Interactions of Ca^{2+} with Sphingomyelin and Dihydrosphingomyelin

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ABSTRACT The changes induced by Ca^{2+} on human lens sphingolipids, sphingomyelin (SM), and dihydrosphingomyelin were investigated by infrared spectroscopy. Ca^{2+}-concentration-dependent studies of the head group region revealed that, for both sphingolipids, Ca^{2+} partially dehydrates some of the phosphate groups and binds to others. Ca^{2+} affects the interface of each sphingolipid differently. In SM, Ca^{2+} shifts the amide I′ band to frequencies lower than those in dehydrated samples of SM alone. This could be attributed to the direct binding of Ca^{2+} to carbonyl groups and/or strong tightening of interlipid H-bonds to levels beyond those in dehydrated samples of SM only. In contrast, Ca^{2+} induces relatively minor dehydration around the amide groups of dihydrosphingomyelin and a slight enhancement of direct lipid-lipid interactions. Temperature-dependent studies reveal that 0.2 M Ca^{2+} increases the transition temperature T_m from 31.6 ± 1.0°C to 35.7 ± 1.1°C for SM and from 45.5 ± 1.1°C to 48.2 ± 1.0°C for dihydrosphingomyelin. Binding of Ca^{2+} to some phosphate groups remains above T_m. The strength of the interaction is, however, weaker. This allows for the partial rehydration of these moieties. Similarly, above T_m, Ca^{2+}-lipid and/or direct inter-lipid interactions are weakened and lead to the rehydration of amide groups.

INTRODUCTION

The interaction of cations with natural membrane lipids is of paramount relevance in the induction of biological processes such as membrane fusion (Niles et al., 1996; Ohki, 1993; Ravoo et al., 1999), enzyme regulation, and signal transduction (Chun Peng et al., 1994; Zicha et al., 1999). It is thus critical to establish not only the sites of binding but also possible alterations in the conformation/structure of lipids as these modifications will affect the biophysical properties of the membrane.

Extensive studies focused on the interaction of physiologically important cations with glycerophospholipids, such as phosphatidic acid (Bicknell-Brown et al., 1986), phosphatidylserine (PS) (Casal et al., 1987, 1989; Choi et al., 1991; Coorssen and Rand, 1995; Holwerda et al., 1981; Leckband et al., 1993; Morillo et al., 1998), phosphatidylcholine (Baeza et al., 1994; Grdadolnik and Hadzi, 1993; Petersheim et al., 1989), and phosphatidylglycerol (PG) (Garidel et al., 2000a,b; Khalil et al., 2000). However, relatively little is known about the impact of ion binding to sphingolipids (SLs). It has been reported that Ca^{2+} partially dehydrates the sulfate group and reduces hydrogen bonds established by the sugar hydroxy groups in cerebroside sulfates (Menikh et al., 1997). Unlike these SLs, sphingophospholipids (SPLs) contain a phosphate-based head group that has the potential to interact with cations.

Sphingophospholipids constitute the most abundant SLs in mammalian membranes. Sphingomyelin (SM) (*N*-acylsphingosine-1-phosphorylcholine or ceramide-1-phosphorylcholine) is present in most membranes of animal tissue. Its structural and conformational characterization has been the theme of numerous reports (Hoffmann et al., 2000; Kan et al., 1991; Khare and Worthington, 1978; Kumar and Gupta, 1985; Lamba et al., 1991; Levin et al., 1985; MacKay et al., 1980; Maulik and Shipley, 1996; Sarmientos et al., 1985; Talbott et al., 2000). Among the tissues with high SM levels, nuclear fiber cells of ocular lenses of large mammals exhibit the highest content (~70% of all phospholipids) (Ferguson-Yankey et al., 1998). Intriguingly, the major SL in human lens membranes is not SM but rather D-*erythro* dihydrosphingomyelin (DHSM) (Byrdwell et al., 1994; Ferguson et al., 1996). Unlike SM, DHSM does not possess the 4,5 *trans* double bond in the sphingoid base. Membranes of other mammalian lenses and other tissues contain only minuscule amounts of DHSM. Only recently has our work begun to reveal the conformational differences imparted by this critical double bond (Ferguson-Yankey et al., 2000; Li et al., 2002; Talbott et al., 2000).

