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Effect of sphingosine and stearylamine on the interaction of phosphatidylserine with calcium. A study using DSC, FT-IR and $^{45}Ca^{2+}$-binding

Francisco López-García, José Villalaín, Juan C. Gómez-Fernández *

Departamento de Bioquímica y Biología Molecular (A), Edificio de Veterinaria, Universidad de Murcia, Apdo. 4021, E-30080 Murcia, Spain

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Abstract

The lamellar gel to lamellar liquid-crystalline phase transition of dipalmitoylphosphatidylserine (DPPS) multilamellar membranes is abolished by the presence of Ca^{2+} at DPPS/Ca^{2+} molar ratios of 2:1 or lower. However, when equimolar sphingosine (SPH) or stearylamine (SA), which are positively charged at the pH studied in this work, were included in DPPS vesicles, the phase transition of DPPS was still observed by differential scanning calorimetry, even in the presence of very high Ca^{2+} concentrations such as a DPPS/Ca^{2+} molar ratio of 1:10. According to that, ΔH was similar for samples formed by equimolar DPPS and SPH and SA, either in the presence or in the absence of Ca^{2+}, whereas no phase transition was observed for the pure phospholipid in the presence of Ca^{2+} at molar ratios lower than DPPS/Ca^{2+} 2:1. $^{45}Ca^{2+}$-binding experiments showed that for DPPS/SPH or DPPS/SA molar ratios of 2:1, only half of the Ca^{2+} was bound to DPPS with respect to pure DPPS, i.e., in the absence of SPH or SA. At concentrations of SPH or SA equimolar with DPPS, the Ca^{2+} binding was nearly abolished. The effect of SPH and SA on the apparent pK_{app} of the carboxyl group of DPPS was also studied in the presence and in the absence of Ca^{2+} by using Fourier transform infrared spectroscopy. The dehydration of the phosphate group of DPPS induced by the binding of Ca^{2+} was followed through the observation of the PO_2^- antisymmetric stretching, and the percentage of dehydrated PO_2^- groups quantitatively assayed. It was again confirmed that, in the presence of equimolar concentrations of SPH or SA, Ca^{2+}, at concentrations which are saturating for pure DPPS, was not bound at all to DPPS. It was also found that the pK_{app} was considerably shifted to lower values in the presence of the amino bases, decreasing from 4.6 in pure DPPS to 2.1 and 2.2 for the equimolar mixtures of DPPS with SPH and SA, respectively. These results show that SPH and SA, being positively charged molecules anchored in the membrane, are able of preventing the binding of positively charged ions such as Ca^{2+} through an electrostatic charge neutralization.

Keywords: DSC; Sphingosine; Stearylamine; Phosphatidylserine; FT-IR; ^{45}Ca-binding

1. Introduction

Sphingosine (SPH) is a long-chain amino base which is the main constituent of the sphingolipid molecules. Sphingosine and other sphingolipids have been recognized as important mediators affecting many cellular functions, such as inhibition of platelet aggregation, activation of neutrophils, inhibition of growth factor action, modulation of receptor function and inhibition of phorbol ester-induced responses, etc. [1–8]. The interest in SPH has recently increased after it has been shown that SPH is a potent and reversible inhibitor of protein kinase C [2,3,9], an enzyme that mediates cellular responses to numerous hormones, growth and differentiation factors, tumor promoters, etc. [10–12]. This enzyme, which is only active when it is associated to the membrane [13–15], requires Ca^{2+}, diacylglycerols and phosphatidylserine [16,17]. SPH also exhibits other protein kinase C independent activities such as inhibition of tissue factor [18], inhibition of insulin receptor tyrosine kinase [19], biphasic effects on diacylglycerol

Abbreviations: DPPS, 1,2-dipalmitoylphosphatidylserine; DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared spectroscopy; Mes, 2-(*N*-morpholino)ethane sulfonic acid; SPH, sphingosine; SA, stearylamine; ΔH, enthalpic change of the gel to liquid-crystalline phase transition; T_c, onset temperature of the gel to liquid-crystalline phase transition; pK_{app}, apparent dissociation constant; pK_i, intrinsic dissociation constant; pK_{el}, electrostatic contribution to pK_i; pK_h, hydration contribution to pK_i; Ψ_O, membrane surface potential.

* Corresponding author. Fax: +34 68 364147.

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kinase [20], inhibition of phosphatidic acid phosphohydrolase [21,22], inhibition of CTP-phosphocholine cytidiltransferase [23] and activation of phospholipase D [24]. Moreover, it has been also shown recently that SPH containing membranes bind DNA and RNA, this interaction being abolished by acidic phospholipids [25,26].

Hannun and coworkers described that SPH inhibits protein kinase C competitively with diacylglycerols, phorbol esters and calcium and also that the inhibition is of a mixed type with respect to phosphatidylserine [2,3]. These studies also indicated that the effect of SPH was not through the inhibition of the catalytic or membrane domain of protein kinase C, but by the inhibition of the protein kinase C phorbol esters binding domain, requiring its hydrophobic character and its positive charge [2,3]. However, other studies [27] have suggested that SPH inhibits protein kinase C by limiting the availability of phosphatidylserine, that is, the phospholipid that produce the maximal activation of protein kinase C [17,28], rather than by a direct interaction with the phorbol ester domain of protein kinase C. SPH also inhibits the translocation of protein kinase C from the cytosol to the membrane fraction upon stimulation with agonists and the down-regulation of protein kinase C [29]. The presence of a charged amino group and an aliphatic side chain have been shown to be necessary for the action of SPH in the inhibition of protein kinase C [30]. The creation of a positive vesicle surface charge by SPH has been suggested to be the mechanism of inhibition of CTP-phosphocholine cytidiltransferase activity [23].

