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Diacylglycerol, phosphatidylserine and Ca^{2+}: a phase behavior study

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Abstract

The interaction of 1,2-dipalmitoylglycerol (1,2-DPG) with dipalmitoylphosphatidylserine (DPPS) has been studied in the presence and in the absence of Ca^{2+} by using differential scanning calorimetry (DSC) and ^{31}P-nuclear magnetic resonance (^{31}P-NMR). In the absence of Ca^{2+}, DSC showed that 1,2-DPG increased the phase transition of DPPS, effect already noticed at very low 1,2-DPG concentrations, whereas lipid immiscibilities were detected at concentrations of 1,2-DPG higher than about 30 mol%. ^{31}P-NMR indicated that lamellar phases were always present at concentrations of 1,2-DPG lower than about 35 mol%, but at higher concentrations non-lamellar phases may be present in the fluid phase. As observed by DSC, the apparent pK_a of the carboxyl group of DPPS was increased slightly in the presence of 1,2-DPG. In the presence of Ca^{2+}, the effect of 1,2-DPG was to further increase the temperature of the onset of the phase transition, indicating an stabilization of the most rigid phase in the DPPS/1,2-DPG/Ca^{2+} samples. Even concentrations of 1,2-DPG as low as 1 mol% of the total lipid already produced a noticeable effect. Moreover, lipid immiscibilities were apparent at concentrations of 1,2-DPG higher than 20 mol%. Furthermore, the transition of the DPPS/Ca^{2+} complex observed by DSC at 155°C was perturbed by the presence of 1,2-DPG, indicating a change in the structure of the crystalline complex. Interestingly, the effect of non-saturating Ca^{2+} concentrations on the DPPS phase transition was enhanced by the presence of 1,2-DPG. The effect reported here may be significant for a number of situations where Ca^{2+}, phosphatidylserine and diacylglycerols are involved, such as fusion of membranes, where diacylglycerol may facilitate Ca^{2+}-induced fusion, or the activation of enzymes such as protein kinase C and phospholipases.

Key words: Diacylglycerol; Phosphatidylserine; NMR, ^{31}P-; DSC

1. Introduction

Diacylglycerols (DAGs) are important lipid mediators which activate the enzyme protein kinase C when it is incorporated into membranes [1,2]. Since this enzyme is recognized as a key element of many biological functions, such as cell transformation, DAGs are attracting the attention of an increasing number of investigators. The mechanism of activation of the enzyme by DAGs might be through a direct interaction between the protein and a lipid effector or through alterations of membrane properties. Although direct interaction is suggested by the activation of the enzyme when incorporated into Triton X-100 micelles [3], this does not exclude that DAGs may also modulate the activity of the enzyme when present in membranes, through the alteration of the membrane structure. There is some evidence to support that DAGs may promote the insertion of the regulatory domain of the protein into the hydrophobic core of the membrane [4–6], possibly through changes in membrane bilayer properties related to lipids with hexagonal phase propensity [7].

Apart from that, DAGs have been shown to favor membrane fusion [8,9] and this could be also related to the formation of domains with altered structure in their polar/lipid interface.

Therefore, it is interesting to study in detail the interaction of DAGs with membranes, and particularly DAGs in mixtures with phosphatidylserine (PS) and Ca^{2+}, since PS is known to be the preferred phospho-

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Abbreviations: 1,2-DPG, *sn*-1,2-dipalmitoylglycerol; DPPS, *sn*-1,2-dipalmitoylphosphatidylserine; DPPC, *sn*-1,2-dipalmitoylphosphatidylcholine; DAG, *sn*-1,2-diacylglycerol; PS, *sn*-1,2-diacylphosphatidylserine; DSC, differential scanning calorimetry; ^{31}P-NMR, phosphorus nuclear magnetic resonance; T_c, onset temperature of the gel to liquid-crystalline phase transition; ΔH, enthalpy change of the gel to liquid-crystalline phase transition.

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lipid for activation of protein kinase C [10], and Ca^{2+} is required to activate the enzyme [11,12].

Diacylglycerol–phospholipid interactions have been already the subject of a number of studies, but these studies have been concentrated specially in phosphatidylcholine [13–22] and phosphatidylethanolamine membranes [14,23–26]. However, much less studies have been done using PS membranes [14,27].

We report here our results on the complex formed by *sn*-1,2-dipalmitoylphosphatidylserine (DPPS), *sn*-1,2-dipalmitoylglycerol (1,2-DPG) and Ca^{2+} using differential scanning calorimetry (DSC) and ^{31}P-nuclear magnetic resonance (^{31}P-NMR). Even small concentrations of DAGs perturb the structure of the DPPS vesicles and these perturbations are enhanced significantly in the presence of Ca^{2+}.

