose column, at a flow rate of 0.4 ml/min, equilibrated with buffer E, and eluted with a linear salt gradient (0.3–1.5 M NaCl). Fractions containing PKC α activity were pooled and loaded onto a 10 ml phenyl-Sepharose column and PKC α was then eluted in the same buffer with a linear descending salt gradient (1–0 M NaCl). Pure PKC α was obtained, as determined by SDS/PAGE. Protein was aliquoted and stored at -80 °C in the presence of 10 % (w/v) glycerol and 0.05 % (v/v) Triton X-100.

Assay of PKC activity

Mixed micellar assay

Mixed micelles were prepared in the following way. 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. Lipids were solubilized in 3 % (v/v) Triton X-100 by vortexing for 1–2 min and then incubating at 37 °C for 5 min. POPS was added to a final concentration of 375 μM and the concentration of diacylglycerols varied from 0–427 μM. The reaction mixture (final volume 250 μl) contained 20 mM Tris/HCl, pH 7.5, 0.2 mg/ml of histone III-S, 20 μM [γ-^{32}P]ATP (300000 c.p.m./nmol), 5 mM $MgCl_2$, 200 μM $CaCl_2$, 0.3 % (v/v) Triton X-100 and 0.5 μg of PKC α. The reaction was ended after 10 min at 25 °C by the addition of 1 ml of ice-cold 25 % (v/v) trichloroacetic acid and 1 ml of ice-cold 0.05 % (w/v) BSA. After precipitating on ice for 30 min, the protein was collected on a 2.5 cm glass-fibre filter (Sartorius, Göttingen, Germany) and washed with 10 ml of ice-cold 10 % (v/v) trichloroacetic acid. The incorporation of ^{32}P into histone III-S was measured by scintillation counting. Activity in the absence of diacylglycerols has been subtracted for each experiment. Data are expressed as the mean $\pm$ S.D. of triplicate determinations. The linearity of the assay was confirmed by a 25 min time course for histone phosphorylation.

Standard assay

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. Lipids were suspended in 20 mM Tris/HCl (pH 7.5)/0.05 mM EGTA by vortex mixing vigorously. The multilamellar vesicles formed gave more reproducible results in the kinase assay and were used for all the assays described. POPS, or POPC/POPS (4:1, mol/mol), was added to the reaction to a final concentration of 50 μM (40 μg/ml) and the concentration of diacylglycerols varied from 0–60 μM. A 100 μl sample of the dispersed lipids was added to the reaction. The reaction mixture and the procedure were identical to those described for the mixed micellar assay. The linearity of the assay was also confirmed.

PKC α membrane binding assay

PKC α (10 ng/μl) was incubated with multilamellar vesicles (500 μM total lipid in 0.4 ml) in a buffer containing 20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 200 μM $CaCl_2$ and 0.3 mg/ml of BSA at 25 °C. Vesicle-bound enzyme was separated from the

free enzyme by centrifuging the mixture at 100000 ***g*** for 30 min at 4 °C. PKC α bound to lipid vesicles was detected by Western blotting. Aliquots from supernatants and pellets were separated by SDS/PAGE (7% gel). After electrophoresis the proteins were transferred on to a nitrocellulose membrane. PKC α was detected using an anti-PKC α polyclonal antibody (Boehringer-Mannheim, Barcelona, Spain) followed by a peroxidase-conjugated secondary antibody. Visualization was carried out using chemiluminescence.

Differential scanning calorimetry (DSC)

Samples containing 3 μmol of total lipid (218 nmol of which was diacylglycerols) and ionophore A23187, at a molar ratio of ionophore to phospholipid of 1:1000, 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 mixing the dried lipid in 10.9 ml of 20 mM Tris/HCl (pH 7.5)/5 mM $MgCl_2$/200 μM $CaCl_2$ (same conditions as used in the assay) extensively by vortex. Samples were centrifuged at 16000 ***g*** for 30 min and the pellets were transferred to small aluminium pans. Thermograms were recorded using a Perkin-Elmer (Norwalk, CT, U.S.A.) DSC-4 calorimeter using an empty sample pan as a reference. The DSC instrument was calibrated using indium as a standard. The samples were scanned over an appropriate temperature range, at a heating rate of 4 °C/min and a sensitivity of 4.2 mJ/s [1 mcal/s; occasionally a 2.1 mJ/s (0.5 mcal/s) rate was used]. Successive scans yielded identical thermograms and the second scans are shown.

Fourier-transform infrared (FTIR) spectroscopy

Infrared spectra were obtained using an FTIR Philips PU-9800 infrared spectrometer equipped with a deuterated triglycine sulphate (DTGS) detector which was continuously purged with dry air in order to remove water vapour bands from the bands of interest. Samples were prepared by hydrating them in 100 mM NaCl/10 mM Hepes, pH 7.4. Samples were examined in a thermostatted Specac 20710 cell equipped with CaF_2 windows and using 25 μm Teflon spacers (Specac, Kent, U.K.). Each spectrum was obtained by collecting 100 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization using a sample shuttle accessory in order to average each consecutive scan. Samples were scanned at 25 °C. Underlying water bands were subtracted interactively by computation using the program Spectra-Calc (Galactic Industries, Salem, U.S.A.).