Despite its remarkable effects, very little is known about the behavior of SPH and its interaction with other lipids. The same applies to other positively charged lipids such as stearylamine (SA), which also inhibits protein kinase C [31] apart from other biological activities such as permeabilization of lysosomal membranes [30]. We have studied recently the phase behavior of SPH and SA in mixtures with DPPS [32] showing that both compounds form azeotropic mixtures with DPPS, at molar ratios of DPPS/SPH and DPPS/SA of 2:1 and 1:1, respectively. It was further shown that at physiological pH, both SPH and SA were protonated in a large extent, with apparent pK_{app} values of 9.1 and 8.9, respectively. We have also studied the phase behavior of mixtures of SPH with dipalmitoylphosphatidylcholine and dielaidoylphosphatidylethanolamine [33] showing that SPH forms an azeotropic point at 30 mol% of SPH with dipalmitoylphosphatidylcholine, whereas no azeotrope was found in mixtures of dielaidoylphosphatidylethanolamine and SPH. In this last case the L_α to hexagonal H_{II} transition of dielaidoylphosphatidylethanolamine was eliminated by SPH concentrations higher than 33 mol%.

It is the aim of this work to study the interaction of SPH and SA with phosphatidylserine in the presence of Ca^{2+}, i.e., a negatively charged phospholipid of primary importance in animal membranes, which produce the maximum activation of protein kinase C [28] together with Ca^{2+} which is also essential for the activation of protein kinase C [34,35]. These molecules have been chosen in order to compare the effect of the free hydroxyl groups of SPH, which are absent in SA, and therefore, discriminate between two possible types of interaction, electrostatic or hydrogen bonding. The interactions taking place in this system have been examined by different techniques such as differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FT-IR) and $^{45}Ca^{2+}$-binding experiments. Our results show that both SPH and SA interact electrostatically with DPPS, involving charge neutralization of the phospholipid, with alteration of the apparent dissociation constant of the carboxyl group of DPPS.

2. Materials and methods

Dipalmitoylphosphatidylserine (DPPS) was obtained from Avanti Polar Lipids (Birmingham, USA), stearylamine (SA), sphingosine (SPH) and D_2O (99.8%) were obtained from Sigma (Madrid, Spain), ionophore A23187 from Boehringer-Mannheim (Barcelona, Spain) and $^{45}CaCl_2$ from Amersham (Amersham, UK). 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, SA and SPH were checked before and after the measurements by thin-layer chromatography where they showed only one spot.

2.1. Sample preparation

DPPS in chloroform/methanol 2:1 (v/v) (2 μmol for DSC and FT-IR and 1 μmol for $^{45}Ca^{2+}$-binding), the appropriate amount of SA or SPH and 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 3 h. The samples were hydrated for 30 min at 70° C in buffer (50 mM Mes, 100 mM NaCl, pH 6.0), with occasional mixing in a vortex mixer until obtaining a homogeneous and uniform suspension. For the FT-IR titration of the carboxyl group of DPPS in the different samples either in the absence or in presence of Ca^{2+}, buffers in D_2O of constant ionic strength were used ($I = 0.2$). After obtaining a homogeneous and uniform suspension of membranes of DPPS and SA or SPH, a specific volume of 50 mM Mes, 100 mM NaCl, 100 mM $CaCl_2$ (containing 750 cpm/nmol Ca^{2+} for binding experiments), pH 6.0, was added to the samples to obtain the specific DPPS/Ca^{2+} ratio required. The final DPPS concentration was 2 mM. The samples were incubated 1 h at 70° C followed by 1 h at room temperature and pelleted at high speed in a bench microfuge at room temperature. For $^{45}Ca^{2+}$-binding experiments the samples were centrifuged at 75 000 × g for 60 min at 25° C. Supernatants were

measured for $^{45}Ca^{2+}$ in a Beckman LS1701 liquid scintillation counter.

The concentration of ionophore used to allow a rapid equilibrium with Ca^{2+} in the multilamellar vesicles did not affect the phase transition of pure DPPS as observed by DSC measurements [32].

Quantitative assay of the incorporation of SA and SPH into the mixtures

After centrifugation of the liposomes, the incorporation of SA and SPH was measured by combining a phosphorus assay [36] and the determination of the amine groups in the sediments by the method of Benson and Hare [37]. The incorporation of SA and SPH in the membranes was higher than 95% in all the samples used in this work.

2.2. Differential scanning calorimetry

Pellets for DSC were collected and placed into small aluminum pans, sealed and scanned in a Perkin-Elmer DSC-4 calorimeter using buffer as reference. The instrument was calibrated using DPPC and indium as standards. The samples were scanned with a heating and cooling rate of 4 C°/min at 1 mcal/s of sensitivity over a range of 30–90° C. The third scan was used for display unless otherwise stated. After the measurements, the pans were carefully opened and the samples were dissolved in chloroform/methanol 1:1 (v/v). The amount of phospholipid originally present was determined after subsequent perchloric acid hydrolysis [36].