2. Material and methods

sn-1,2-Dipalmitoylphosphatidylserine (DPPS) and *sn*-1,2-dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids (Birmingham, AL, USA), *sn*-1,2-dipalmitoylglycerol (1,2-DPG) was obtained from Sigma (Poole, UK) and ionophore A23187 from Boehringer-Mannheim (Barcelona, Spain). All other reagents used were of analytical grade. Water was twice distilled and deionized in a Milli-Q apparatus from Millipore. The purity of DPPS and 1,2-DPG before and after the measurements, specially those done at high temperatures, were checked by thin-layer chromatography using as solvents $CHCl_3/CH_3OH/$ 30% NH_4OH (10 : 10 : 3, v/v/v) and petroleum ether/diethyl ether/acetic acid (80 : 20 : 1, v/v/v). They showed only one spot in all the cases. We have not detected the formation of 1,3-DPG from 1,2-DPG under our experimental conditions.

Sample preparation

DPPS in chloroform/methanol (2 : 1, v/v) (2 μmol for DSC and 30 μmol for ^{31}P-NMR), the appropriate amount of 1,2-DPG in chloroform/methanol (2 : 1, v/v) and the appropriate amount of ionophore A23187 in ethanol to give a phospholipid/ionophore ratio of 500 : 1 were mixed and dried under a stream of O_2-free N_2 and the last traces of solvent were removed by high vacuum for more than three hours. The samples were dispersed for 30 min at 70°C, temperature above the gel to liquid-crystalline phase transition of the mixture, in 1000 μl of 0.1 mM EDTA, 10 mM Mops, 100 mM NaCl buffer (pH 7.4), with occasional mixing in a vortex mixer until obtaining a homogeneous and uniform suspension. Calcium containing samples were prepared by adding a specific volume of calcium containing buffer to the hydrated samples in order to obtain the phospholipid/calcium ratio required and incubated 1 h at 70°C followed by 1 h at room temperature. Subsequently, the samples were spun and pelleted at high speed in a bench microfuge. DSC samples containing no Ca^{2+} were either scanned immediately after preparation or incubated for 25 days at 4°C before scanning. Some samples were prepared at different pH values, so that the appropriate buffer was used (ionic strength equal to 0.1). It was observed, after DSC measurements, that the concentration of ionophore used to allow a rapid equilibration with Ca^{2+} of the multilamellar vesicles, did not affect the phase transition of pure DPPS.

Differential scanning calorimetry

Samples were scanned in a Perkin-Elmer DSC-4 calorimeter using buffer as reference. The instrument was calibrated using indium and pure DPPC as standards. The samples were scanned with a heating and cooling rate of 4 C°/min at 1 mcal/s except were noted. The range of temperatures studied was from 30°C to 90°C. The first and the second heating scans were used for transition enthalpy calculations and displays. Peak areas were measured by weighing paper cut-outs of the peaks. Data was obtained from the thermograms as previously described by Eliasz et al., 1976 [28].

After the measurements, the pans were carefully opened and the samples were dissolved in chloroform/methanol (1 : 1, v/v). After subsequent perchloric acid hydrolysis, the amount of phospholipid originally present was determined as described before [29].

The incorporation of 1,2-DPG into liposomes was measured by combining gas-chromatography and phosphorus assay. Aliquots were spun down and the pellets analyzed for phosphorus and also for fatty acids. In the case of fatty acids, quantitative analysis were done by using internal standards through gas-liquid chromatography [15]. The incorporation of 1,2-DPG in the membranes was found to be higher than 90–95% in all the samples used in this work.

^{31}P-nuclear magnetic resonance

^{31}P-NMR spectra were recorded in the Fourier transform mode on a Varian Unity 300 spectrometer (121 MHz for phosphorus) and the temperature was controlled to ±0.5 C°. All chemical shift values are quoted in parts per million (ppm) from pure lysophosphatidylcholine micelles (0 ppm), positive values referring to low-field shifts. All spectra were obtained in the presence of a gated-broad band decoupling (5 W input power during acquisition time) and accumulated free induction decays were obtained from up to 2000 transients. A spectral width of 25 kHz, a memory of 8 K data points, a 1.3 s interpulse time and a 80° radio frequency pulse were used. Prior to Fourier transformation an exponential multiplication was applied resulting in a 100 Hz line broadening. The residual chemical shift anisotropy, $\Delta\sigma$, was measured as 3-times the chemical shift difference between the high-field peak and the position of isotropically moving lipid molecules at 0 ppm [30].

3. Results and discussion

Differential scanning calorimetry

The effect of incorporating different amounts of 1,2-DPG on the thermotropic phase transition of DPPS in the absence of Ca^{2+} is shown in Figs. 1 and 2. Fig. 1 depicts the heating and cooling thermograms corresponding to mixtures which contained up to 30 mol% of 1,2-DPG. Similarly to what was observed for mixtures of DPPC/1,2-DPG [15], the incorporation of increasing concentrations of 1,2-DPG produced an increase in the gel to liquid-crystalline phase transition of DPPS. This was already perceptible at the lowest concentration of 1,2-DPG studied in this work, i.e., 1 mol%. As the concentration of 1,2-DPG increased up to 20 mol%, the gel to liquid-crystalline transition was broadened, indicating that the transition cooperativity was lower than in the pure phospholipid. However, at greater concentrations up to 35 mol% it became sharper, indicating the formation of a complex between DPPS and 1,2-DPG (see below).