If all of the Ca^{2+} ions were unbound and distributed uniformly within the volume of the adult human lens, as in a homogeneous solution, the concentration would be 2.6 mM (Duncan and van Heyningen, 1977; Hightower and Reddy, 1982; Jedziniak et al., 1976; Rasi et al., 1992). This concentration is much higher than that of the free calcium, measured electrochemically to be 10 μM in young lenses and between 12 and 20 μM in older lenses (Duncan et al., 1989). The two orders of magnitude difference in the levels of Ca^{2+} suggests that either most of the ions are bound or reside in intracellular compartments that are only present in the elongating fibers of the outer region of the lens cortex (Brown and Bron, 1996). These possibilities have been addressed by our group and, based on the Ca^{2+} and phospholipid contents, it was estimated that 99% of the calcium is bound to the plasma membrane (unpublished results). Disruption in calcium homeostasis in lens fibers has been

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implicated in the development of human cataracts (Duncan and Bushell, 1975; Hightower and Reddy, 1982; Jedziniak et al., 1976; Rasi et al., 1992). In particular, experimentally induced (Sanderson et al., 2000) as well as natural (Duncan and Bushell, 1975) cataracts developed in the cortex exhibit elevated levels of Ca^{2+}.

Although a few studies on the interaction of SPLs with Ca^{2+} have been published (Shah and Shulman, 1967; Yuan et al., 1995, 1996; Zhao et al., 1995), there are no comparative reports on the impact of Ca^{2+} binding on the conformation of SM versus DHSM. Given the differences in the conformational preferences of the two lipids, it is likely that Ca^{2+} may affect the head group and interface regions of SM and DHSM in distinct fashions.

The aim of this work is to localize Ca^{2+}-binding sites in SM and DHSM and to elucidate the changes that are induced by these interactions in the head group, interface, and hydrocarbon regions of the two lipids. The informational richness of the infrared spectrum of SLs in aqueous media makes Fourier transform infrared spectroscopy an ideal tool to carry out these studies.

MATERIALS AND METHODS

Sample preparation

SM, 99% from bovine brain, and deuterium oxide, D_2O, were obtained from Sigma (St. Louis, MO) and used without further purification. H_2O was used as a solvent for the study of head group (phosphate stretching bands). The interface region investigation was performed in D_2O to avoid spectral interferences from H_2O in the amide I′ band region. The hydrophobic region (CH_2-symmetric stretching band) was investigated using both solvents. Calcium nitrate was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ) and used without further purification. DHSM was prepared from bovine brain SM by catalytic hydrogenation of SM with hydrogen over platinum oxide in ethanol at room temperature and atmospheric pressure, as was described previously (Ferguson et al., 1996). For both SPLs, a molecular weight of 780 g/mol was assumed, as this value represents the median of the range of molecular weights provided by Sigma for SM.

Each of the two lipids was introduced into Ca^{2+}-aqueous solutions to achieve lipid concentrations between 1 and 2 M and Ca^{2+} levels ranging from 0.2 to 2 M. The mixture was vortexed and heated for one-half hour in a 50°C water bath. This step was repeated twice, and the sample was then allowed to come back to room temperature.

For the Ca^{2+}-concentration-dependent studies, aliquots of the sample were placed between AgCl windows, and Fourier transform infrared spectra were acquired at room temperature. To study dehydration of the SPLs in the absence of the Ca^{2+}, samples were placed on a single AgCl window and allowed to dry in the sample chamber of the instrument while Fourier transform infrared spectra were acquired.

The same sample preparation steps were followed for the variable-temperature analysis. A temperature-controlled cell with a special heat-transfer adapter allowed spectral acquisition over a temperature range from 20°C to 45°C for SM and 30°C to 55°C for DHSM. Each sample was equilibrated for 30 min at every given temperature of the running sequence. CaF_2 windows were used in these temperature-dependent studies because they are not as soft as AgCl windows and can withstand higher temperatures. No spectral changes were observed for the bands of interest whether CaF_2 or AgCl windows were used.

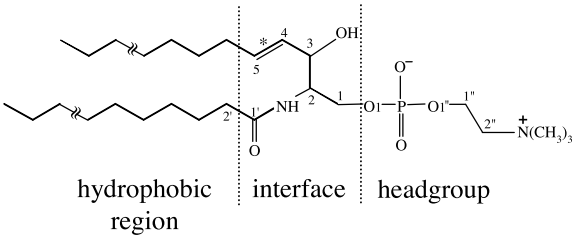

FIGURE 1 Components and regions of SM. * In DHSM the *trans*-double bond is absent.