2.3. Fourier transform infrared spectroscopy

Infrared spectra were obtained in a Philips PU9800 Fourier transform infrared spectrometer equipped with a DTGS detector. Samples were examined in a thermostated Specac 20710 cell equipped with CaF_2 windows and using 25 μm teflon spacers (all from Specac, Kent, UK). The samples were made either in H_2O or D_2O buffers. When D_2O was used instead of H_2O, buffers and pD values were corrected for the isotope effect [38]. Samples were equilibrated at 40° C in the infrared cell for 20 min before acquisition. Each spectrum was obtained by collecting 100 interferograms with a nominal resolution of 2 cm^{-1} and a triangular apodization using a sample shuttle accessory in order to average background spectra between consecutive samples spectra over the same time period. The spectrometer was continuously purged with dry air in order to remove atmospheric water vapor from the bands of interest. Subtraction was performed interactively using Spectra-Calc software (Galactic Industries, Salem, USA). Deconvolution was performed as previously described [39]. Quantitative analysis of the PO_2^- asymmetric stretching vibration band of DPPS was made by using a partial least-squares multivariate statistical method. For this study we have used the algorithms for calibration and prediction develops by Haaland an Thomas [40] and implemented by Galactic Industries in the PLSplus program (see [39]). Reference spectra and the absortivities of the PO_2^- hydrated and dehydrated bands of DPPS for each DPPS/amino base molar ratio were obtained by taking infrared spectra of samples, all of them containing the same phospholipid amount, either in the absence of Ca^{2+} or in the presence of saturating Ca^{2+} concentrations [39].

3. Results

As described in Materials and methods, the incorporation of SPH and SA into the different mixtures used in this work has been quantitatively studied. The results have shown that these amino bases partition preferentially into the membrane subphase, because more than 95% of the nominal SPH or SA were localized in the membrane in all the samples studied in this work.

3.1. Differential scanning calorimetry

Differential scanning calorimetry has been used to study the effect of different DPPS/Ca^{2+} molar ratios on the phase transition of pure DPPS and on mixtures of DPPS plus either SPH or SA. We have studied specifically mixtures of DPPS and either SPH or SA at molar ratios of DPPS/SPH 2:1 and DPPS/SA 1:1, because these specific relationships between the phospholipid and the amino bases correspond to azeotropic mixtures as it has been previously described [32], as well as a sample containing DPPS and SPH at a DPPS/SPH molar ratio of 1:1 in order to compare it with the SA containing sample. The thermograms recorded in heating scans at different DPPS/Ca^{2+} molar ratios for the different samples studied in this work are represented in Fig. 1. It should be remarked that the samples containing DPPS and SPH or SA were formed before Ca^{2+} was added to the medium.

For pure DPPS (Fig. 1A) T_c increased with increasing Ca^{2+} concentration indicating a stabilization of the gel phase in agreement with previous results using FT-IR that indicated an increase in the rigidification of DPPS induced by Ca^{2+} [41]. At DPPS/Ca^{2+} molar ratios of 2:1 or lower no phase transition was observed at temperatures between 30° C and 80° C (see below), due to the formation of cochleate structures [42]. This is in agreement with previous observations which concluded that the phosphatidylserine/Ca^{2+} binding stoichiometry is 2:1 [43] as it has been also confirmed by later measurements of Ca^{2+}-binding between phosphatidylserine lamellae [44] and infrared spectroscopy [39].

However, in the presence of SPH at DPPS/SPH molar ratios of 2:1 and 1:1 (Fig. 1B and C, respectively) the results were significantly different to those of pure DPPS. In both mixtures, and with increasing Ca^{2+} concentration, T_c decreased slightly. However, and at variance with pure

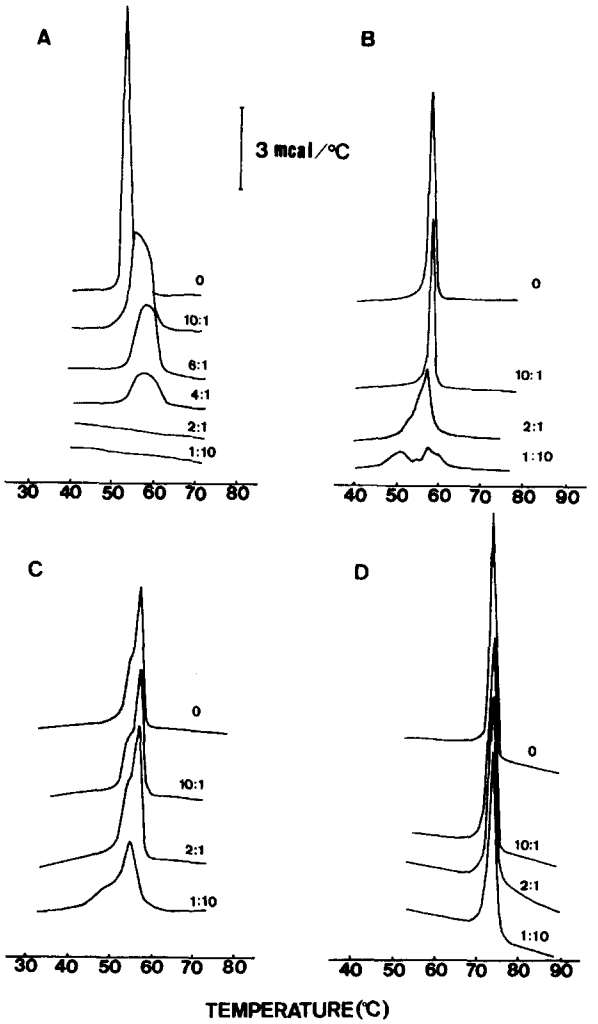

Fig. 1. DSC heating thermograms of pure DPPS (A), DPPS containing SPH at DPPS/SPH molar ratios of 2:1 (B) and 1:1 (C), and DPPS containing SA at a DPPS/SA molar ratio of 1:1 (D) at the indicated DPPS/Ca^{2+} molar ratios.