It is known that 1,2-DPG presents thermal metastability, so that the β' structure is the most stable form [31,32]. Upon rapid cooling the molten 1,2-DPG is transformed to the α form. As described by Kodali et al. [31], if the molten form is rapidly cooled to temperatures below T_c and then rapidly heated, only one endotherm is observed corresponding to the melting of the α form. In order to obtain the β' form of 1,2-DPG it was necessary to incubate the samples for 25 days at 4°C as stated in Materials and methods.

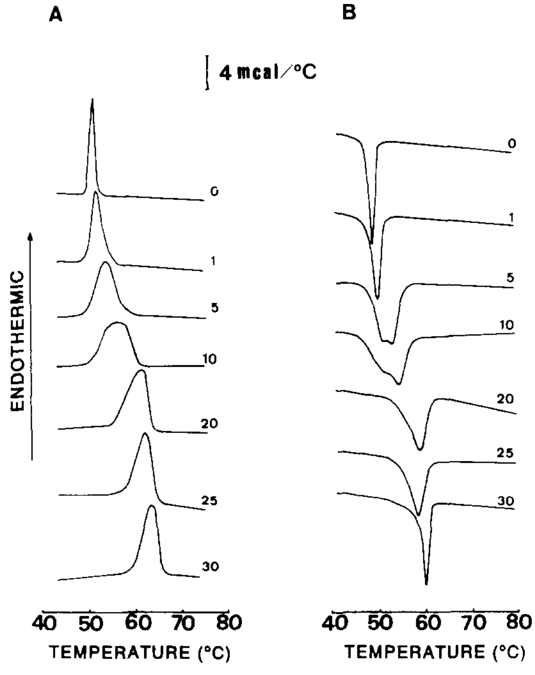

Fig. 1. Differential scanning calorimetric thermograms of mixtures of DPPS/1,2-DPG up to 30 mol% of 1,2-DPG. The molar percentage of 1,2-DPG in the mixture is indicated on each of the (A) heating and (B) cooling thermograms.

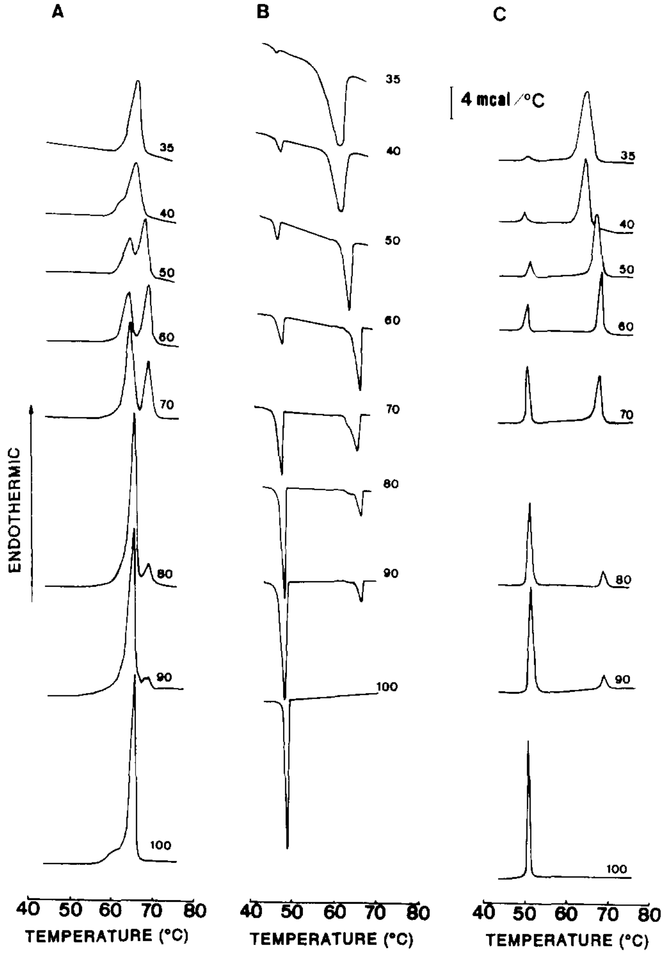

Fig. 2. Differential scanning calorimetric thermograms of mixtures of DPPS/1,2-DPG from 35 mol% to pure 1,2-DPG. The molar percentage of 1,2-DPG in the mixtures is indicated on each of the (A) first heating, (B) first cooling and (C) the second heating thermograms. These samples were incubated for 25 days at 4°C. See text for details.