RESULTS

Activation assays using POPS/Triton X-100 mixed micelles

The activation of PKC α by different diacylglycerols was studied by using the micellar assay in which phosphatidylserine is solubilized in Triton X-100 micelles. When this mixed micellar system was used to activate PKC α, in the absence of diacylglycerol, the resulting activity was 19.3 ± 0.9 nmol of $^{32}P_i$ incorporated into histone III-S $\cdot min^{-1} \cdot mg^{-1}$ of protein. Figure 1 shows that the dependence of the activity of the enzyme on the concentration of 1,2-diacylglycerol was hyperbolic in the cases of 1,2-DOG, 1,2-DPG, 1,2-DMG, 1,2-DCG, 1,2-POG and 1,2-SAG. The behaviour of 1,2-DSG was an exception since it produced maximum activity at low concentrations, and when its concentration was increased, a decrease in enzymic activity was observed. This was most likely caused by destabilization of the micellar structure by this diacylglycerol. In all the cases studied the activities obtained in the presence of 1,3-isomers were lower than those in the presence of 1,2-isomers. Activities were plotted

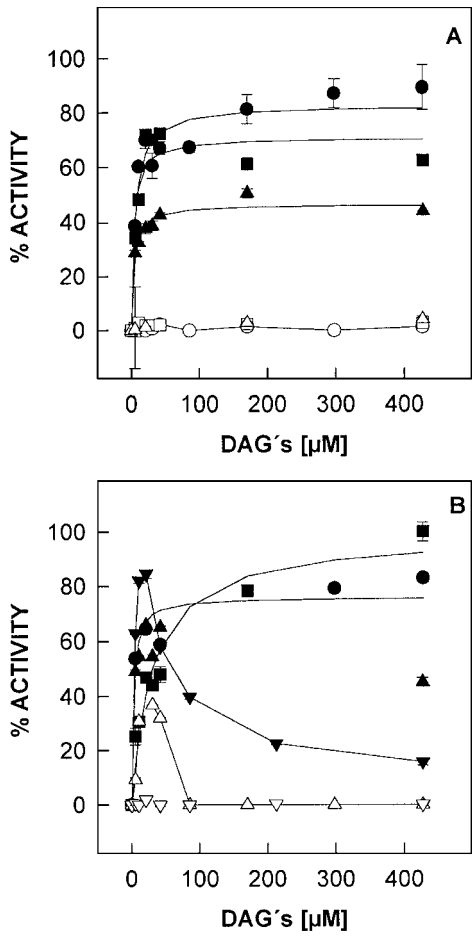

Figure 1 Activation of PKC α by 1,2-diacylglycerols and 1,3-diacylglycerols incorporated into POPS/Triton X-100 mixed micelles

Activities are expressed as a percentage of the activity of 1,2-POG. See Table 1 for absolute values. Diacylglycerols (DAG's) in (**A**): 1,2-DOG (●); 1,3-DOG (○); 1,2-DPG (■); 1,3-DPG (□); 1,2-DMG (▲); 1,3-DMG (△). Diacylglycerols in (**B**): 1,2-SAG (●); 1,2-POG (■); 1,2-DCG (▲); 1,3-DCG (△); 1,2-DSG (▼); 1,3-DSG (▽).

Table 1 Activation of PKC α by POPS/Triton X-100 mixed micelles containing 1,2-diacylglycerols and 1,3-diacylglycerols having various saturated and unsaturated fatty acyl moieties

The V_{max} values are expressed as a percentage of the activity of 1,2-POG. The V_{max} for 1,2-POG was 52.2 ± 3.5 nmol of $^{32}P_i$ incorporated into histone III-S $\cdot min^{-1} \cdot mg^{-1}$ of protein at 37 °C. The activity in the absence of diacylglycerol was 19.3 ± 0.9 nmol of $^{32}P_i$ incorporated into histone III-S $\cdot min^{-1} \cdot mg^{-1}$ of protein.

Diacylglycerol	V_{max} (%)	K_a (μM)
1,2-POG	100 ± 3.5	30.2
1,2-DOG	89.3 ± 8.3	5.9
1,2-DSG	84.5 ± 1.6	0.1
1,2-SAG	82.9 ± 0.2	2.8
1,2-DPG	72.4 ± 1.3	3.8
1,2-DCG	65.0 ± 0.3	0.5
1,2-DMG	50.5 ± 2.4	4.1
1,3-DCG	36.8 ± 0.1	1
1,3-DMG	4.1 ± 1.2	0.01
1,3-DPG	2.6 ± 0.4	0.01
1,3-DOG	2.3 ± 0.1	0.01
1,3-DSG	1.9 ± 0.2	0.01