DPPS, at a DPPS/Ca^{2+} molar ratio of 2:1 a phase transition peak was observed (compare Fig. 1A, B and C). At a DPPS/Ca^{2+} molar ratio of 1:10, i.e., at a very high concentration of Ca^{2+} which exceeds considerably the ratio for the stoichiometric compound Ca(phosphatidylserine)$_2$, a complex transition peak presenting more than one component was observed (Fig. 1B and C). In all the cases the width of the transition increased at increasing DPPS/Ca^{2+} molar ratios, indicating a decrease in the cooperativity. However, in the presence of SA at a DPPS/SA molar ratio of 1:1, no significant effect was observed neither in the phase transition temperatures of DPPS nor in the width of the peak even in the presence of very high Ca^{2+} concentrations (Fig. 1D).

The phase transition temperatures of the different mixtures studied, in both heating and cooling scans, are shown in Fig. 2. For pure DPPS and at increasing Ca^{2+} concentrations, an increase in the T_c was observed, both in heating and in cooling scans, as previously described [45,46] (Fig. 2A). At very high concentrations of Ca^{2+} (DPPS/Ca^{2+} molar ratio of 2:1 or lower) no transition peaks were observed, presumably due to the formation of crystalline structures (cochleates) in the presence of such Ca^{2+} concentrations (see above) [42]. However, in the presence of SPH, at DPPS/SPH molar ratios of either 2:1 or 1:1 no increase in T_c was observed at the different Ca^{2+} concentrations used in this study (Fig. 2B and C). It is interesting to note that, in cooling scans, nearly no effect was observed at increasing Ca^{2+} concentrations, whereas a decrease in T_c was observed for the heating scans, so that for a DPPS/Ca^{2+} molar ratio of 1:10, T_c suffers a shift to lower temperatures, of approx. 15° C for DPPS/SPH at a molar ratio of 2:1 and of 9° C for a 1:1 molar ratio. A different behavior was observed for the SA containing sample since a much small effect was observed in T_c either in cooling or in heating scans (Fig. 2D), indicating that the interaction between DPPS and SPH is somehow different than that of DPPS and SA.

The effect of increasing Ca^{2+} concentrations on the enthalpy of the transition of DPPS (ΔH) in the presence or in the absence of SPH or SA is shown in Fig. 3. For pure DPPS, ΔH decreases with increasing Ca^{2+} concentrations until reaching a DPPS/Ca^{2+} molar ratio of 2:1, for which no transition was observed (see Fig. 1A). In the presence of SPH and SA and in the absence of Ca^{2+}, a slight decrease in ΔH was observed as previously reported [32]. At increasing Ca^{2+} concentrations and in the presence of either SPH or SA, a decrease in ΔH was observed, but, even so, ΔH was not nullified at very high Ca^{2+} concentrations such as DPPS/Ca^{2+} molar ratios of 1:10, at variance with pure DPPS in the presence of Ca^{2+}

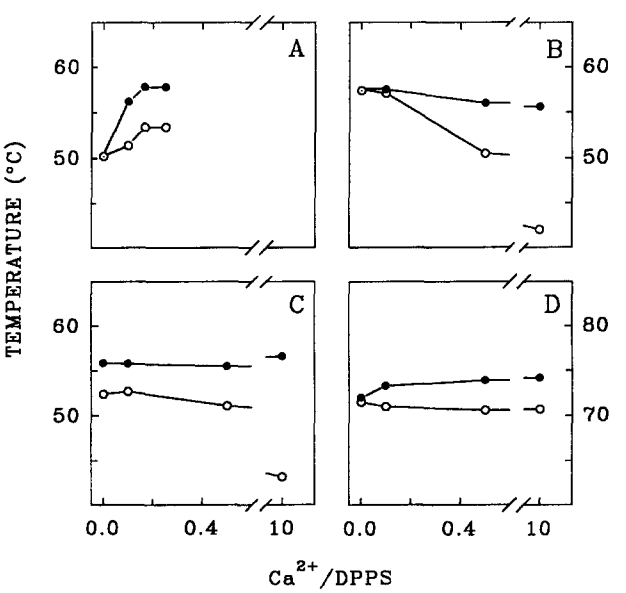

Fig. 2. Dependence of the transition temperatures with the DPPS/Ca^{2+} molar ratio on heating (○) and cooling (●) scans for pure DPPS (A), DPPS containing SPH at a DPPS/SPH molar ratios of 2:1 (B) and 1:1 (C), and DPPS containing SA at a DPPS/SA molar ratio of 1:1 (D).

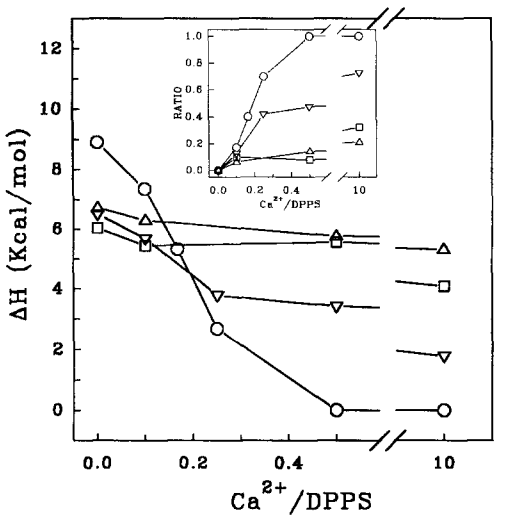

Fig. 3. Dependence of enthalpy (ΔH) with the Ca^{2+}/DPPS molar ratio for pure DPPS (○), DPPS containing SPH at a DPPS/SPH molar ratios of 2:1 (▽) and 1:1 (△), and DPPS containing SA at a DPPS/SA molar ratio of 1:1 (□). The inset shows the ratio of ΔH in the presence of Ca^{2+} to ΔH in its absence for each different sample.