Samples containing 30 mol% of 1,2-DPG or less, presented the same thermal pattern after incubation for 25 days at 4°C as when they were immediately scanned after being rapidly cooled from the molten form (Fig. 1). On the other hand, the pattern observed was the same for the first scan and for other successive scans up to five, leaving the sample at low temperatures for less than 2 min before rescanning. However, the behavior of samples containing ≥ 35 mol% of 1,2-DPG (Fig. 2) was considerably different since these samples gave place to transitions which were attributable to pure 1,2-DPG. The origin of these new transitions was confirmed to be due to pure 1,2-DPG by their metastability behavior. After incubation for 25 days at 4°C, samples containing 35 mol% and 40 mol% of 1,2-DPG showed a broad peak at about 62°C but two well resolved peaks at higher concentrations (Fig. 2A). When the molten samples were subsequently cooled they gave place to two clearly resolved transitions, one of them at about the same temperature in which a transition was detected in the heating process, and another one at approx. 49°C (Fig. 2B). It is then evident that, during the preincubation, the β' form of 1,2-DPG has been obtained, giving place then to a transition at about 62°C. This transition considerably overlaps with the transition due to the DPPS/1,2-DPG complex at 35 mol% and 40 mol% of 1,2-DPG, but both transitions were well resolved at higher concentrations of 1,2-DPG because the transition temperature of the complex was increased by some degrees. Upon cooling, the α form of 1,2-DPG was formed showing a transition at about 50°C. The transition corresponding to the α form was then observed when the sample was rescanned without any incubation (Fig. 2C). In order to construct the phase diagram (see below) the second heating scan is more convenient than the first, since the transition temperature corresponding to the DPPS/1,2-DPG complex is better resolved in the second heating scan.

The increase in transition temperature induced by the association of 1,2-DPG and DPPS is similar to that previously observed for samples containing DPPC and 1,2-DPG [15]: the interposition of 1,2-DPG molecules between DPPS molecules will reduce the repulsion effect of the bulky and negatively charged polar groups of the phospholipids (at pH 7.4) thus leading to a stabilization of the gel phase, i.e., an increase in the transition temperature. This effect could be also due, at least in part, to changes in surface hydration. In fact, changes in hydration have been found previously by FT-IR [27].

^{31}P-nuclear magnetic resonance

We have also studied by ^{31}P-NMR the phase behavior of different DPPS/1,2-DPG mixtures at different temperatures (results not shown). At 40°C, all the samples, either pure DPPS or containing different amounts of 1,2-DPG, presented spectra which were rather similar, indicating that they were in the lamellar gel phase. At 60°C, some of the samples were in the gel phase but other were already fluid as seen through DSC (see Figs. 1 and 2). However, samples containing 35 mol%, 50 mol% and 70 mol% of 1,2-DPG still presented spectra characteristic of the lamellar gel phase. A small isotropic component could be observed in the samples containing 50 mol% and 70 mol% of 1,2-DPG (not shown).

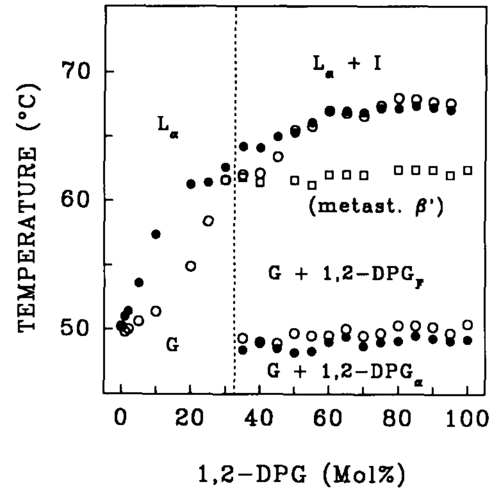

Fig. 3. Phase diagram of mixtures of DPPS and 1,2-DPG based on DSC and ^{31}P-NMR data. Closed and open circles were obtained from DSC heating and cooling scans, respectively.

At 75°C, all the samples were in the fluid condition (see Figs. 1 and 2 above). Whereas pure DPPS and the samples containing 20 mol% and 35 mol% of 1,2-DPG showed spectra characteristic of the L_α phase, samples containing 50 mol% and 70 mol% of 1,2-DPG, gave place to spectra where an isotropic peak was superimposed on the lamellar powder pattern. The origin of this isotropic peak is not known with certainty since it may correspond to the formation of small vesicles, regions of the bilayer surface with a relatively high curvature or it could be also originated by a separate phase, either cubic or an isotropic melt. Furthermore, ^{31}P-NMR results are to be considered with caution, since ^{31}P-NMR lineshape does not unambiguously sense phospholipid phase structure because changes in headgroup conformation can, at least theoretically, affect the spectral shape in a manner consistent with a phase change [33].

