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Interaction of sphingosine and stearylamine with phosphatidylserine as studied by DSC and NMR

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The interaction of sphingosine (SP) and stearylamine (SA) with dipalmitoylphosphatidylserine (DPPS) has been studied by using differential scanning calorimetry (DSC) and phosphorus nuclear magnetic resonance (^{31}P-NMR). DSC showed that SP and SA rigidified the membranes, forming an azeotropic mixture with DPPS. The azeotropic mixture which was formed between DPPS and SP was found at a DPPS/SP molar ratio of 2:1 whereas SA and DPPS formed an azeotropic mixture at a DPPS/SA molar ratio of 1:1. An eutectic point was observed at 85 mol% of SP and 90 mol% of SA in DPPS. ^{31}P-NMR showed the presence of a lamellar phase at DPPS/SP and DPPS/SA molar ratios lower than 1:1, whereas at higher molar ratios and at high temperatures, besides the lamellar phase, an isotropic component was detected. It was found that, at physiological pH, both SP and SA were protonated in a large extent, i.e., positively charged, since their apparent pK in the membrane were 9.1 and 8.9, respectively. The results reported in this work may be relevant to understand a number of biological effects produced by these positively charged molecules, due to their electrostatic interaction with negatively charged phospholipids.

Introduction

Sphingosine (SP) and other sphingolipids have been recently recognized as important biological mediators affecting many cellular functions [1,2]. In many cases they have been shown to exert their function by inhibiting protein kinase C [3] and this activity appears to mediate many of the biologic effects of SP [1,2]. However, SP also exhibits other protein kinase C independent activities such as inhibition of tissue factor [4], inhibition of insulin receptor tyrosine kinase [5], biphasic effects on diacylglycerol kinase [6], inhibition of phosphatidic acid phosphohydrolase [7,8], inhibition of CTP-phosphocholine cytidyltransferase [9] and activation of phospholipase D [10,11]. Thanks to its ability to inhibit protein kinase C, SP has been described as a cancer preventive agent [12]. A unique property of SP with respect to other membrane lipids lies in its positive charge. SP, although a minor lipid component in biomembranes, is the main lipid to have positive charge in normal cells, since sphinganine exists in much smaller amounts [2] and other lysosphingolipids have been only detected in certain pathological conditions but not in normal cells [1]. Hence, SP and lysosphingolipids may be involved in the regulation of protein kinase C under normal and/or pathological conditions.

With respect to the molecular mechanism of action of SP, its positive charge has been shown to correlate with its capacity to inhibit protein kinase C [13,14]. It has been suggested that this effect may be produced by charge neutralization of phosphatidylserine, an essential phospholipid for protein kinase C activity [15–17], possibly by preventing the interaction of the enzyme and/or its protein substrate with the lipid [13].

Despite the remarkable importance of SP in health and disease, very little is known about the behavior of this lipid in membranes and its interaction with other lipids. And the same apply to other positively charged lipids such as stearylamine (SA), which is also able of

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Abbreviations: $\Delta\sigma$, chemical shift anisotropy; ΔH, enthalpy change of the gel to liquid-crystalline phase transition; ^{31}P-NMR, phosphorus-31 nuclear magnetic resonance; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine; DSC, differential scanning calorimetry; EDTA, ethylenediamine tetraacetic acid; Mes, 3-(*N*-morpholino)ethane sulfonic acid; ppm, parts per million; SA, stearylamine; SP, sphingosine; T_c, onset temperature of the gel to lipid-crystalline phase transition; $\overline{T}_c^6$, calculated average T_c value at pH = 6; $\overline{T}_c^{10}$, calculated average T_c value at pH = 10.

inhibiting protein kinase C [18] apart from having other biological activities such as permeabilization of lysosomal membranes [19].

It is the aim of this work to study the interaction of SP and SA with phosphatidylserine, i.e., a negatively charged phospholipid of primary importance in animal membranes and which is the phospholipid which produces the maximum activation of protein kinase C [15–17]. Our purpose is to understand the mechanism of action of these lipids which must obviously being localized in cell membranes. Differential scanning calorimetry (DSC) and phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) have been used, showing that both compounds are able of forming homogeneous mixtures with DPPS with molar ratios DPPS/SP of 2:1 and DPPS/SA of 1:1. According to the phase diagrams these mixtures corresponded to azeotropic points. Important effects on the dissociation of DPPS and on its phase transition were produced by the presence of these long-chain amines at pH values at which they were fully protonated, i.e., positively charged.