(Fig. 3). The ratio of ΔH for each mixture in the presence of Ca^{2+} with ΔH of the same mixture but in the absence of Ca^{2+} can be observed in the inset of Fig. 3. For pure DPPS this ratio increased as the Ca^{2+} concentration increased until a maximum at a DPPS/Ca^{2+} molar ratio of 2:1, i.e., until no ΔH was observed. The presence of SPH at a DPPS/SPH molar ratio of 2:1 decreased this ratio to nearly a half of its value without amino base, whereas it was kept close to 0 in the presence of SPH or SA at DPPS/amino base molar ratios of 1:1 (Fig. 3, inset). These results indicated very clearly that the presence of either SPH or SA in DPPS membranes prevents the interaction between DPPS and Ca^{2+}. This was supported by the fact that equimolar amounts of either SPH or SA abolished the effect of Ca^{2+} on DPPS, even at DPPS/Ca^{2+} molar ratios of 1:10, whereas half the quantity of SPH in DPPS reduced ΔH to approximately a half (see Fig. 3 and inset).

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 [47]. In order to study the effect of the incorporation of SPH and SA in the crystalline structure of the complex DPPS/Ca^{2+} we have made a study in the high temperature region (130–170° C). When equimolar quantities of SA or SPH were included in DPPS and in the presence of very high calcium concentrations, no transition was observed at 155° C, demonstrating that the crystalline complex, i.e., the cochleate structure, was not formed (not shown for brevity).

3.2. ^{45}Ca-binding experiments

In order to confirm that SPH and SA prevented the interaction between DPPS and Ca^{2+}, binding experiments were carried out by using $^{45}CaCl_2$. The experimental points of the binding experiments, shown in Fig. 4, were fitted to the following Michaelis-Menten type equation [39],

$$\frac{[Ca^{2+}]_{bound}}{[DPPS]} = \frac{n([Ca^{2+}]_{free}/[DPPS])}{m + ([Ca^{2+}]_{free}/[DPPS])} \quad (1)$$

The binding of Ca^{2+} by DPPS was saturable as observed in Fig. 4. For pure DPPS, and at saturating Ca^{2+} concentrations, we found previously a value of 0.51 for n which is the maximum value of Ca^{2+} bound per molecule of DPPS [39]. This result agrees with the generally observed stoichiometry of about 2 molecules of phosphatidylserine bound to each Ca^{2+} ion [32,39,44,48]. However, in the presence of SPH or SA, and at a DPPS/amino bases molar ratio of 2:1, n was found to be 0.24 and 0.23 for the SPH and SA containing samples, respectively. These values are nearly a half of the value found for pure DPPS, and hence these mixtures will bind about one Ca^{2+} ion per four DPPS molecules. In this case, half of the DPPS molecules would be interacting electrostatically with the free amino bases, whereas the other half of the molecules would be bound to Ca^{2+}. In the case of DPPS/amino bases molar ratio of 1:1 (see Fig. 4), n was 0.05, i.e., nearly nullified, for both SPH and SA containing samples indicating that, in this case, almost all DPPS molecules would be interacting with the amino bases, so that there is almost no binding of Ca^{2+} to the DPPS molecules in the membrane.

3.3. FT-IR experiments

We have shown previously using DSC and ^{31}P-NMR that SPH and SA alter the phase behavior of DPPS [32]. In this work we have examined these interactions at a microscopic level, trying to find by means of FT-IR whether there are or not specific interactions between DPPS and these amphiphatic aminobases as well as the influence of Ca^{2+} on these interactions. Different groups located both

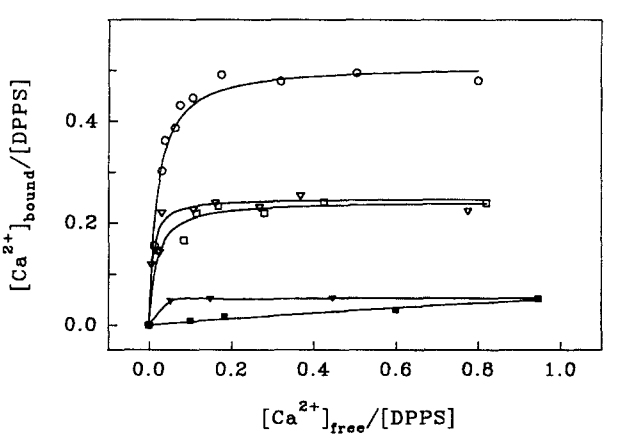

Fig. 4. ^{45}Ca-binding to pure DPPS (○), DPPS/SPH 2:1 (▽), DPPS/SPH 1:1 (▼), DPPS/SA 2:1 (□) and DPPS/SA 1:1 (■).

at the polar head of the phospholipid and in the hydrophobic matrix of the membrane have been examined by FT-IR. In this way the phase transition of the samples, as detected by the CH_2 asymmetric and symmetric stretching bands located at 2918 cm^{-1} and 2850 cm^{-1}, increased in the presence of SPH and SA (not shown for brevity), similarly as it was previously found by DSC [32].

The ester C=O stretching mode showed a broad band appearing at 1734 cm^{-1} which, after deconvolution, yielded two components at 1740 and 1728 cm^{-1} [39,49]. The presence of either SPH or SA did not change the maximum nor the width of this phospholipid band and their components, neither in the presence nor in the absence of Ca^{2+} (not shown for briefness), suggesting that the amino bases do not establish hydrogen bonding with the C=O group nor affect its hydration state.