Binary phase diagram

A phase diagram was elaborated on the basis of the DSC data (Fig. 3). Solidus and fluidus lines were based on the onset of heating and cooling scans, respectively [34]. ^{31}P-NMR results were also taken into account. Two regions could be distinguished. The first one corresponded to mixtures up to 35 mol% of 1,2-DPG. There seems to be a good miscibility between DPPS and 1,2-DPG in both gel and fluid conditions. According to ^{31}P-NMR, L_β and L_α lamellar phases seem to occur below and above the phase transition. The second region corresponded to mixtures containing 1,2-DPG between 35 mol% and pure 1,2-DPG. In this region, pure 1,2-DPG is found to be laterally separated of a DPPS/1,2-DPG complex. It can be postulated that the complex has a DPPS/1,2-DPG molar ratio of approx. 2:1 which is the last composition at which no

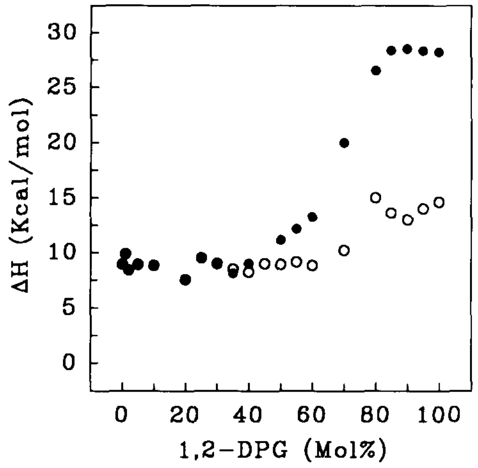

Fig. 4. Dependence of the enthalpy change (ΔH) with the amount of 1,2-DPG in DPPS. (●) Samples incubated for 25 days at 4°C (β' form of 1,2-DPG) and (○) samples without preincubation (α form of 1,2-DPG).

pure 1,2-DPG was detected. However, the transition temperature of the complex increased from about 60°C at 35 mol% of 1,2-DPG to 66°C at 60 mol% of 1,2-DPG. At compositions greater than 60 mol% the transition temperature of the complex remained constant. This seems to indicate a limited solubility of 1,2-DPG in the 2:1 DPPS/1,2-DPG mixture. In this region, and after ^{31}P-NMR measurements, the transition is from a lamellar gel to a lamellar fluid plus isotropic phase. No hexagonal H_{II} phase was detected here, in contrast to the dimyristoylphosphatidylcholine/1,2-dimyristoylglycerol system [22], although in coincidence with the last system, isotropic phases were detected at high 1,2-DPG concentrations.

It should be noted that the melting of the β' form of 1,2-DPG found at approx. 63°C (Fig. 3) could be observed only at the first heating scan as it was commented above.

Molar enthalpies

The molar enthalpies of the endothermic transitions presented in Figs. 1 and 2 are plotted in Fig. 4 versus the molar percentage of 1,2-DPG. ΔH was calculated by adding the areas corresponding to all of the peaks detected in each sample. It can be observed that ΔH remains fairly constant and equal to that found for pure DPPS up to about 35 mol% of 1,2-DPG, i.e., the concentration at which a complex between 1,2-DPG and DPPS is supposed to occur. For concentrations ≤ 35 mol% the same ΔH values were obtained during the first and the second heating scans. Moreover, the same value of ΔH was found without preincubation and after preincubation for 25 days at 4°C. This was not the case, however, for samples containing concentrations greater than 35 mol% of 1,2-DPG, in which different ΔH values were found depending on the thermal history of the samples. Preincubated samples, where the β' form of pure 1,2-DPG was induced, displayed a gradual increase of the ΔH values from 35 mol% of 1,2-DPG up to approx. 80 mol%, approaching the value of the β' form of pure 1,2-DPG. From 80 mol% of 1,2-DPG until pure 1,2-DPG the same ΔH values were found (approx. 26.5 kcal/mol). However, samples not preincubated or immediately heated after cooling, so that the α form of 1,2-DPG was present, showed a fairly constant value of ΔH up to 70 mol% (approximately equal to the ΔH value of pure DPPS). An increase of approx. 5 kcal/mol was observed at concentrations greater than 70 mol% of 1,2-DPG, approaching the ΔH value corresponding to the α form of pure 1,2-DPG (Fig. 4). These results reinforce the conclusions obtained from the phase diagram of Fig. 3. The complex with a 2:1 DPPS/1,2-DPG molar ratio has a ΔH similar to pure DPPS. Since the β' form has a much higher ΔH, when it is present it will dominate the total ΔH, giving place to very high values of ΔH in the mixture.