Materials and Methods

Dipalmitoylphosphatidylserine (DPPS) was obtained from Avanti Polar Lipids (Birmingham, AL, USA) and stearylamine (SA), sphingosine (SP) and D_2O (99.8%) were obtained from Sigma (Madrid, 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, SA and SP before and after the measurements, specially those experiments done at high temperature, were checked by thin-layer chromatography where they showed only one spot in all cases.

Sample preparation. DPPS in chloroform/methanol (2:1, v/v) (2 μmol for DSC and 30 μmol for ^{31}P-NMR) and the appropriate amount of either SA or SP in chloroform/methanol (2:1, v/v) were mixed and dried under a stream of O_2-free dry N_2 and the last traces of solvent were removed at high vacuum overnight. The samples were kept and dispersed for 30 min at 70°C, temperature above the gel to liquid-crystalline phase transition of the mixture, in 1 ml for DSC and 15 ml for ^{31}P-NMR of 0.1 mM EDTA, 50 mM Mes, 100 mM NaCl buffer (pH 6.0), with occasional mixing in a vortex mixer until obtaining a homogeneous and uniform suspension. For the pH titration of the phase transition temperature of the mixtures, buffers of constant ionic strength were used ($I = 0.2$). Subsequently the samples for DSC were centrifuged at high speed in a bench microfuge for 30 min and the samples for ^{31}P-NMR were centrifuged at $30\,000 \times g$ for 30 min in a Beckman LB-55M ultracentrifuge.

Differential scanning calorimetry. Pellets for DSC were collected and placed into small aluminium pans, sealed and incubated for 48 h at 20°C before scanning in a Perkin-Elmer DSC-4 calorimeter using 10 μl of buffer as reference. The instrument was calibrated using indium as standard. The samples were scanned with a heating and cooling rate of 4 C°/min at 1 mcal/s and the first scan was used for display except where noted. The range of temperatures studied was from 20°C to 90°C.

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 as described before [20].

The incorporation of SA and SP into liposomes was measured by combining phosphorus assay and the determination of amine groups by the method of Benson and Hare [21]. The incorporation of SA and SP in the membranes was higher than 95% in all the samples used in this work.

^{31}P-Nuclear magnetic resonance. Samples for NMR were introduced in a small tube (5 cm length, 8 mm outer diameter) and put inside a conventional 10 mm NMR tube with external D_2O. ^{31}P-NMR spectra were recorded in the Fourier transform mode on a Bruker AC-200 spectrometer (81 MHz) equipped with an Aspect 3000 computer. Temperature was controlled to ± 1 C° with a standard Bruker B-VT-1000 variable temperature control unit. 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 (4 W input power during acquisition time) and accumulated free induction decays were obtained from up to 2000–2500 transients. A spectral width of 25 kHz, a memory of 8K 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 [22]. Aliquots of the NMR tubes were also measured by DSC as described above. In all cases the alterations in the NMR spectra were reversible upon recooling the samples.

Results

The effect of incorporating different amounts of SP on the gel to liquid-crystalline phase transition of DPPS at pH = 6 is shown in Fig. 1A. This pH was specifically chosen because the amino group of SP was fully protonated (see below). Aqueous dispersions of DPPS can

undergo a gel to liquid-crystalline ($L_\beta \rightarrow L_\alpha$) phase transition ($T_c$) in the lamellar phase as it is shown in the thermograms of pure DPPS (Fig. 1, upper part). The $L_\beta \rightarrow L_\alpha$ phase transition of DPPS occurred at 50.6°C in agreement with previous data [23].

As the concentration of SP was increased, the transition of DPPS was shifted to higher temperatures until a concentration of 35 mol% of SP in DPPS was reached. At 40 mol% of SP a shoulder was beginning to be observed in the main transition peak, and at the same time, the width of the endothermic peak increased as the concentration of SP increased. At 55 mol% of SP a broad peak was observed at a temperature lower than that of T_c of DPPS. This low-temperature transition became the main peak between 60 mol% and 80 mol% of SP. At 85 mol% this peak, centered at about 35°C, was the only one to remain. Only one peak was observed in the thermograms until pure SP.

The effect of SP on ΔH of the transition of DPPS is shown in Fig. 2A. ΔH of the phase transition of pure DPPS was found to be 8.8 kcal/mol in agreement with previous data [24]. Significantly, ΔH of the $L_\beta \rightarrow L_\alpha$ transition undergo a gentle decrease at increasing concentrations of SP until reaching pure SP. The observed decrease of ΔH was linear going from pure DPPS to pure SP.

The effect of the incorporation of different amounts of SA on the gel to liquid-crystalline phase transition of DPPS is shown in Fig. 1B. Similarly to the SP samples, the experiments were carried out at pH = 6, in order to have the amine group of SA fully protonated (see below). SA concentration as low as 1.9 mol% already broadened the transition peak in comparison to pure DPPS (Fig. 1B). As the SA concentration was increased up to 16.7 mol%, a broad transition peak formed by at least two components was observed.