Spectral acquisition and analysis

Fourier transform infrared data were acquired on a Galaxy Series Fourier transform infrared 5000 spectrometer (Mattson, Madison, WI). Absorbance spectra from 4000 to 400 cm^{-1} were the average of 50 scans collected with a resolution of 4 cm^{-1}.

Data analysis (baseline, Fourier self-deconvolution, curve fitting, integration) was performed with GRAMS/386 software (Galactic Industries, Salem, NH).

RESULTS

To understand the impact of Ca^{2+} on the head group, interface, and hydrophobic regions of SM and DHSM (Fig. 1), the following bands were analyzed: phosphate asymmetric and symmetric stretch, amide I′, and CH_2-symmetric stretch. These bands were monitored as a function of Ca^{2+} concentration and temperature.

Ca^{2+}-concentration-dependent studies

The changes induced by the presence of Ca^{2+} in the vicinity of SPLs were assessed using relatively high concentrations of SPLs and Ca^{2+} to ensure a detectable contribution of Ca^{2+}-bound SPL molecules.

Head group region

To help in the interpretation of spectral changes due to Ca^{2+}-induced dehydration, Fig. 2 *a* shows the changes that took place in a sample of SM alone prepared in H_2O before (SM hydrated) and after (SM partially dehydrated) partial removal of water. It is important to emphasize that even after extended hours of water removal, the samples still retained strongly bound water molecules. Based on our previous nuclear magnetic resonance studies on SM (Talbott et al., 2000) and DHSM (Ferguson-Yankey et al., 2000), it is estimated that between two and six water molecules remain bound to each lipid.

Upon addition of Ca^{2+}, the initial broad phosphate asymmetric stretching band of SM (centered around 1221 cm^{-1}) changed its contour and a new feature emerged at 1251 cm^{-1}, as shown in Fig. 2 *b*. The band at 1251 cm^{-1} increased its relative contribution as the Ca^{2+} concentration

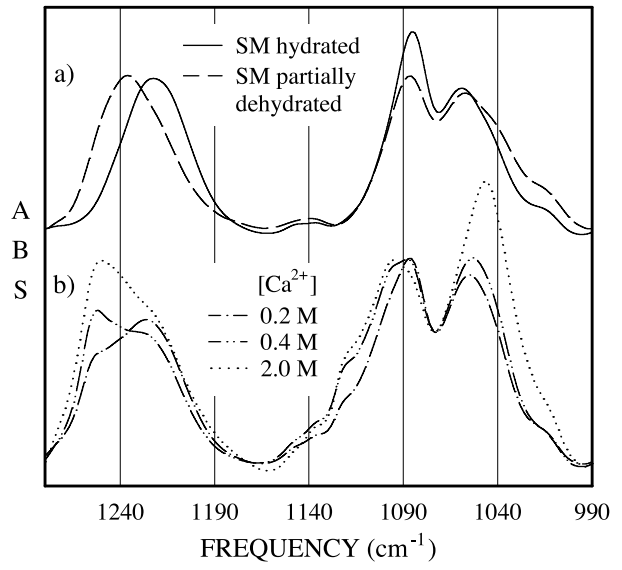

FIGURE 2 Changes in the phosphate and phosphodiester stretching bands of SM caused by (*a*) partial dehydration of SM in the absence of Ca^{2+} and (*b*) increasing Ca^{2+} concentration. The studies were carried out at room temperature.