Exactly the same was the case for the PO_2^- antisymmetric band at 1220 cm^{-1} (not shown for the sake of briefness). It has been shown previously that this band is useful to monitor the hydration state of the polar head group of the phospholipid and specifically the interaction of phosphatidylserine with Ca^{2+} [39,41,50–52]. A frequency of 1220 cm^{-1} characterizes a fully hydrated PO_2^- group, whereas its dehydration is characterized by a frequency of 1240 cm^{-1} [53]. In order to quantify the degree of dehydration of the PO_2^- group of DPPS a partial least-squares multivariate statistical analysis was devised as described previously [39]. Hence, the proportion of the PO_2^- groups of DPPS which are dehydrated or hydrated, as observed by the wide band at 1220 cm^{-1}, can be estimated using this method in any sample containing DPPS and equimolar quantities of SPH or SA in the presence of Ca^{2+} (Fig. 5). Pure DPPS in the presence of Ca^{2+} at a DPPS/Ca^{2+} molar ratio of 2:1 is totally dehydrated (Fig. 5) in agreement with previous observations [39]. However, in the presence of equimolar amounts of either SPH or SA

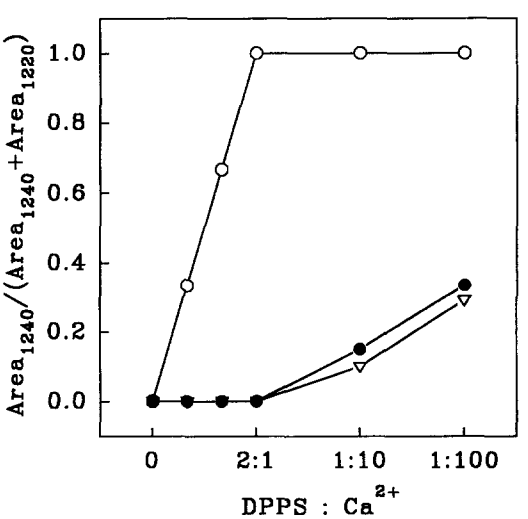

Fig. 5. Extent of PO_2^- dehydration at different DPPS/Ca^{2+} molar ratios for pure DPPS (○), DPPS/SPH 1:1 (●) and DPPS/SA 1:1 (▽).

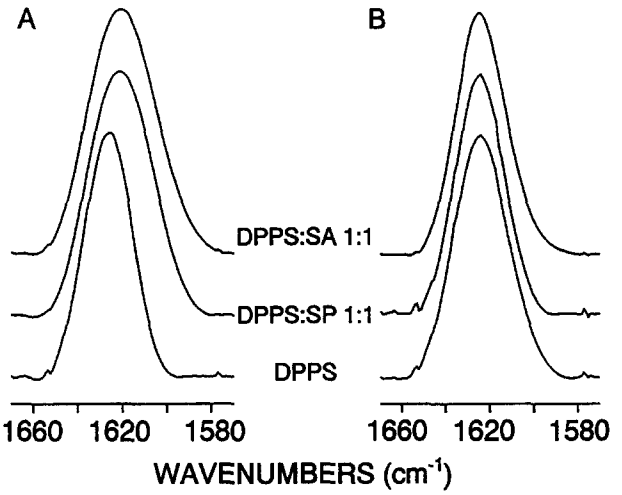

Fig. 6. Infrared spectra of the COO^- carboxyl band of DPPS in (A) the absence and (B) in the presence of Ca^{2+} at a DPPS/Ca^{2+} molar ratio of 2:1 at different DPPS/amino bases molar ratios as indicated.

dehydration of this group was observed only at very high Ca^{2+} concentrations. This dehydration was not affecting to all PO_2^- groups of the DPPS molecules, since the dehydration found for samples containing equimolar amounts of either SPH or SA at a DPPS/Ca^{2+} molar ratio of 1:100 was about 30% compared with pure DPPS at the same DPPS/Ca^{2+} molar ratio (Fig. 5).

The COO^- vibration band appearing at 1622 cm^{-1} was different in the presence of the amino bases either below or above the phase transition temperature of the mixtures. In the presence of equimolar quantities of SPH and SA and in the absence of Ca^{2+} there was a decrease of approx. 4 cm^{-1} in the frequency of the maximum of the COO^- vibration band of DPPS as well as an increase of approx. 6 cm^{-1} in its half band-width as compared with pure DPPS (Fig. 6A). However, in the presence of Ca^{2+} at a DPPS/Ca^{2+} molar ratio of 2:1 no significant changes were observed in the COO^- vibration band (see Fig. 6B). A shift of the COO^- vibration band to higher wavenumbers should be expected if a hydrogen bonding would be established between the amino bases and the phospholipid [41]. Therefore, as shown by the shift to lower wavenumbers in the presence of the amino bases, only electrostatic and/or hydration effects should be the origin of this effect, as discussed below.

The COO^- antisymmetric stretching vibration of DPPS appears at approx. 1622 cm^{-1} whereas the COOH band appears at approx. 1740 cm^{-1} [54]. Therefore, it would be possible to titrate directly the carboxylic group of DPPS measuring the intensity of the COOH and COO^- bands, because, as bulk pH is increased, the band corresponding to the COOH group of DPPS decreases in intensity, whereas at the same time, the band corresponding to the COO^- group increases in intensity as observed in the inset of Fig. 7 [54]. In this way we obtained the apparent pK (pK_{app}) of the carboxyl group of DPPS either pure or in