Effect of pH on the transition temperatures

The effect of pH on the transition temperature of DPPS, in presence and in absence of 1,2-DPG, was studied in order to gain information about the effect of 1,2-DPG on the lipid/water interface. It has been shown before that the membrane chain melting properties are regulated by the polar surface of the lipid bilayer, and the apparent pK_a of different groups of phospholipids like PS can be observed through the transition temperature dependence on pH as observed by DSC [35,36]. Fig. 5 shows, in agreement with previous authors [37], a decrease in the transition temperature of pure DPPS at a pH value of approx. 4 caused by the dissociation of the carboxyl group of PS. The apparent dissociation constant were deduced from the

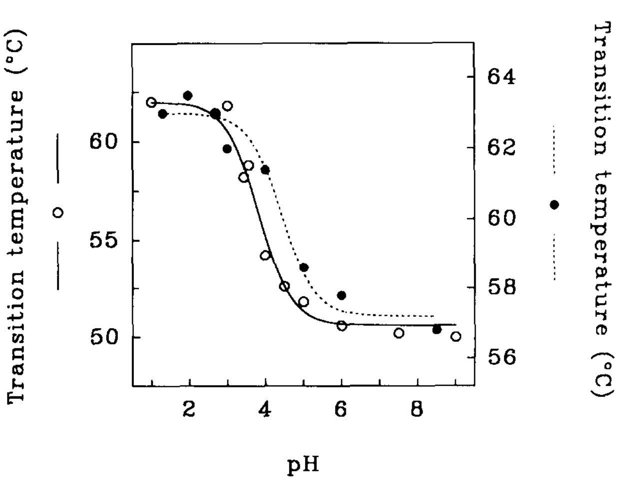

Fig. 5. pH titration of the transition temperature of (○) DPPS and (●) DPPS/25 mol% of 1,2-DPG.

midpoints of the transition temperature titrations [37]. A value of 3.8 for the apparent pK_a of DPPS was obtained. Fig. 5 shows a similar plot for a DPPS sample containing 25 mol% of 1,2-DPG. In this case, the value obtained for the apparent pK_a was 4.4, i.e., a higher value than that found for pure DPPS (approx. 0.6 pH units).

At higher pH values the amine group of DPPS should be expected to dissociate but this was not measured since, in our hands, the consistency of the samples was drastically changed at pH values higher than 11, so that it was not possible to sediment the pellets in order to prepare the samples to be measured by DSC, and it seemed like a change to a non-lamellar phase had taken place.

The change observed for the apparent pK_a of the carboxyl group in the presence of pure 1,2-DPG was modest, but it certainly deserves some discussion because it informs about the effect occasioned by 1,2-DPG in the lipid/water interface. It is well known that the apparent pK_a of any membrane group differs from the apparent pK_a values of the same group in the bulk solution, and this is the case of the carboxyl group of PS (see [36] for a review of this field), where the apparent pK_a found for this group is in the range 3.2–5, depending on the experimental conditions and techniques used to make the measurements [37], compared to a value of 2.65, which is the value reported for phosphoserine in bulk aqueous solution [36]. This may be justified by several reasons, such as the effect of surface electrostatics and by a hydration effect [36]. The incorporation of 1,2-DPG makes the apparent pK_a shift to be even greater. However, if the electrostatic effect is considered alone, the opposite should be expected. According to the Gouy-Chapman theory, the membrane potential (Ψ_0) should decrease upon a decrease of the surface charge density [36] since the electrostatic pK shift in the apparent pK_a will be [36]:

$$\Delta pK_{\mathrm{el}} = -\frac{e \cdot \Psi_{\mathrm{el}}}{2.3 \cdot kT}$$

where pK_{el} refers to the pK_a shift due to electrostatic effects, Ψ_{el} is the electrostatic field potential and e, k and T have their usual meanings. Hence a decrease in the apparent pK_a of the carboxyl group of DPPS should be expected considering electrostatic effects only. Therefore, something is also happening in this case which counteracts the electrostatic effect. A possibility could be that the hydration is perturbed since this is another possible reason for an apparent pK shift [36]. However, judging by our previous infrared results [27], no important frequency shift was observed on the carboxylate vibration band as should be expected for dehydration effects as they take place for example, in the case of the binding of Li^+ [38]. Instead, only small changes were detected [27], which could be interpreted as small conformational changes or changes in the location of the carboxyl group so that it might be displaced to a more apolar environment. These changes could then be invoked in order to explain the apparent pK_a shift detected in this case by DSC. Hence, in the presence of 1,2-DPG, a change in the lipid/water interface structure could take place, so that the carboxyl group of DPPS changed its conformation and/or location, going probably to a more apolar microenvironment.

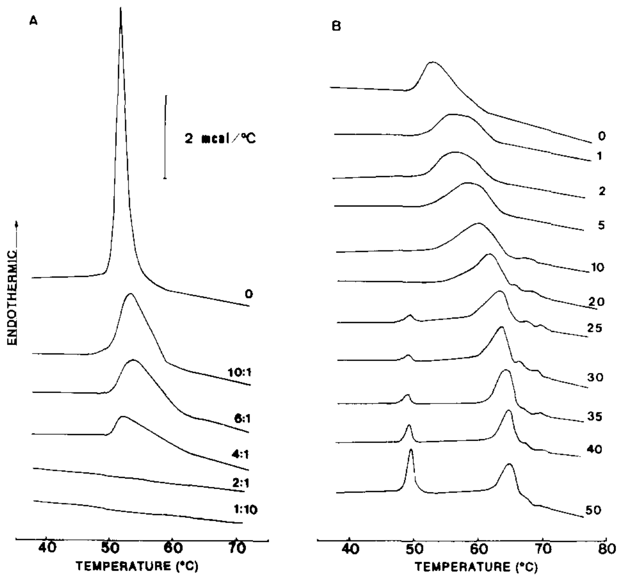

Fig. 6. Differential scanning calorimetric heating thermograms of (A) pure DPPS in the presence of Ca^{2+} at different DPPS/Ca^{2+} molar ratios as indicated and (B) DPPS containing different amounts of 1,2-DPG as indicated at a DPPS/Ca^{2+} molar ratio of 4:1.