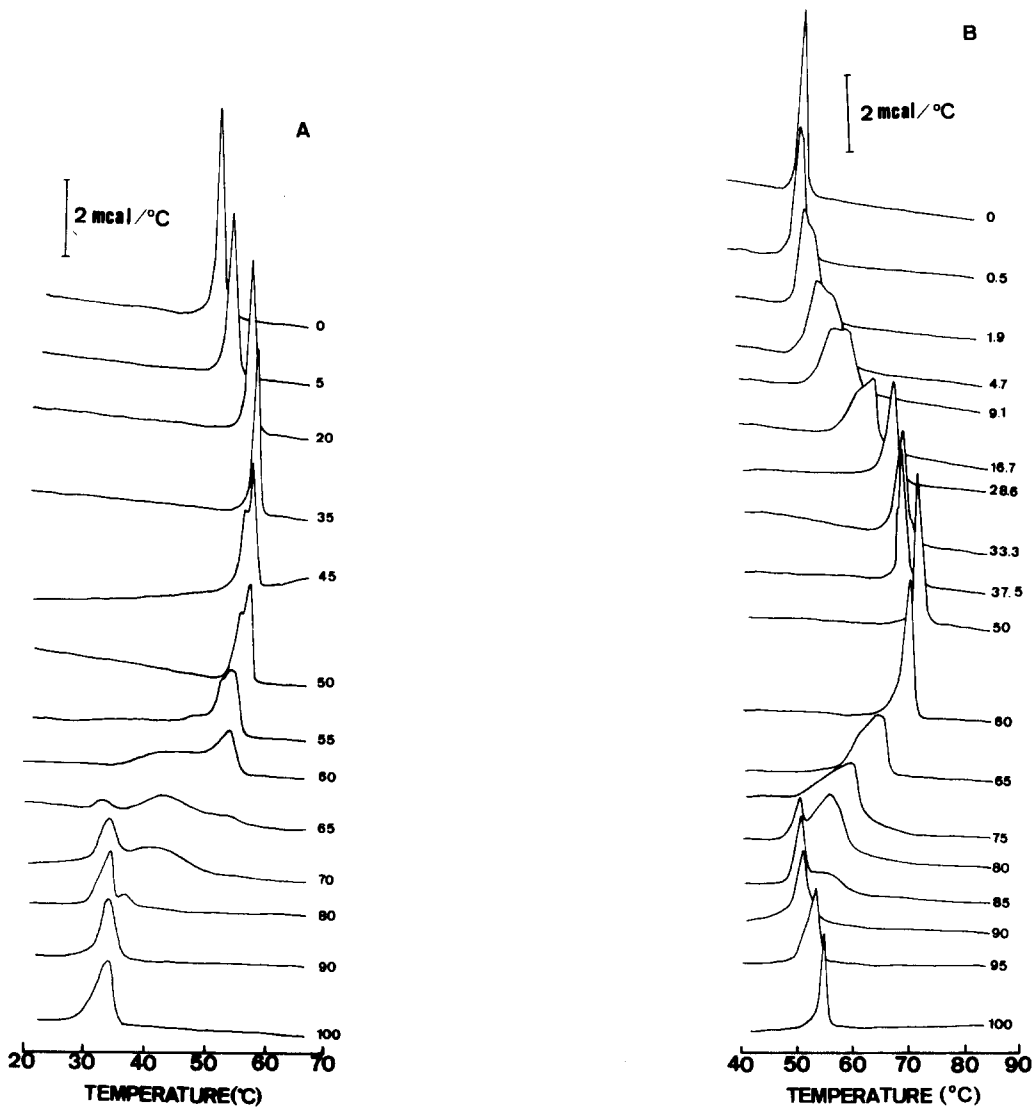

Fig. 1. DSC calorimetric curves for systems containing DPPS and (A) sphingosine or (B) stearylamine at pH = 6. Molar percentages are indicated on the curves. The profiles correspond to heating scans.