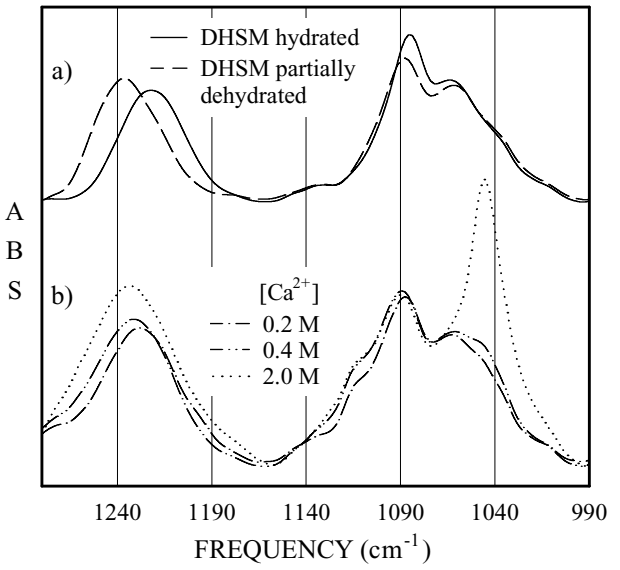

FIGURE 3 Changes in the phosphate and phosphodiester stretching bands of DHSM caused by (*a*) partial dehydration of DHSM in the absence of Ca^{2+} and (*b*) increasing Ca^{2+} concentration. The studies were carried out at room temperature.

was raised from 0.2 to 2.0 M. A new component appeared at 1121 cm^{-1}, and its relative contribution was also enhanced with increasing Ca^{2+} concentrations. This band was never observed for samples that contained the SPL alone. The phosphate symmetric stretching band also exhibited a Ca^{2+}-concentration-dependent shift, from 1085 cm^{-1} (no Ca^{2+}) to 1096 cm^{-1} (2.0 M Ca^{2+}). A significant Ca^{2+}-dependent increase in intensity was observed in the region extending from 1000 cm^{-1} to 1070 cm^{-1} with maximal signal at 1048 cm^{-1}. This spectral region includes the complex overlap of C—O and P—O stretches corresponding to the diester linkage (C1—O1—P—O—C′1) of the head group.

The spectral traces of hydrated and partially dehydrated DHSM (without Ca^{2+}) are included in Fig. 3 *a* to aid in the later discussion of dehydration effects. With increasing calcium concentrations, the phosphate asymmetric stretching band presented a smaller (compared with SM), yet significant shift from 1223 to 1233 cm^{-1} (Fig. 3 *b*). A new Ca^{2+}-dependent band was observed at 1115 cm^{-1}. The presence of Ca^{2+} also increased the frequency of the phosphate symmetric stretching band of DHSM but only from 1085 to 1090 cm^{-1}. As for SM, a Ca^{2+}-dependent increase in intensity was observed in the phosphodiester stretch region and reached its maximum at 1046 cm^{-1}.

Interface region

The impact of dehydration on the amide I′ band of samples of only SM or DHSM prepared in D_2O is shown in Figs. 4 *a* and 5 *a*, respectively. The partially dehydrated samples of SM alone exhibited two components centered at 1650 and 1628 cm^{-1} (Fig. 4 *a*). For DHSM, partial dehydration resulted in a minor shift to higher frequencies from 1631 and 1634 cm^{-1} (Fig. 5 *a*).

The very different effect of Ca^{2+} on the amide I′ band of SM and DHSM is evident in the corresponding Figs. 4 *b* and 5 *b*. In the case of SM, there was an overall shift toward lower frequencies, reaching 1620 cm^{-1} for the highest Ca^{2+} concentration. Conversely, for DHSM, the frequency of

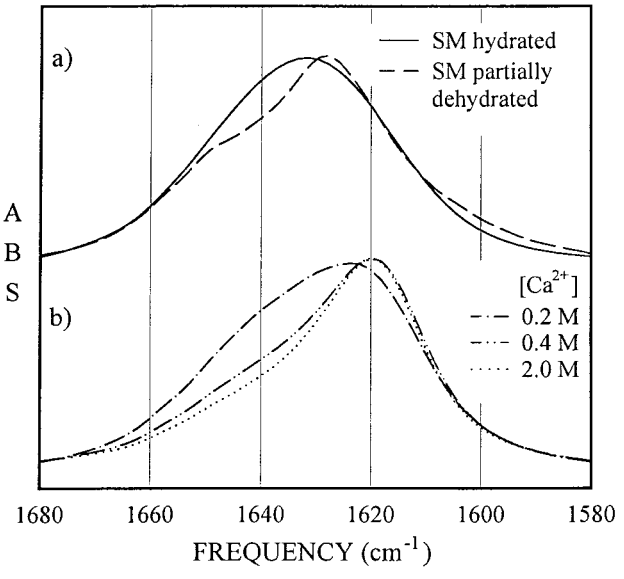

FIGURE 4 Changes in the amide I′ band of SM caused by (*a*) partial dehydration of SM in the absence of Ca^{2+} and (*b*) increasing Ca^{2+} concentration. The studies were carried out at room temperature.