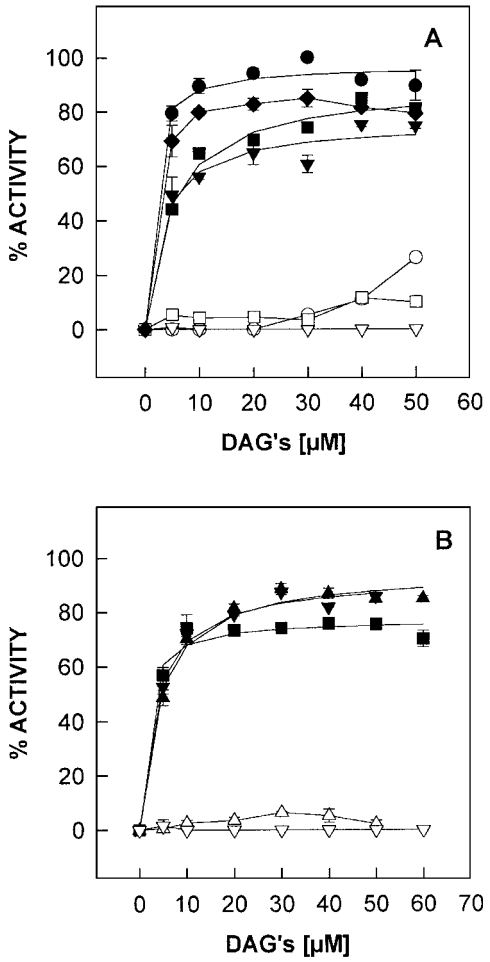

Figure 2 Activation of PKC α by 1,2-diacylglycerols and 1,3-diacylglycerols incorporated into POPC/POPS (4:1, molar ratio) vesicles

Activities are expressed as a percentage of the activity of 1,2-DOG. See Table 2 for absolute values. Diacylglycerols (DAG's) in (**A**): 1,2-DOG (●); 1,3-DOG (○); 1,2-DPG (■); 1,3-DPG (□); 1,2-DSG (▼); 1,3-DSG (▽); 1,2-SAG (◆). Diacylglycerols in (**B**): 1,2-DMG (▲); 1,3-DMG (△); 1,2-POG (■); 1,2-DCG (▼); 1,3-DCG (▽).

as percentages of the diacylglycerol with the maximum activity, which in this case was 1,2-POG, amounting to 52.2 ± 3.5 nmol of $^{32}P_i$ incorporated into histone III-S $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein. As shown in Table 1, the maximum activity was induced by 1,2-POG, followed by the other unsaturated 1,2-isomers, 1,2-DOG and 1,2-SAG. The saturated isomers produced lower PKC α activities, although 1,2-DSG was again an exception, with a maximum activity intermediate between 1,2-DOG and 1,2-SAG. It should also be noted that the maximum activity of 1,3-DCG was relatively high and close to the activities of some of the 1,2-isomers. The other saturated diacylglycerols, 1,2-DPG and 1,2-DMG, had maximum activities intermediate between the unsaturated isomers and the 1,3-isomers. With respect to the K_a values (where K_a is the enzyme activation constant, i.e. the diacylglycerol concentration giving the half maximum reaction velocity), it should be observed that whereas 1,2-POG produced the maximum activity, its K_a was the highest at 30.2 μM. The value shown for 1,2-DSG is very low but again its peculiar behaviour should be taken into account.

Table 2 Activation of PKC α by POPC/POPS (4:1) vesicles containing 1,2-diacylglycerols and 1,3-diacylglycerols having various saturated and unsaturated fatty acyl moieties

The V_{max} values are expressed as a percentage of the activity of 1,2-DOG. The V_{max} of 1,2-DOG was 34.9 ± 2.8 nmol of $^{32}P_i$ incorporated into histone III-S $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein at 37 °C. The activity in the absence of diacylglycerol was 14.7 ± 0.3 nmol of $^{32}P_i$ incorporated into histone III-S $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein.

Diacylglycerol	V_{max} (%)	K_a (μM)
1,2-DOG	100 ± 1.3	0.9
1,2-DCG	98 ± 0.9	4.0
1,2-DMG	95.7 ± 2.0	3.4
1,2-DPG	93.0 ± 0.1	4.8
1,2-SAG	87.9 ± 3.2	1.5
1,2-POG	79.5 ± 0.6	1.3
1,2-DSG	78.3 ± 0.1	3.1
1,3-DOG	27.2 ± 0.1	40.0
1,3-DPG	11.9 ± 1.9	10.0
1,3-DMG	6.6 ± 0.4	10.0
1,3-DCG	0.0	0.0
1,3-DSG	0.0	0.0

Activation assays using POPC/POPS vesicles

Multilamellar vesicles were chosen for the enzymic assays. These type of vesicles were preferred over small unilamellar vesicles, obtained by sonication, because extensive sonication may result in isomerization of the diacylglycerols giving acyl migrations with transformation of 1,2- to 1,3-isomers which are structurally more stable [25]. In addition to that, it has been described that multilamellar vesicles are more reproducible than the sonicated ones, and the preparation of extruded large unilamellar vesicles is difficult when diacylglycerols have to be included in the system [26]. It is an additional advantage in our case that DSC experiments, which have been used here for the correlation between activity and physical properties, are more informative with multilamellar vesicles, since the phase transitions which can be observed when using small vesicles are less cooperative than those of multilamellar vesicles.

When these vesicles were used in the absence of diacylglycerol, the activity observed was 14.7 ± 0.3 nmol of $^{32}P_i$ incorporated into histone III-S $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein. As seen in Figure 2(A) and 2(B) the maximum activities reached in the presence of 1,2-isomers were higher than those obtained in the presence of 1,3-isomers. However, similar maximum activities were observed for all 1,2-isomers. Table 2 shows that the maximum activity for 1,2-DOG was 34.9 ± 2.8 nmol of $^{32}P_i$ incorporated into histone III-S $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein. The activities of the rest of the 1,2-isomers were at least 80 % of the maximum activity. Only 1,2-DSG was below this level with 78 %. The K_a values were also similar for all the 1,2-isomers, the lowest being that of 1,2-DOG (0.9 μM). Among the 1,3-isomers the only isomer which presented significant activity was 1,3-DOG (27 %); it also had a very high K_a of 40 μM.