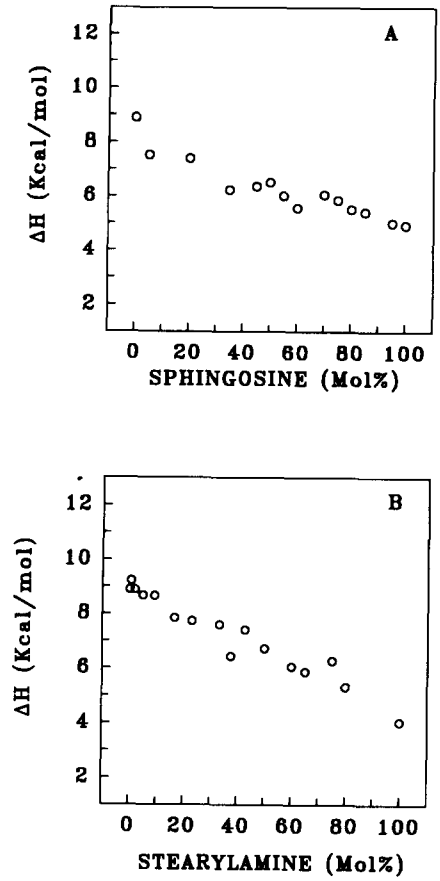

Fig. 2. Dependency of the enthalpy of the main gel to liquid-crystalline phase transition of DPPS upon incorporation of (A) sphingosine or (B) stearylamine.

At the same time the temperature of the transition was progressively shifted to higher temperatures and the component occurring at the highest temperature was growing at the expense of the other (Fig. 1B). Between 23.1 and 33.3 mol% of SA, only one peak was observed. However, at 37.5 and 42.8 mol%, the corresponding peaks showed shoulders again, whereas at 50 mol% only one narrow peak was detected. At higher SA compositions than 50 mol% the transition peaks were shifted to lower temperatures and broad peaks were observed, containing more than one component, except at 90 mol% and higher concentrations of SA, which showed a narrow peak.

The effect of SA on ΔH of the transition of DPPS is shown in Fig. 2B. Similarly to the DPPS/SP mixture, ΔH decreased steadily as the concentration of SA increased until pure SA. Again, and as it was found before for SP, this decrease was linear despite the fact that thermogram peaks with different widths were found at different DPPS/SA ratios (see Fig. 1B).

The effect of SA and SP on the thermotropic phase behavior of DPPS was investigated by means of ^{31}P-NMR. Membrane phospholipids when organized in bilayer structures give rise to a characteristic asymmetrical ^{31}P-NMR line-shape with a high-field peak and a low-field shoulder presenting a residual chemical shift anisotropy ($\Delta\sigma$) of approx. 64 ppm in the gel state and approx. 45 ppm in the liquid-crystalline state due to increased ^{1}H-^{31}P dipolar interactions [25,26]. At temperatures between 20 and 85°C pure DPPS presents an

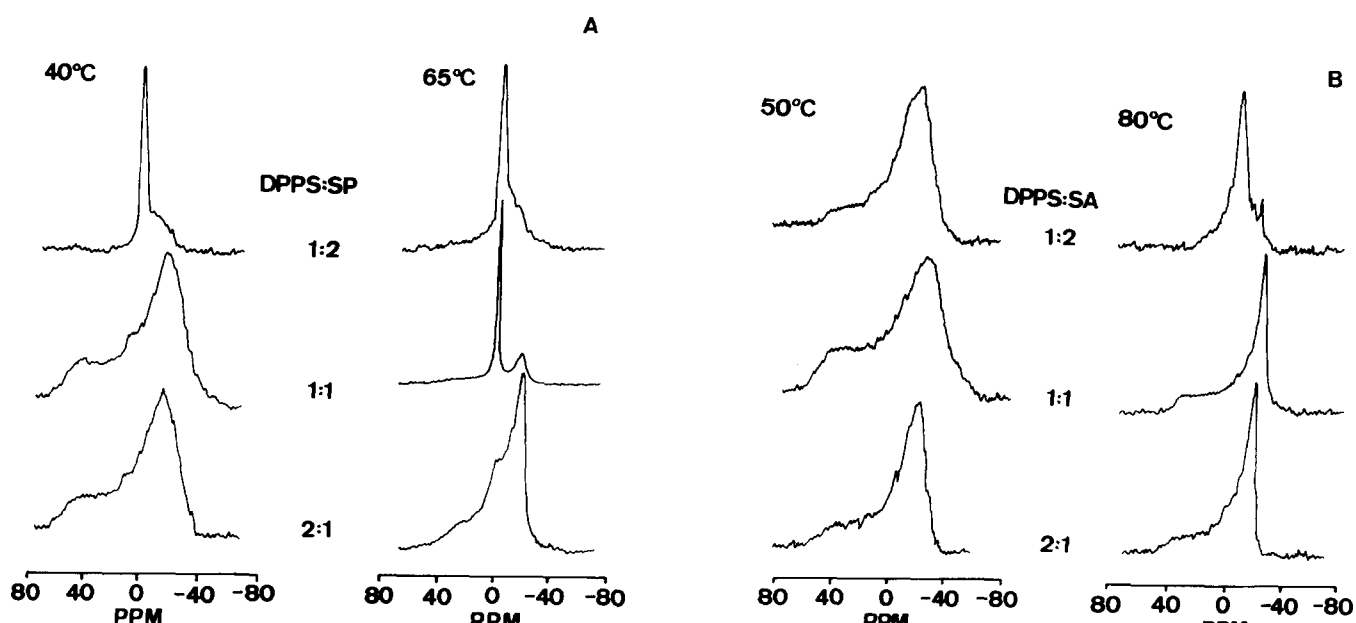

Fig. 3. ^{31}P-NMR spectra of DPPS containing different amounts of (A) sphingosine or (B) stearylamine at different temperatures as indicated. The spectra have been normalized to the same signal height.