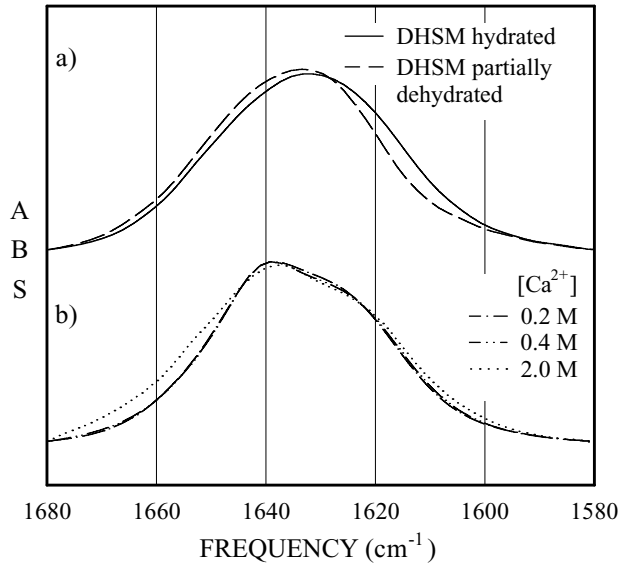

FIGURE 5 Changes in the amide I′ band of DHSM caused by (*a*) partial dehydration of DHSM in the absence of Ca^{2+} and (*b*) increasing Ca^{2+} concentration. The studies were carried out at room temperature.

maximal absorption increased from ~1631 (no Ca^{2+}) to 1638 cm^{-1} with addition of Ca^{2+}.

Temperature studies

Hydrophobic region

Changes in the frequency of the CH_2-symmetric stretching band allowed the determination of the gel to liquid-crystalline phase midpoint-transition temperature T_m for each SPL in the absence and presence of Ca^{2+}. For SM, the values of T_m increased from 31.6 ± 1.0°C (no Ca^{2+}) to 35.7 ± 1.1°C when the lipid was prepared in 0.2 M Ca^{2+}. Under similar conditions, the values of T_m for DHSM changed from 45.5 ± 1.1°C to 48.2 ± 1.0°C.

Head group region

Figs. 6 and 7 show spectral traces of the phosphate and phosphodiester stretching bands of SM and DHSM, respectively, for temperatures above (upper traces) and below (lower traces) T_m and in the presence (dotted lines) and absence (solid lines) of Ca^{2+}.

As indicated previously, the presence of Ca^{2+} resulted in the appearance of new bands in the phosphate and phosphodiester stretching band regions of both SPLs at low temperatures. At temperatures above T_m, however, these spectral contours resembled more closely those of the corresponding bands of SM (Fig. 6, upper traces) or DHSM (Fig. 7, upper traces) in the absence of Ca^{2+}. At elevated temperatures, the bands present at 1121 cm^{-1} for SM and at 1115 cm^{-1} for DHSM broadened and, although present, diminished their relative contributions. For both lipids, the

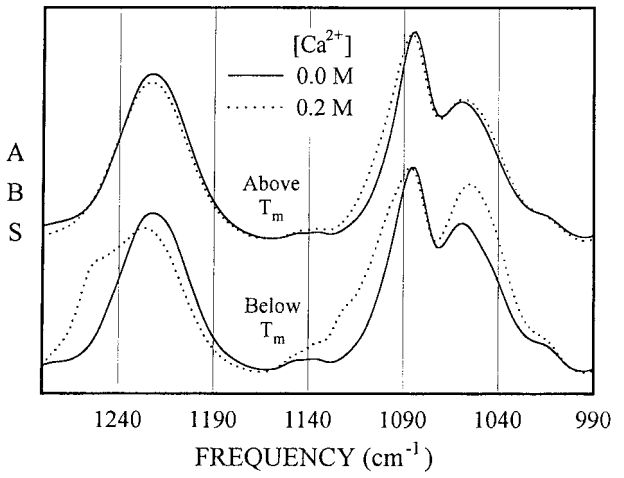

FIGURE 6 Temperature-dependent changes in the phosphate and phosphodiester stretching region of SM samples, in the presence (*dotted lines*) and the absence (*solid lines*) of Ca^{2+}. T = 43°C for upper traces (above T_m) and T = 24°C for lower traces (below T_m).

phosphate symmetric stretching band frequency decreased slightly with temperature. The intensity of the band(s) between 1040 and 1060 cm^{-1}, more prominent in SM, diminished at temperatures above T_m.