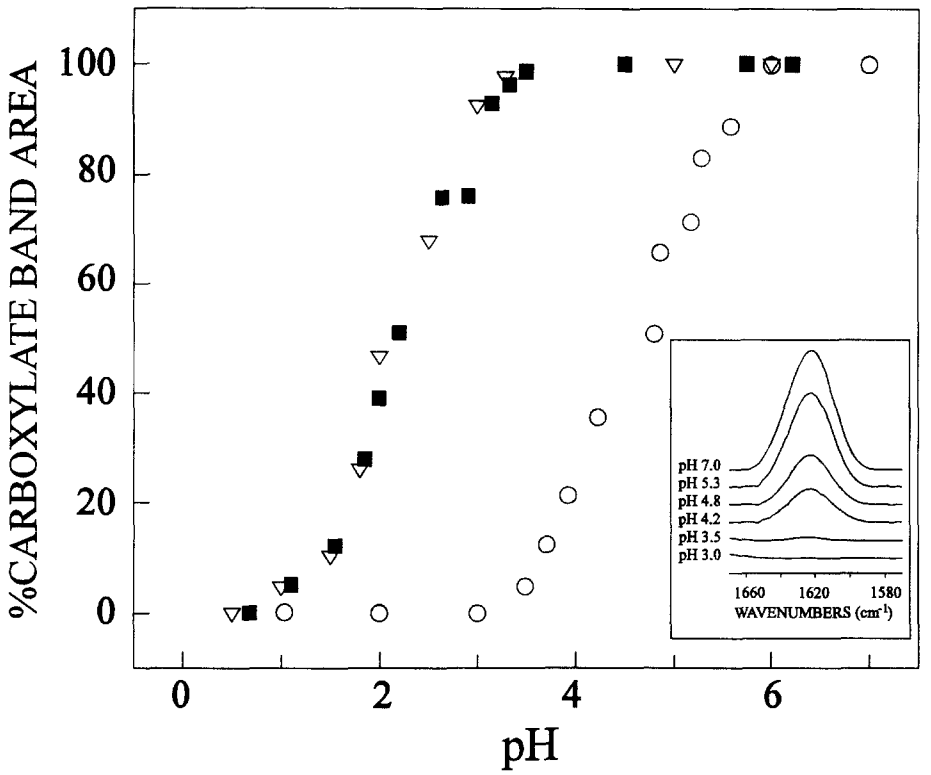

Fig. 7. FT-IR titration of the carboxyl group of pure DPPS (○), DPPS/SPH 1:1 (▽) and DPPS/SA 1:1 (■). The inset shows the COO^- vibration band of pure DPPS at different pH values as indicated.

the presence of SPH or SA, both in the absence and in the presence of Ca^{2+}. The corresponding titration curves for the COO^- group of DPPS, i.e., increase in band area as pH is increased, in the different mixtures studied in this work and at 40° C are shown in Fig. 7. The pK_{app} values were obtained from the midpoints of the titration curves as previously described [32,55].

The pK_{app} of the carboxylate group of pure DPPS was found to be 4.6, in agreement with previous results [55]. On the other hand, and in the presence of equimolar amounts of SPH or SA, the pK_{app} of the carboxylate group was 2.1 in the presence of SPH and 2.0 in the presence of SA.

4. Discussion

There is much interest nowadays in the characterization of the occasional association to membranes of several important proteins, such as protein kinase C or phospholipases, since these membrane associating proteins are involved in cellular signal transmission mechanisms [10–12]. Moreover, it has been shown that the association of these proteins to the membrane (a prerequisite for their activation, [13–15]) is mediated by acidic phospholipids, specifically phosphatidylserine, and Ca^{2+} [16,17], and inhibited, for example, by SPH and SA [1–8]. We studied previously the interaction of SPH and SA with phosphatidylserine in the absence of Ca^{2+} [32]. In this work we have extended this previous work and studied the effect of the incorporation of SPH and SA on phosphatidylserine membranes in the presence of Ca^{2+} by using DSC, FT-IR and $^{45}Ca^{2+}$-binding. The combination of these techniques and the use of Ca^{2+}, which, together with phosphatidylserine, is implicated in the activation of protein kinase C, has allowed us to reveal the strong interaction established between phosphatidylserine and the aminobases SPH and SA.

The results presented in this work clearly illustrates the type of alterations that SPH and SA introduce in the DPPS bilayers. As shown through the DSC experiments, the interaction of Ca^{2+} with DPPS is prevented by the presence of these amino bases, i.e., the cochleate is not formed. The behavior of the azeotropic mixtures of DPPS and SPH or SA (molar ratios of 2:1 and 1:1, respectively), as well as the mixture DPPS/SPH at a molar ratio 1:1, show that the interaction of DPPS and Ca^{2+} is not taking place, even at very high concentrations of Ca^{2+}. As it was pointed out recently by us [32] and another group [56], the main effect of SPH and SA on DPPS (in the absence of Ca^{2+}) is mainly of electrostatic origin, i.e., lies on their amino polar groups. The maintenance of the enthalpic values for the DSC thermograms of the mixtures of DPPS and SPH or SA in the presence of Ca^{2+}, as shown in this work, would strengthen this conclusion. Therefore the binding of Ca^{2+} to DPPS is completely prevented by the interposition of

the charged amino bases, whose pK_{app} values in DPPS membranes are 9.12 and 8.94 for SPH and SA, respectively [32]. The lack of interaction between Ca^{2+} and DPPS is furthermore demonstrated by the $^{45}Ca^{2+}$-binding experiments, were the binding ratio of DPPS/Ca^{+} is dramatically altered in the presence of the amino bases. These data suggest then that Ca^{2+} does not bind almost at all to DPPS molecules in the presence of equimolar amounts of SPH or SA, and at the range of molar ratios used in these experiments (see Fig. 4).