DSC was used for the study of the vesicles in order to correlate their physical properties with the activation by diacylglycerols. Thermograms (Figure 3) were obtained in the presence of the same concentrations of Ca^{2+} (200 μM) and Mg^{2+} (5 mM) as were used in the enzymic assays. The mixtures scanned contained the corresponding diacylglycerol at a concentration of 20 μM (7 mol % of the total lipid). Several phase separations were observed, although at the temperature used for the assays (25 °C) all the systems were in the fluid state, except the system corresponding to 1,2-DMG where the transition ended at 27 °C,

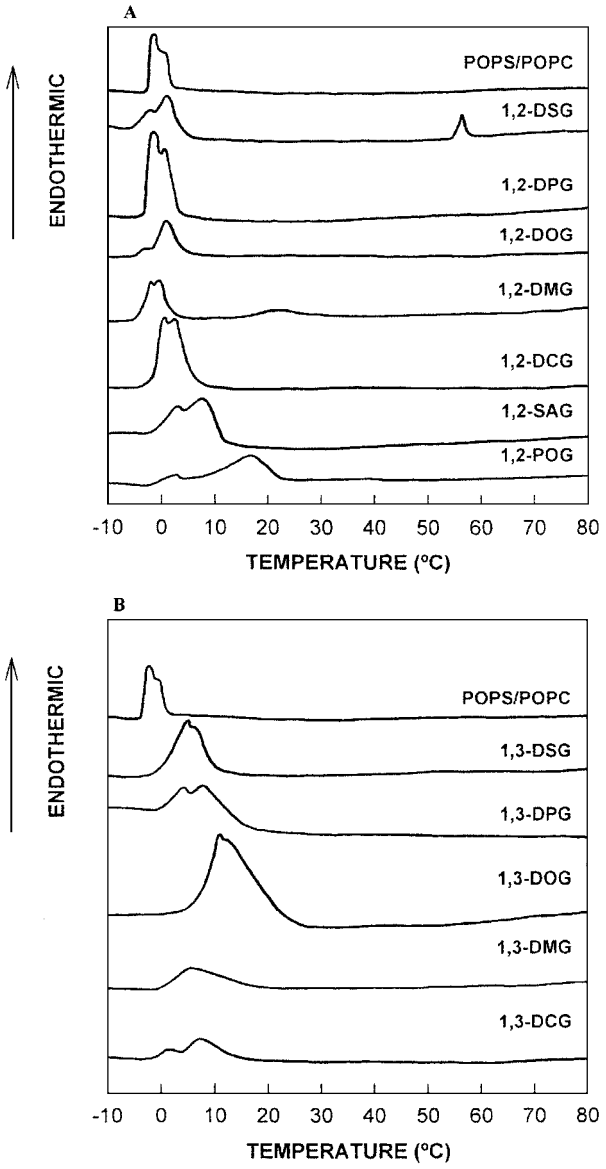

Figure 3 DSC thermograms of aqueous dispersions of mixtures of POPC/POPS (4:1, molar ratio) multilamellar vesicles containing different diacylglycerols

The concentration of diacylglycerol was 20 μM (7 mol% of the total lipid) in each case. (**A**) 1,2-Diacylglycerols. (**B**) 1,3-Diacylglycerols.

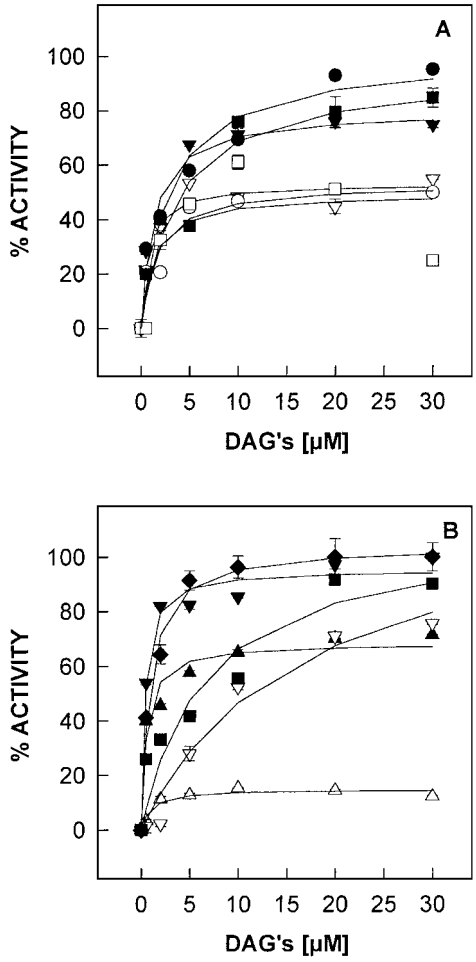

Figure 4 Activation of PKC α by 1,2-diacylglycerols and 1,3-diacylglycerols incorporated into POPS vesicles