asymmetrical ^{31}P-NMR line-shape with a high-field peak and a low-field shoulder indicating that it is in the lamellar phase (not shown).

The effect of the incorporation of SP on the ^{31}P-NMR spectra of DPPS at different temperatures is shown in Fig. 3A. At 40°C and for DPPS/SP samples containing either 33 mol% or 50 mol% of SP, a typical lamellar gel phase component was obtained ($\Delta\sigma = 76$ ppm). However, at a 66 mol% of SP, the NMR spectra resulted in the superposition of two different signals, one of them corresponding to a spectral component with resonance position at 0 ppm, i.e., an isotropic component, and the other one corresponding to a lamellar component (Fig. 3A). The appearance of an isotropic component in the presence of SP indicated that part of the phospholipid molecules experience a rapid motion that leads to a nearly complete averaging of the chemical shift anisotropy. This isotropic component could be due to either phospholipids present in membrane structures or phospholipids in micelles.

At higher temperatures a different behavior was observed. At 65°C and at a SP content of 33 mol% a small isotropic component was apparent. Increasing the SP content to 50 and 66 mol% produced a progressive enhancement of the isotropic component at the expense of the lamellar component (Fig. 3A). In this case the isotropic component was the major one, but a lamellar component was still visible at all temperatures. The $\Delta\sigma$ values decreased with increasing SP content, from 58 ppm at a DPPS/SP ratio of 2:1 to 36 ppm at a DPPS/SP ratio of 1:2.

Incorporation of SA in DPPS induced different effects on the ^{31}P-NMR spectra of DPPS (Fig. 3B). At 50°C and at either 33, 50 or 66 mol% SA, the ^{31}P-NMR spectra showed a component typical of the lamellar phase but no isotropic component was observed (Fig. 3B). In contrast to what was found before for the DPPS/SP mixtures, the $\Delta\sigma$ values of the DPPS/SA mixtures at 50°C were slightly lower (approx. 70 ppm), indicating a more restricted motion of the phosphate group of DPPS. At higher temperatures a different behavior was observed. Whereas at 33 mol% and 50 mol% of SA and 80°C, a bilayer type component was observed ($\Delta\sigma = 64$ ppm), higher concentrations of SA such as 66 mol%, induced the appearance of a broad component centered at 0 ppm (Fig. 3B).

Phase diagrams have been constructed on the basis of DSC and ^{31}P-NMR results. DSC onset temperatures for the gel to liquid-crystalline phase transition have been used as the primary data. Heating and cooling DSC scans were used to produce the solidus and fluidus lines respectively, as described before [27].

The phase diagram corresponding to mixtures of SP with DPPS is shown in Fig. 4A. It can be seen that a good mixing takes place up to 35 mol% of SP, concentration which corresponds to an azeotropic point. At higher molar fractions of SP, solid-solid phase separations were observed and an eutectic point could be observed at 85 mol% of SP. Note that the main transition temperature is higher for a number of DPPS/SP mixtures than for either of the pure components.

The phase diagram corresponding to mixtures of SA with DPPS is shown in Fig. 4B. It is very similar to that of DPPS/SP, but in this case an azeotropic point was found at 50 mol% of SA. In this phase diagram and in contrast to what has been seen in the case of SP, a convergence of the solidus and liquidus curves are observed until a clear maximum at 50 mol%. Apart from that, and as it was found before for SP, an eutectic point was detected at 90 mol% SA.

Since both SP and SA bear an amine group which should be dissociated at physiological pH, it is interesting to investigate their apparent pK in order to calibrate their possible effect on biomembranes.