The effects seen here with respect to Ca^{2+} will operate similarly in relation to other ions or molecules approaching the membrane from the water medium. This can be the case of proteins and nucleic acids. For example, SPH incorporated in lipidic membranes containing acidic lipids, lowers the binding to the membrane of cationic proteins such as cytochrome *c* and phospholipase A_2 as well as small molecules such as adriamycin [57]. Other authors also showed that SPH prevents the binding of histones to phosphatidylserine in mixed Triton-phosphatidylserine micelles [27]. Recently, it has been also shown the binding of both DNA and RNA to SPH-containing membranes and its reversal by acidic phospholipids such as egg phosphatidic acid [25,26].

In order to know the way in which SPH and SA exert this effect we have investigated by FT-IR the type of alteration that they introduce in DPPS bilayers, and particularly in the polar headgroups with which Ca^{2+} interacts [42]. Previous FT-IR experiments have shown that Ca^{2+} interacts specifically with phosphatidylserine altering significantly several vibrational bands of the phospholipid, such as the C=O ester and PO_2^- bands, whereas no dehydration effects were observed in the COO^- carboxylate stretching band [39,41]. Significantly and in the presence of the amino bases, it was really remarkably that Ca^{2+} did not affect in a dramatic way the C=O ester and PO_2^- vibration bands of DPPS, indicating, first, that Ca^{2+} did not bind to the polar head group of DPPS and, second, that the phospholipid and the amino bases, either in the absence or in the presence of Ca^{2+}, did not present hydrogen bonding interactions, at least in those groups mentioned above. By observing the PO_2^- antisymmetric stretching of DPPS (Fig. 5), it can be concluded that the presence of SPH and SA, in equimolar ratios with DPPS, completely prevents the binding of Ca^{2+} to the PO_2^- group at DPPS/Ca^{2+} molar ratios of 2:1, and, at molar ratios of 1:100, only about 30% of the total molecules are dehydrated.

However, there were small but significant effects of the amino bases on the carboxyl group of DPPS, both in frequency and band-width. Moreover, FT-IR spectroscopy experiments have shown that the apparent pK_{app} of the carboxyl group of DPPS was dramatically affected by the amino bases, so that the pK_{app} was reduced from 4.6 in pure DPPS to 2.2 in the presence of the amino bases. Certainly SPH and SA alter the organization of the membrane as previously shown through DSC [32,56], but this perturbation does not seem to be due to the establishment of specific molecular interactions between the phospholipid and the amino bases. However, the possibility that the free hydroxyl groups of SPH interact by hydrogen bonding with the amino group of DPPS can not be ruled out on the basis of our FT-IR results, since we have not been able of observing those vibrational bands. We concluded that the main effect of SPH and SA on DPPS is of electrostatic origin (see Ref. [30] and this work) and as a consequence the pK_{app} value of the carboxyl group of the phospholipid was changed.

Let us now examine in detail the origin of the shifts in pK_{app} observed for the mixtures of SPH or SA in DPPS. As pure DPPS forms bilayers spontaneously in water, it is not possible to measure the intrinsic pK directly (pK_i) and therefore the observed pK, i.e., 4.6, is an apparent pK (pK_{app}). As it has been described before, the pK_{app} of the carboxylate group of DPPS differs from the pK_i of the group in water due to electrostatic and hydration effects [58]:

$$pK_{app} = pK_i \pm \Delta pK_{el} + \Delta pK_h \quad (2)$$

where ΔpK_{el} is the electrostatic-induced shift and ΔpK_h is the hydration-induced shift. The hydration-induced shift is positive or negative if the number of surface charges increases or decreases, respectively, upon titration [59]. On the other hand, the electrostatic shift, ΔpK_{el}, is [58]:

$$\Delta pK_{el} = \frac{-e\psi_o}{2.3\kappa T} \quad (3)$$

where Ψ_o is the membrane surface potential and e, κ and T have their usual meanings. Since increasing the degree of ionization of DPPS, the surface potential becomes negative [60], ΔpK_{el} must be positive and as the number of charges increase upon titration, pK_{app} must be bigger than pK_i, which has been estimated of about 2.65 [48,60]. In the presence of SPH or SA at equimolar concentrations with DPPS, the pK_{app} of the carboxyl group of DPPS decreased to 2.1 and 2.2, respectively. This change in pK_{app} in the presence of SPH or SA compared with pK_{app} of pure DPPS is in accordance with, first, the introduction of one positive charge throughout the titration and, second, the decrease in the membrane surface potential upon the inclusion of the amino bases in the membrane.

4.1. Concluding remarks

In this work we show that SPH and SA prevent completely the binding of Ca^{2+} to DPPS membranes at Ca^{2+}/DPPS molar ratios which are saturating for pure DPPS. Our findings reveal that the presence of positively charged molecules in membranes such as SPH or SA, anchored in them due to their hydrophobic character, affects the dissociation of the carboxyl group of DPPS,

shifting its apparent pK_{app} to lower values, and interacting electrostatically with the phospholipid. Moreover, the association of both SPH and SA with DPPS involves similar mechanisms as shown by the above results. Apart from that, the incorporation of SPH and SA alter the thermotropic behavior of the phospholipids in the membrane through electrostatic effects as shown before [32,56]. Therefore, SPH and SA are able of preventing the binding of other external positively charged molecules, such as Ca^{2+}, through an electrostatic charge neutralization (this work) and it can be suggested that this is also the reason to prevent the binding of proteins [57] and nucleic acids [25,26]. As pointed out recently [7], SPH can be envisaged as a lipid second messenger in cell membranes, interacting with cell receptors, protein kinases and other fundamental sites of the cell, and thereby affecting many cellular functions. The data provided in this work could be useful when trying to explain the biological effects of these amino bases.

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