Activities are expressed as a percentage of the activity of 1,2-SAG. See Table 3 for absolute values. The diacylglycerols (DAG's) shown in (**A**) are: 1,2-DOG (●); 1,3-DOG (○); 1,2-DPG (■); 1,3-DPG (□); 1,2-DSG (▼); 1,3-DSG (▽). The diacylglycerols shown in (**B**) are: 1,2-DMG (▲); 1,3-DMG (△); 1,2-DCG (▼);1,3-DCG (▽); 1,2-SAG (◆) and 1,2-POG (■).

and also that of 1,2-DSG in which a phase separation appeared with a transition at 55 °C (Figure 3A). This peak most probably corresponded to a phospholipid–diacylglycerol complex as described for similar systems previously [27–29]. It is, however, remarkable that clear differences could be observed when comparing the effects of 1,2-DSG and 1,3-DSG (Figure 3B), since for 1,3-DSG no clear phase separation could be discerned, and hence the transition at high temperature in the sample, corresponding to 1,2-DSG, was absent.

Activation assays using POPS vesicles

The activity observed when these vesicles were used in the absence of diacylglycerols was 14.7 nmol of $^{32}P_i$ incorporated into histone III-S$\cdot$min$^{-1}\cdot$mg^{-1} of protein. Figure 4 shows that the activation produced by the diacylglycerols tested fitted the hyperbolic kinetics as well as it did for the data from the mixed POPC/POPS vesicles. However, the differences between the 1,2-isomers and the 1,3-isomers were not as great as those observed for micelles or POPC/POPS vesicles. 1,2-Isomers unsaturated in both acyl chains, such as 1,2-DOG, and those unsaturated in only one acyl chain, such as 1,2-SAG and 1,2-POG, had activities ranging between 91.6 and 100% (Table 3). The maximum value which corresponded to 1,2-SAG, was 51.5 ± 6.7 nmol of $^{32}P_i$ incorporated into histone III-S$\cdot$min$^{-1}\cdot$mg^{-1} of protein. Maximum activities for 1,2-diacylglycerols bearing long and saturated acyl chains (1,2-DPG, 1,2-DSG and 1,2-DMG) were found to be between 71.4 and 84.5%. 1,2-DCG showed the maximum activity in this case and was similar to the unsaturated diacylglycerols. Table 3 also shows that 1,3-DCG produced a maximum activity of 75.5%, close to that of some of the 1,2-isomers, although with a very high K_a of 16.2 μM. 1,3-DOG, 1,3-DPG and 1,3-DSG had maximum activities ranging from 54.7–66.5%. Only 1,3-DMG had a distinctly lower maximum activity of 15.3%, although this is still very high compared to the activity produced by the same

Table 3 Activation of PKC α by POPS vesicles containing 1,2-diacylglycerols and 1,3-diacylglycerols having various saturated and unsaturated fatty acyl moieties

The V_{max} values are expressed as a percentage of the activity of 1,2-SAG. The V_{max} for 1,2-SAG was 51.5 ± 6.7 nmol of $^{32}P_i$ incorporated into histone III-S · min^{-1} · mg^{-1} of protein at 37 °C. The activity in the absence of diacylglycerol was 25.6 ± 6.7 nmol of $^{32}P_i$ incorporated into histone III-S · min^{-1} · mg^{-1} of protein.

Diacylglycerol	V_{max} (%)	K_a (μM)
1,2-SAG	100 ± 6.7	2.5
1,2-DCG	99.8 ± 1.1	0.4
1,2-DOG	92.9 ± 0.3	2.9
1,2-POG	91.6 ± 0.2	6.5
1,2-DPG	84.5 ± 3.6	3.7
1,2-DSG	75.9 ± 1.6	1.3
1,3-DCG	75.5 ± 0.5	16.2
1,2-DMG	71.4 ± 0.2	0.5
1,3-DOG	66.5 ± 1.3	1.6
1,3-DPG	61.0 ± 0.7	1.2
1,3-DSG	54.7 ± 0.3	0.7
1,3-DMG	15.3 ± 0.2	0.9

diacylglycerol in the other lipid membrane systems. K_a values were similar for all the diacylglycerols studied except 1,3-DCG, mentioned above, and 1,2-POG with 6.5 μM. DSC revealed in this case that, in the presence of the same concentrations of Ca^{2+} and Mg^{2+} used in the enzymic assays, no phase transitions were observed for either pure POPS, or POPS in the presence of the different diacylglycerols (results not shown). It is known that phosphatidylserine systems associate with divalent cations to form dehydrated cochleate phases. They do not show phase transitions in the range of temperatures studied in this work, although it has been shown that they do undergo a transition at temperatures higher than 100 °C [30].

The effect of Ca^{2+} and Mg^{2+} on the state of hydration of the lipid membranes, as measured by FTIR