Samples with a DPPS/SP molar ratio of 1:1 were studied by DSC at different pH values and the T_c

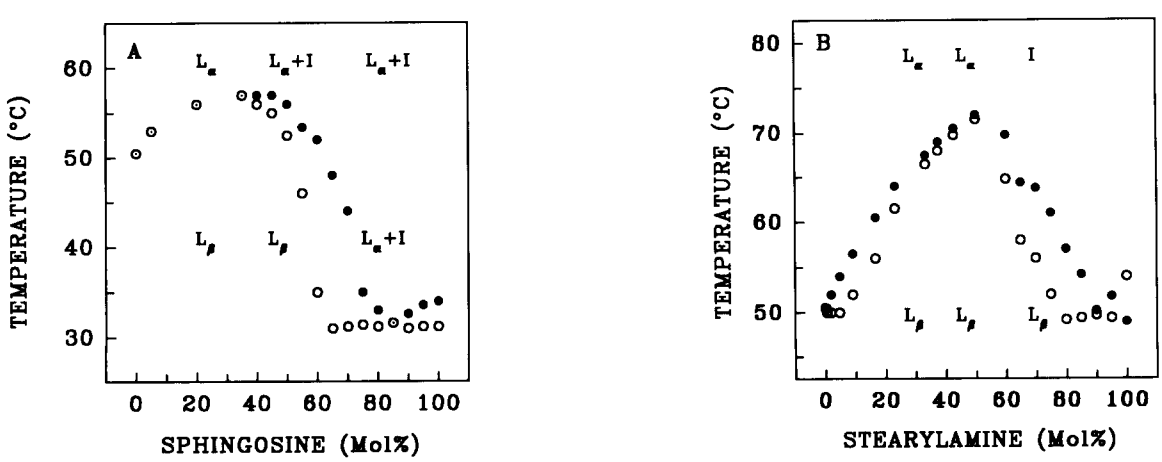

Fig. 4. Partial phase diagrams for systems containing DPPS and (A) sphingosine and (B) stearylamine. Closed and open symbols were obtained from DSC heating and cooling scans, respectively. L, lamellar and I, isotropic phases.

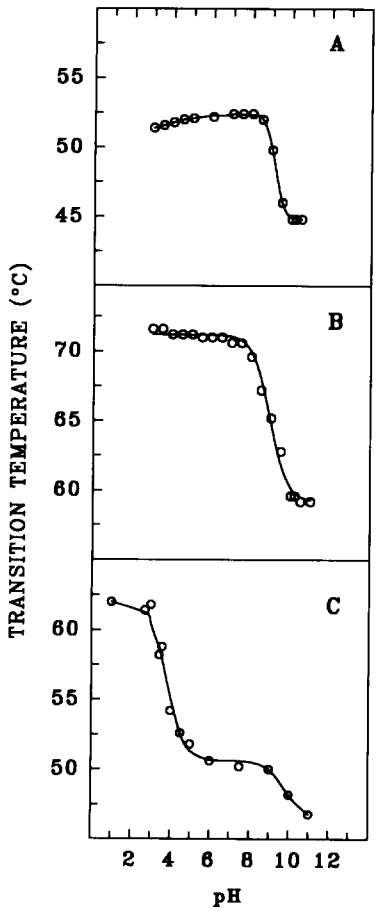

Fig. 5. DSC titration plot at different pH values of (A) an equimolar mixture of DPPS/SP, (B) an equimolar mixture of DPPS/SA and (C) pure DPPS.

transition temperatures found are shown in Fig. 5. The T_c transition temperatures of the DPPS/SP mixture were in the range of 51–52°C at pH 3–8.5 and decreased to approx. 45°C at pH values of 10–11 (Fig. 5A). Fitting the data to the Henderson-Hasselbach equation, the apparent pK of the group which was dissociated was found to be 9.12 ± 0.15. A similar plot corresponding to a DPPS/SA mixture with a 1:1 molar ratio is shown in Fig. 5B. In this case, the temperature decreased from 71°C at low pH (below 6) to 58°C at high pH (above 10), with an estimated apparent pK of 8.94 ± 0.1.

For the sake of comparison, the pH titration plot of pure DPPS is shown also in Fig. 5C. It can be appreciated that a decrease in T_c took place at pH 3, decreasing from 62°C to 50°C, so that an apparent pK of 3.83 ± 0.12 could be estimated, corresponding to the dissociation of the carboxyl group of DPPS. The samples containing either SA or SP did not show any dissociation attributable to the carboxyl group of DPPS. Indeed, infrared studies showed that the carboxyl group was already fully deprotonated at pH 3 in the samples containing either SP or SA, indicating that the apparent pK of the carboxyl group of DPPS in the presence of SA or SP, was lower than 3 (López-García, F., Villalaín, J. and Gómez-Fernández, J.C., unpublished results). In order to explain these results, it has to bear in mind that the presence of the positive charge of SP or SA will favor the dissociation of the carboxyl group at a lower pH than in pure DPPS. This could be expected, since the introduction of new positive charges in the DPPS bilayer will stabilize the DPPS molecule with two negative (phosphate and carboxylate groups) and one positive (amine group) charges, so that taken into account both DPPS and SP or SA molecules, a neutral charge will be obtained.