Effect of Ca^{2+} on DPPS / 1,2-DPG mixtures

DSC experiments were carried out using certain DPPS/Ca^{2+} molar ratios in the absence of 1,2-DPG and the results are shown in Fig. 6A at concentrations of Ca^{2+} up to a DPPS/Ca^{2+} molar ratio of 1:10, i.e., saturating Ca^{2+} concentrations. The phase transition of pure DPPS is broadened as the DPPS/Ca^{2+} molar ratio is decreased, so that the transition is no longer observed at a molar ratio of DPPS/Ca^{2+} of 2:1. This is in agreement with previous observations which concluded that the PS/Ca^{2+} binding stoichiometry is 2:1 mol/mol [39], as was also confirmed by later measurements of Ca^{2+} binding between PS lamellae [40] and infrared spectroscopy [27].

The DSC pattern which was found for DPPS/1,2-DPG mixtures in the presence of Ca^{2+} was different from either pure DPPS in the presence of Ca^{2+} or DPPS/1,2-DPG mixtures in the absence of Ca^{2+}. Fig. 6B shows the DSC thermograms of different DPPS/1,2-DPG mixtures at a DPPS/Ca^{2+} molar ratio of 4:1, i.e., at subsaturating Ca^{2+} concentrations. It can be

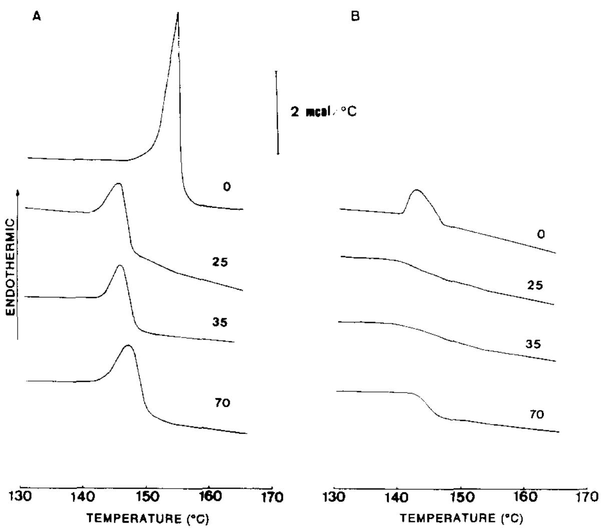

Fig. 7. Differential scanning calorimetric heating thermograms of the Ca^{2+} complex of DPPS containing different amounts of 1,2-DPG as indicated at a DPPS/Ca^{2+} molar ratio of (A) 1:10 and (B) 4:1.

appreciated that the phase transition of DPPS/Ca^{2+} was clearly affected by 1,2-DPG, even at very low concentrations such as 1 mol%, since the peak of the transition was shifted towards higher temperatures, indicating a stabilization of the structure of the DPPS/1,2-DPG complex. 1,2-DPG shifted the transition further, and at concentrations of 20 mol% additional 1,2-DPG peaks could be detected in the region 65–70°C. Apart from that, a peak, corresponding to pure 1,2-DPG laterally segregated in the membrane, was detected at concentrations ≥ 25 mol% of 1,2-DPG. The size of this peak increased as the amount of 1,2-DPG present increased. It is evident that the miscibility of 1,2-DPG in DPPS was lower in the presence than in the absence of Ca^{2+}, since as it was shown in Fig. 2, pure 1,2-DPG was detected only at concentrations higher than about 35 mol% in the absence of Ca^{2+}.