It is known that membranes formed with phosphatidylserine as the only constituent become dehydrated as $Ca^{2+}$and/or Mg^{2+} are added. Full dehydration was reached at a phosphatidylserine/Ca^{2+} molar ratio of 2:1, with the formation of the so called cochleate phases [31–33]. FTIR was well suited to monitoring these cation–phospholipid interactions since it measured the vibrational absorbance of groups present in lipid molecules, and this could then be used as a diagnostic tool to monitor the state of hydration of the bilayer membrane. The most useful vibrations for phosphatidylserine were the asymmetric vibration of the phosphate group (1220–1240 cm^{-1}) and the methyl umbrella region (1378–1386 cm^{-1}) [34]. The asymmetric phosphate double-bond stretching band in hydrated phosphatidylserine appeared with a maximum absorption at approx. 1223 cm^{-1}. However it shifted to about 1238 cm^{-1} upon full dehydration by Ca^{2+} binding [35]. Non-saturating concentrations of Ca^{2+} gave rise to intermediate situations in which the 1223 cm^{-1} and the 1238 cm^{-1} bands coexisted [36]. Figure 5(A, trace 2) shows that 200 μM Ca^{2+} and 5 mM Mg^{2+} induced partial dehydration in POPS vesicles but that there was still a predominance of the dehydrated component that had its maximum absorbance at 1238 cm^{-1}. The addition of 25 μM 1,2-DOG (32.8 mol% of the total lipid) favoured the hydration of the phosphate group, since the 1223 cm^{-1} component appeared to be predominant over the 1238 cm^{-1} component (Figure 5A, trace 3). However, the addition of the same concentration of 1,3-DOG produced a higher proportion of dehydrated component with respect to pure POPS (Figure 5A, trace 4). On the other hand, in POPC/POPS vesicles, this band was slightly wider than in pure POPS vesicles, and the maximum appeared at 1225 cm^{-1}. The addition of 200 μM Ca^{2+} and 5 mM Mg^{2+}did not apparently affect the hydration state of the phosphatidylserine membranes (Figure 5B).

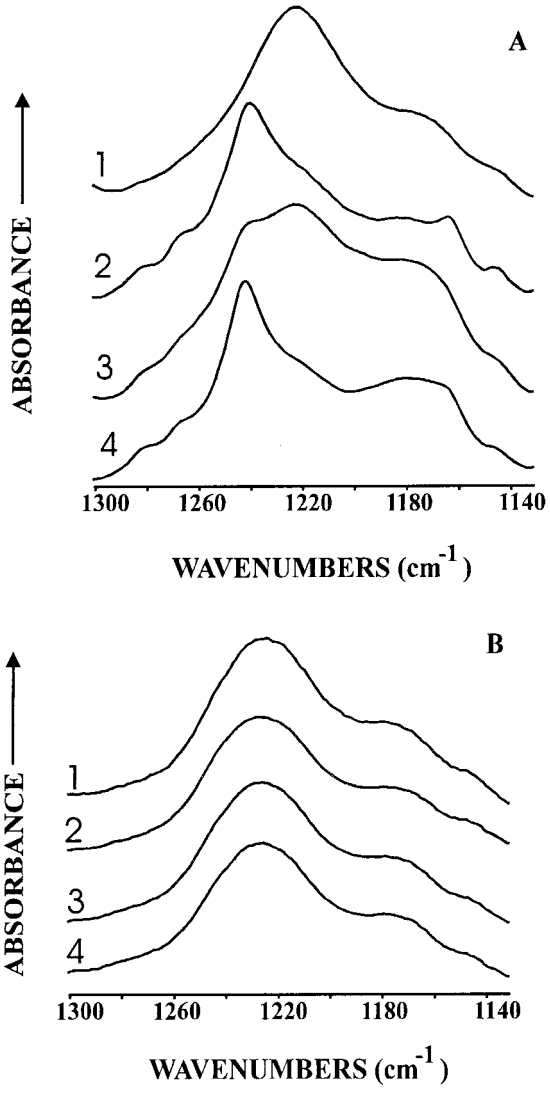

Figure 5 FTIR spectra of the asymmetric phosphate double-bond stretching region

(**A**) Pure POPS vesicles: trace 1, in the absence of Ca^{2+} or Mg^{2+}; trace 2, in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; trace 3, containing 25 μM 1,2-DOG (32.8 mol% of the total lipid) in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; trace 4, containing 25 μM 1,3-DOG (32.8 mol% of the total lipid) in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}. (**B**) POPC/POPS (4:1, molar ratio) multilamellar vesicles: trace 1, in the absence of Ca^{2+} and Mg^{2+}; trace 2, in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; trace 3, containing 25 μM 1,2-DOG (32.8 mol% of the total lipid) in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; trace 4, containing 25 μM 1,3-DOG (32.8 mol% of the total lipid) in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}.

The methyl umbrella region of the FTIR spectrum of phosphatidylserine has also been shown to be a very good marker for cochleate phases [34]. Whereas a main band appeared at 1378 cm^{-1} in fully hydrated phosphatidylserine bilayers, in the