Discussion

We have studied in this work the interaction of two positively charged molecules, SP and SA, with DPPS by DSC and ^{31}P-NMR. These molecules share the characteristic of having only one hydrocarbon chain compared to the common phosphoglycerides present in biomembranes which have two hydrocarbon chains.

It can be deduced from the phase diagrams that the cationic forms of both SP and SA produced homogeneous mixtures with DPPS at DPPS/SP molar ratios of 2:1 and DPPS/SA molar ratios of 1:1. All systems studied here, DPPS/SP and DPPS/SA, exhibit higher phase transition temperatures for mixed-lipid samples than for samples of their pure components. Hence these homogeneous compositions with higher transition temperatures corresponded to azeotropic points. In this aspect SP and SA behaved similarly to other long-chain and charged compounds as free fatty acids in phospholipid membranes [28–30] and also to other cationic amphiphiles of one [31] or two hydrophobic chains [32].

The marked elevation in the transition temperature of the lipid mixtures could be due in principle to hydrogen bonding and/or electrostatic interactions. By studying the phase transition of mixtures of DPPS with SP and SA at different pH values, it was possible to investigate in detail the interactions established in the mixtures and the origin of the observed increase in T_c.

The observed apparent pK for the dissociation of the amine groups of SP and SA in DPPS membranes were found to be higher than other previously reported values. It has been described recently that the apparent pK of the amino group of SP in Triton X-100 micelles was as low as 6.7 [18]. These authors suggested then that, although the presence of a free amino group and an aliphatic side chain were required for sphingoid bases to inhibit protein kinase C, positive charge was not clearly needed for protein kinase C inhibition. This low apparent pK was later disputed by other authors [14] who claimed that the apparent pK in Triton micelles was 7.7 and the calculated intrinsic pK in

Triton micelles, after correcting for electrostatic repulsion, was 8.5. It should be noted that the apparent pK of SP is expected to be different in solution, in micelles and in bilayers because differences in hydration and or hydrogen bonding shift the pK of the amino group [18].

It should be taken into account that, when making a pH titration using membrane models, the Henderson-Hasselbach equation is not applicable to describe titrations at the bilayer surface. Because of that all reported values are stated as apparent pK values. The value of the apparent pK for SA in membranes has not been reported so far, to the best of our knowledge. As shown in Fig. 5, the apparent pK of SP in DPPS was 9.12 and the apparent pK of SA in DPPS was 8.94. The apparent pK values obtained in this work for SP and SA might be different if these compounds were mixed with other phospholipids different from DPPS. It should be also considered that the DPPS amino group may become at least partially deprotonated at these pH values but, unfortunately, DPPS as other phosphatidylserines degrades very rapidly at high pH [33], precluding to obtain by DSC the apparent pK value of the DPPS amino group either in the presence of SP or SA.

Let us now examine the origin of the shifts in T_c observed for the mixtures of DPPS with SP and SA. Considering firstly the DPPS/SP mixture containing 50 mol% of SP at pH = 10, DPPS will bear a single negative charge but SP will be deprotonated. Therefore the effect of SP on the T_c of the mixture will be of a purely non-electrostatic origin. At this pH, the T_c of pure DPPS (see Table I) was 48.2°C and the T_c of pure SP was 39°C (thermograms not shown). Therefore, the average of both values at pH = 10 ($\overline{T}_c^{10}$) is what we should take as a reference for this equimolar mixture. In this case $\overline{T}_c^{10}$(SP) would be 43.6°C. The observed T_c for this mixture at this pH was 44.8°C, and therefore the difference between the average and the experimental values is ΔT_c^{10}(SP) = 1.2 C° (Table I). Similarly, for pure SA and at pH = 10, the T_c was observed to be 59.2°C (thermogram not shown) and the average of the T_c values of both pure DPPS and pure SA would be $\overline{T}_c^{10}$(SA) = 53.7°C. Since the equimolar mixture of DPPS/SA had a experimental T_c of 59.6°C, the difference between the calculated average and the experimental values is ΔT_c^{10}(SA) = 5.9 C° (Table I).