The incorporation of 1,2-DPG into DPPS vesicles also affected to the formation and properties of the DPPS/Ca^{2+} crystalline complex. It is known that above a certain DPPS/Ca^{2+} ratio, a crystalline and dehydrated complex is formed, with a phase transition temperature around 155°C [41]. It can be appreciated in Fig. 7A that the phase transition corresponding to the crystalline phase formed in the presence of saturating Ca^{2+} (DPPS/Ca^{2+} molar ratio of 1:10) was shifted to lower temperatures by the presence of 1,2-DPG, from 151°C in the absence of 1,2-DPG to 143°C in its presence. On the other hand, at higher DPPS/Ca^{2+} ratios, like 4:1, i.e., subsaturating Ca^{2+} concentrations, the transition was observed in the absence but not in the presence of 1,2-DPG (Fig. 7B). These changes might be attributed to either the prevention of the formation of the crystalline DPPS/Ca^{2+} complex by 1,2-DPG or to the formation of complexes which do not show a phase transition in this range of temperatures. As it has been shown through the study of the PO_2^- asymmetric vibration band of DPPS by infrared spectroscopy, dehydration of DPPS was enhanced by the presence of 25 mol% of 1,2-DPG, so that total dehydration of the phosphate group was reached when 2 out of 3 molecules of PS were binding Ca^{2+} [27]. Therefore, the presence of 1,2-DPG changed the structure of the DPPS/Ca^{2+} crystalline complex. We conclude that the complex formed by DPPS and Ca^{2+}, in the presence of 1,2-DPG, may be unable to produce a cooperative phase transition in the range of temperatures studied. At lower DPPS/Ca^{2+} molar ratios, such as 1:10, i.e., saturating Ca^{2+} concentrations, the transition was not suppressed by even 35 mol% of 1,2-DPG, but it was shifted to lower temperatures, indicating that 1,2-DPG also destabilized the DPPS/Ca^{2+} complex at this high Ca^{2+} concentrations.

Concluding remarks

The results shown in this work indicate that 1,2-DPG may perturb the phase behavior of DPPS, especially in the presence of Ca^{2+} and even at non-saturating concentrations. It is interesting to consider that the activation of protein kinase C by DAGs also requires the presence of Ca^{2+} and phospholipids, PS being the preferred one [10]. Therefore, the interactions between PS and DAGs in the presence of Ca^{2+} is a valuable element to be considered in order to understand the complex that all these components will give with the enzyme.

We have chosen to study a simple system formed by a saturated phospholipid (DPPS) and a DAG with the same fatty acyl chains (1,2-DPG). This system is convenient since it may be easily studied by using DSC, because their transition temperatures are above ambient temperatures, and the length of the fatty acyl chains is similar to those to be expected in cell membranes. It can be argued that this saturated system is not adequate, since saturated phospholipids and saturated DAGs are not good activators of protein kinase C. Nevertheless, it has been found that a saturated PS, like dimyristoylphosphatidylserine, can fully activate protein kinase C although high concentrations of DAGs are necessary [42]. On the other hand, the experimental evidence indicating that saturated DAGs are less effective activators of protein kinase C than unsaturated ones [43], has been obtained in mixtures with unsaturated phospholipids, and at temperatures (30°C) at which some DAGs, like 1,2-DPG, are below of its phase transition, hence phase separation can be suspected. In fact, saturated DAGs with shorter chains, which are fluid at this temperature, were found to fully activate the enzyme [43]. Apart from that, we have observed than the interaction of 1,2-dioleoylglycerol with dioleoylphosphatidylcholine (unpublished) is very similar to that of 1,2-DPG with DPPC [15]. Therefore, we are looking in this paper to the effects of DAGs in the lipid/water interphase, since the DAG and phospholipid used here will differ only in their parts situated at this interface. This is probably the more interesting effect, since DAGs formed in cell membranes will have, most likely, a fatty acyl composition similar to that of the phospholipids present in the membrane. In any case, we are not studying here other complex effects which may arise from mixing saturated with unsaturated lipids, or more generally, DAGs and phospholipids with different fatty acyl chain compositions.

It is shown here that 1,2-DPG changed the apparent pK_a of the carboxyl group of DPPS, confirming our previous results obtained through infrared spectroscopy which indicated a change in the structure of the lipid/water interface in the presence of 1,2-DPG [27]. Apart from that, changes in the hydrophobic part of the membrane were also apparent through changes in the phase transition. It is important to note that the effect of non-saturating Ca^{2+} concentrations is greater on DPPS/1,2-DPG mixtures than in pure DPPS (Fig. 7B), emphasizing that the formation of a complex capable of activating surface-bound enzymes, or causing membrane fusion, may be facilitated in points of the membrane with high local concentrations of DAGs.

It should be pointed out that, in some in vitro assays reported for protein kinase C in the literature, these high concentrations of DAGs are found not in local points but in the whole of the membrane. For example, 18 mol% [43], 19 mol% [44] and 25 mol% [42] of DAGs are widely used for protein kinase C assays, giving the maximum activation of the enzyme. As we have shown in this work very significant effects should be expected for these concentrations of DAGs in the presence of Ca^{2+}.

Finally, we want to point out that, although the physiological levels of DAGs are not expected to be higher than about 2 mol% of the total lipid in special cases [45], this does not mean that local concentrations could not be found in biomembranes, for example near the point of action of phospholipases. This will depend on membrane heterogeneity, rate of formation of DAGs, lifetime of DAGs before being metabolized, etc. Therefore in vitro experiments where relatively high concentrations of DAGs are used might be taken as models, not of the whole biomembrane, but of the local points where DAGs could accumulate and play their physiological role. In any case we have described here significative effects of DAGs even at very low concentrations such as 1–2 mol%, and we think that our results should be taken into account when trying to interpret the biological mechanism of action of DAGs.

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5. References

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