Interface region

Spectral changes as a function of temperature for the amide I′ band of both SM and DHSM without (solid lines) and with (dotted lines) Ca^{2+} are shown in Figs. 8 and 9, respectively. As described earlier, at temperatures below T_m, Ca^{2+} addition shifted in opposite directions the frequencies of the amide I′ bands of SM and DHSM. At temperatures above

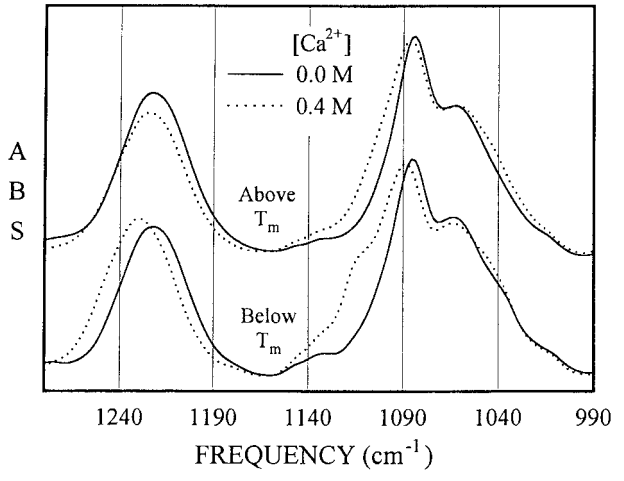

FIGURE 7 Temperature-dependent changes in the phosphate and phosphodiester stretching region of DHSM samples, in the presence (*dotted lines*), and the absence (*solid lines*) of Ca^{2+}. T = 52°C for upper traces (above T_m) and T = 31°C for lower traces (below T_m).

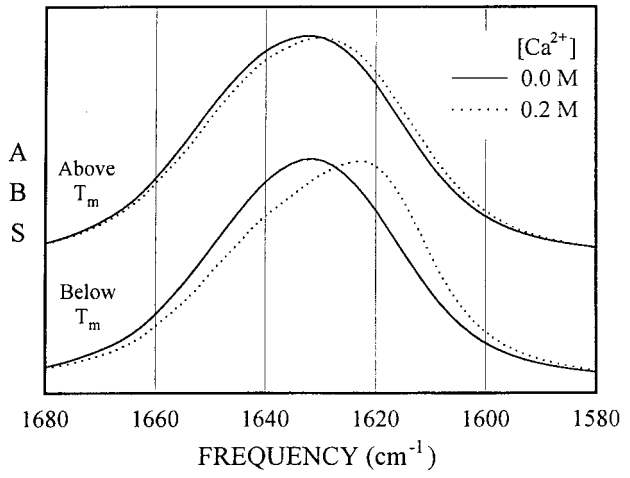

FIGURE 8 Temperature-dependent changes in the amide I′ region of SM samples, in the presence (*dotted lines*), and the absence (*solid lines*) of Ca^{2+}. $T = 43°C$ for upper traces (above T_m) and $T = 24°C$ for lower traces (below T_m).

T_m, the differences between the traces with and without Ca^{2+} were less pronounced for each lipid. However, there continued to be a shift to lower frequencies in the amide I′ of SM in the presence of Ca^{2+}. In the case of DHSM, there was also a minor shift to lower frequencies.

DISCUSSION

Fourier transform infrared spectra provide snapshots of all infrared-absorbing conformers present in a molecular ensemble. Therefore, different components of a given band may be attributed to different populations in which the molecule under investigation is present in a different conformation or environment. Because our goal was to investigate the changes that take place in SPLs when they bind Ca^{2+}, we ensured that Ca^{2+}-bound SPLs contributed significantly to the collected spectral traces by using high concentrations of SPLs and Ca^{2+}. The Ca^{2+} levels are higher that those of free Ca^{2+} measured in the extracellular space of human lenses, as stated earlier. However, because most of the Ca^{2+} is bound to the plasma membrane of lens fibers, the microregional concentration of Ca^{2+} at the membrane surface is expected to be high enough for binding. In vitro studies carried out by our group indicate that lens lipids are capable of binding most of the Ca^{2+} present in human lenses (unpublished results).