On the other hand, and at pH = 6, SP and SA were protonated, i.e., positively charged, and DPPS beared a single negative charge (Fig. 5). Therefore, and at this pH, electrostatic effects are to be expected. It should be reminded that the hydrogen-bonding capacities of the amine group of both SA and SP and the amino group of DPPS are also altered as the pH is changed. The phase transition of pure DPPS at this pH was 50.6°C, whereas those of pure SP and pure SA were 30°C and 53.8°C, respectively (Table I). Therefore, the average values for equimolar mixtures of DPPS/SP and DPPS/SA at pH = 6 are $\overline{T}_c^6$(SP) = 40.3°C and $\overline{T}_c^6$(SA) = 52.2°C, respectively. Since the experimentally observed value for an equimolar mixture of DPPS/SP was 52°C, the difference in T_c compared to the average value is ΔT_c^6(SP) = 11.7 C°. Similarly T_c of an equimolar ratio of DPPS/SA was found to be 71°C and the difference in T_c compared to the average value is ΔT_c^6(SA) = 18.8 C°.

These results deserve several comments. Firstly, the deprotonation of pure SP and pure SA produced an increase in T_c; this is what is expected from the stabilization induced by loss of electric repulsions. Secondly, calculating the difference between the calculated average and experimental T_c values at the two different pH values, $\Delta T_c^{el} = \Delta T_c^6 - \Delta T_c^{10}$, it would be possible to deduce which is the disturbance of the inclusion of SP and SA on DPPS which could be due to either electrostatic effects and/or changes in hydrogen-bonding capacity. For an equimolar ratio of DPPS/SP ΔT_c^{el} was found to be 10.5 C°, whereas for an equimolar ratio of DPPS/SA it was 12.9 C° (Table I).

A possible explanation for the increase in T_c of both DPPS and SP or SA at pH = 6 and at other pH values at which these bases were protonated may be the reorientation of the polar head group due to effects similar to those observed for phosphatidylcholines in the presence of cationic amphiphiles [34]. This reorientation could decrease the effective cross-sectional area of the polar headgroup by modifying steric and/or

TABLE I

T_c (°C) phase transition temperatures of different lipid samples at pH = 6 (T_c^6) and pH = 10 (T_c^{10})

For details see text. ΔT_c values are given in C°.

	T_c^6	$\overline{T}_c^{6\,a}$	$\Delta T_c^6 = T_c^6 - \overline{T}_c^6$	T_c^{10}	$\overline{T}_c^{10\,a}$	$\Delta T_c^{10} = T_c^{10} - \overline{T}_c^{10}$	$\Delta T_c^{el} = \Delta T_c^6 - \Delta T_c^{10}$
Pure SP	30	–	–	39	–	–	–
Pure SA	53.8	–	–	59.2	–	–	–
Pure DPPS	50.6	–	–	48.2	–	–	–
DPPS/SP (1:1)	52	40.3	11.7	44.8	43.6	1.2	10.5
DPPS/SA (1:1)	71	52.2	18.8	59.6	53.7	5.9	12.9

[a] Expected (average) T_c value of an equimolar mixture of DPPS and SP or SA at pH = 6 ($\overline{T}_c^6$) or pH = 10 ($\overline{T}_c^{10}$).

electrostatic (repulsion) effects. This effect would allow a tighter packing of the molecules compared to pure DPPS, and therefore a higher melting temperature should be expected. Similarly, the temperature of the mixture would be also higher than those of pure SP or SA since the intercalation of DPPS molecules will dilute their positive charges and therefore would diminish the repulsion effect.

Since ΔT_c^{el} for SP was 10.5 C°, whereas ΔT_c^{el} for SA was 12.9 C°, we can conclude that there are no significant differences between both of them. However, SP has a bigger polar head than SA whereas SA has a longer hydrophobic chain than SP; atop of that, SP bears a double bond in its hydrophobic part. Although intermolecular hydrogen bonding between DPPS and SP molecules could be established through several combinations, thereby modifying the interaction between both molecules and differing therefore from the interaction of SA with DPPS, these results point out then that the main effect of SP and SA lies in their amino polar group.

The capacity of SP and SA to establish electrostatic interactions with phosphatidylserine has been proposed to be the reason of their capacity to inhibit protein kinase C [13,14]. We certainly find in this work that this electrostatic effect is important to explain the interactions between DPPS and either SP or SA. Other physiological effects of SP and SA may be also attributed to electrostatic interactions of these long-chain amino bases with other lipids and proteins bearing negative charged groups. Apart from that, these bases are also able to induce other types of interactions. Particularly SP, which we suggest to interact at pH = 6 with two DPPS molecules; one of these interactions may be hydrogen bonding through its hydroxyl group. Another important effect of both SP and SA is to rigidify the membrane. Finally, it is important to remark that both SP and SA have apparent pK values high enough to conclude that they will be mostly protonated at physiological pH, and therefore bearing a net positive charge. To conclude it may be worth pointing out that the long chain bases exist at physiological pH predominantly in charged form, which would facilitate movement through an aqueous phase, but also, although in a low proportion, they can be found as neutral species which can readily transverse biological membranes as pointed out by previous authors [18].

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