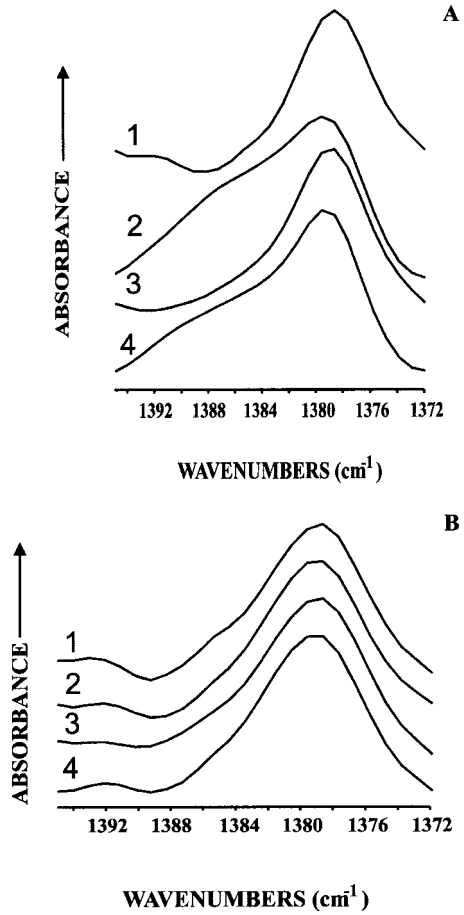

Figure 6 FTIR spectra of the methyl umbrella region

(**A**) Pure POPS vesicles: trace 1, in the absence of Ca^{2+} or Mg^{2+}; trace 2, in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; trace 3, containing 25 μM 1,2-DOG (32.8 mol% of the total lipid) in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; trace 4, containing 25 μM 1,3-DOG (32.8 mol% of the total lipid) in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}. (**B**) POPC/POPS (4:1, molar ratio) multilamellar vesicles: trace 1, in the absence of Ca^{2+} and Mg^{2+}; trace 2, in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; trace 3, containing 25 μM 1,2-DOG (32.8 mol% of the total lipid) in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; trace 4, containing 25 μM 1,3-DOG (32.8 mol% of the total lipid) in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}.

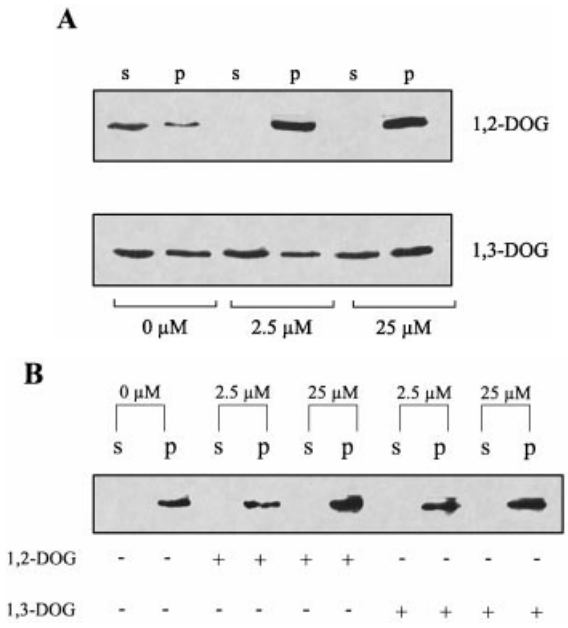

Figure 7 PKC α binding to membrane vesicles

Vesicles were incubated with the enzyme in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+} and centrifuged to pellet the vesicles and the bound protein. The protein was detected by SDS/PAGE and immunoblotting. (**A**) POPC/POPS (4:1, molar ratio) multilamellar vesicles. (**B**) Pure POPS vesicles. s, Supernatant and p, pellet.

presence of excess Ca^{2+} the main band was seen at 1386 cm^{-1} [34]. Figure 6(A) shows the effect of adding 200 μM Ca^{2+} and 5 mM Mg^{2+} to POPS vesicles. A wide peak can be seen with an increased absorbance at 1386 cm^{-1} (Figure 6A, trace 2) as evidence of partial dehydration. 1,2-DOG remarkably reduced the dehydration (Figure 6A, trace 3), however 1,3-DOG had much less effect (Figure 6A, trace 4). These observations were in agreement with those made using the asymmetric phosphate double-bond stretching band. It is interesting to comment here that the consistency of the samples made with POPS and 1,3-DOG was lighter and thus very difficult to sediment by centrifugation, whereas those made of POPS and 1,2-DOG were heavier and more easily sedimented. Samples containing POPC/POPS also behaved as described above for the phosphate band, thus in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+} full hydration was apparent in the methyl umbrella region with no changes if either 1,2-DOG or 1,3-DOG were present. Other works have also shown that in pure POPS vesicles at least a substantial proportion of the phospholipids adopted a dehydrated cochleate structure [9,37]. The DSC results shown above also support this notion, since POPC/POPS vesicles had a phase transition which was indicative of a lamellar structure. Vesicles formed only by POPS did not exhibit any phase transition which is compatible with a predominantly dehydrated cochleate structure. It should be noted that this is in contrast with our previous observations performed with dipalmitoylphosphatidylserine/1,2-DPG [38]. Perhaps this is due to full saturation of the acyl chains of the system used in this study.

Dependence of protein binding to membrane vesicles on diacylglycerols

The binding of PKC α to POPC/POPS multilamellar vesicles was studied by incubating the vesicles with the enzyme, in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}, followed by centrifugation to pellet the vesicles and any protein bound to them. The protein was then detected by immunoblotting (Figure 7) after separation by SDS/PAGE. It was found by this procedure that the presence of 2.5 μM 1,2-DOG induced almost total translocation of PKC α to the membrane (Figure 7A), however 1,3-DOG was less effective in promoting enzyme binding to the membrane.

In another set of experiments the binding of PKC α to lipid was studied using POPS as the only phospholipid in the vesicles. It can be seen in Figure 7(B), that most of the protein was pelleted with the lipid, even in the total absence of diacylglycerols. In the presence of 1,2-DOG and 1,3-DOG, the protein was also bound to the lipid. These results can be explained by the amount of available POPS in this preparation, compared to POPC/POPS vesicles. Note that the total concentration of POPS was the same in both cases, although the total phospholipid concentration was 4 times higher in the case of the POPC/POPS system. However, whereas in the multilamellar POPC/POPS system the only

molecules of POPS available for PKC α were those located at the most external monolayer, in the dehydrated POPS/(Ca^{2+} + Mg^{2+}) system the morphology of the membranes can be expected to be shifted to that of cochleate cylinders, and more molecules of POPS will be exposed to the enzyme.

DISCUSSION

In this paper we have correlated results on enzyme activities with other results obtained using binding and physical techniques such as FTIR and DSC. A limitation to this approach is that whereas PKC α assays were done with very low lipid concentrations (50 μM), membrane binding, FTIR and DSC were performed using concentrations at least 10 times higher, although the lipid to PKC α ratios were kept constant. Ideally, the interactions of PKC α with lipids should be explored using the same concentrations in all the assays. However, this is not easy, due to the different sensitivities of the techniques involved.

It has been shown that biophysical properties of membranes can modulate PKC α activity in lipid bilayers [19–23]. Current models of activation for the conventional PKC isoforms, such as PKC α, suggest its association with membranes containing acidic phospholipids in a calcium-dependent manner, and the role of diacylglycerols is to increase the affinity of PKC α for these membranes [3], but the mechanism involved is still unclear.

In relation to the saturation of the acyl chains, we have found that diacylglycerols containing unsaturated acyl chains are, in general, better activators when they are incorporated into Triton X-100 mixed micelles or into a pure POPS system, but no differences have been found when the assays were based on POPC/POPS vesicles. Nevertheless, saturated diacylglycerols induced significant activities even in Triton X-100 micelles and in pure POPS vesicles, and this is in agreement with other results that indicated that 1,2-DPG significantly activated PKC in a micellar assay [39]. In previous work [9], where the vesicles used were formed using a bovine phosphatidylserine/egg phosphatidylcholine (1:4 molar ratio) system, i.e. an unsaturated lipid mixture, 1,2-DOG and 1,2-DPG were also found to have similar activities, although these were the only 1,2-isomers studied in that work. However, in other studies in which bovine liver phosphatidylcholine, dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylserine were used to form the vesicles, saturated diacylglycerols, such as 1,2-DPG, had less capacity to activate PKC α than other unsaturated diacylglycerols, such as 1,2-DOG [40]. This was attributed to the lack of miscibility of 1,2-DPG with the phospholipid mixture, since, as pointed out previously, a prerequisite for a diacylglycerol to be able to activate PKC α is its capacity to mix with the bulk phospholipid in the lipidic system used to activate the enzyme [41]. If 1,2-DPG is mixed with only unsaturated phospholipids, as it was in the case presented in this paper (see Figure 2), a good mixing of the diacylglycerol with the phospholipids will occur, and hence this may explain why its activating capacity was similar to that of unsaturated diacylglycerols (see Table 2). In a recent work it has also been concluded that phase separations occurring in lipid mixtures facilitate an accumulation of diacylglycerols to one of these domains leading to an increase in PKC α activity [23]. Related to that is the observation that sequestration of diacylglycerols complexed with phospholipids into gel domains resulted in a lack of PKC α-activating capacity, due to the non-availability of the diacylglycerols required for interaction with the enzyme [22]. The activation of PKC α was also attributed by other authors to the coexistence of diacylglycerol-enriched and diacylglycerol-poor domains [42].

Comparing the activating ability of different isomers, it is interesting to note the results obtained when 1,3-isomers were incorporated into pure POPS vesicles. In contrast to the other two systems 1,3-isomers were able to activate PKC α almost to the same extent as 1,2-isomers. Our results differ from previous work which showed a very low capacity for activation by the 1,3-isomers [10]. However, it should be mentioned that in our studies we used unsonicated preparations of multilamellar vesicles whereas the previous study was carried out using sonicated phosphatidylserine vesicles [10]. In another study where 1,2-DOG and 1,3-DOG were used to activate PKC in Triton X-100 mixed micelles, it was shown that 1,2-DOG was the better activator [18] and this is in agreement with our results.

It has been shown previously that there is co-operativity of phosphatidylserine and diacylglycerol concentration in order to produce the binding of PKC α to the membrane and its activation [43–45]. It seems that if the phosphatidylserine concentration is high enough, diacylglycerols can not contribute to increase the binding of PKC α which will already be at 100 % with just the phospholipid. Nevertheless, the activities obtained for the preparations which included 1,2-isomers were higher than those including 1,3-isomers. This emphasizes the fact that apart from increasing binding, diacylglycerols are also necessary to facilitate the full activation of PKC α. Another interesting conclusion that can be obtained from these data is that the dehydrated (cochleate) phase, which was formed due to the presence of Ca^{2+} and Mg^{2+}, somehow increased the specificity of PKC α for 1,3-DOG. A possible reason for this could be that the conformation adopted by the 1,3-isomers when immersed in the cochleate phase makes them capable of producing a certain activation of PKC α.

The general picture emerging from these results is that the structure of the lipid vesicles has a certain influence on the activating capacity of the different isomers of diacylglycerols, probably by changing the conformation of the protein.

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 in the PKC α purification and insect cell culture work. This work was supported by Grants PB95-1022 and PB96-1107 from Dirección General de Enseñanza Superior (Madrid).

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Received 24 June 1998/28 October 1998; accepted 6 November 1998