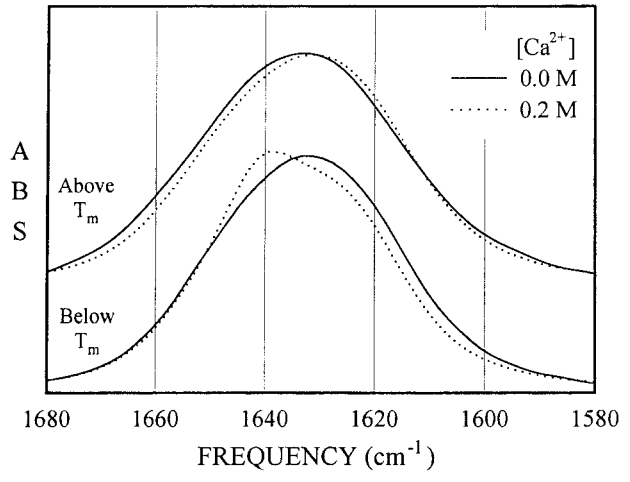

FIGURE 9 Temperature-dependent changes in the amide I′ region of DHSM samples, in the presence (*dotted lines*), and the absence (*solid lines*) of Ca^{2+}. $T = 52°C$ for upper traces (above T_m) and $T = 31°C$ for lower traces (below T_m).

Ca^{2+}-concentration-dependent studies

Head group region

The spectral region encompassing the phosphate and phosphodiester stretching bands of phospholipids, 1300 to 950 cm^{-1}, is crowded and complex due to the overlap of several other bands (Casal et al., 1987; Garidel et al., 2000a; Grdadolnik and Hadzi, 1993; Petersheim et al., 1989). In the paragraphs below, several assignments are proposed for bands whose intensities correlate to the levels of added Ca^{2+}. However, it is important to highlight that to confirm these postulates further experimental and theoretical work with model compounds and other divalent cations is needed.

Effect of Ca^{2+} on SM. The phosphate asymmetric stretching band split into two components centered at 1221 and 1251 cm^{-1} when Ca^{2+} was added to the SM samples. We attribute the 1251 cm^{-1} band to highly dehydrated phosphate groups. This assignment is based on the trend observed upon partial dehydration of SM alone, in which the frequency of the phosphate asymmetric band shifted from 1221 to 1237 cm^{-1} (Fig. 2 *a*). The high frequency of the component at 1251 cm^{-1} and its Ca^{2+}-dependent increase in intensity suggest that Ca^{2+} is capable of abstracting even those tightly phosphate-bound water molecules that are not removed by prolonged dehydration of the lipid alone. The dehydration effect of Ca^{2+} on head groups of other phospholipids has been reported for PS (Casal et al., 1987) and phosphatidylcholine (Grdadolnik and Hadzi, 1993).

The effectiveness of the Ca^{2+}-induced dehydration was also reflected by the large shift, from 1085 (no Ca^{2+}) to 1096 cm^{-1} (with 2.0 M Ca^{2+}) in the phosphate symmetric stretch (Fig. 2 *b*). This shift is larger than that observed in this band upon partial dehydration of SM only. Previous studies (Goni and Arrondo, 1986; Hadzi et al., 1992) also indicated that the dehydration of phosphate groups results in an increase of the frequency of both symmetric and asymmetric phosphate stretching vibrations bands. Quantum-chemical calculations corroborated that this effect is much more pronounced for the asymmetric stretching band than

for the symmetric one (Pohle et al., 1991). Another change in this spectral region is related to the appearance of a shoulder centered at 1121 cm^{-1}. A band of similar frequency has been reported for PS (Dluhy et al., 1983) and was attributed to the splitting of the phosphate symmetric stretch band into four components caused by a lowering of symmetry in the phosphate group upon calcium binding. A similar argument was used to interpret the appearance of a series of six bands, including one at 1122 cm^{-1}, when Ca^{2+} was complexed with PG (Garidel et al., 2000a). However, the shoulder observed for PG at 1120 cm^{-1} in the absence of Ca^{2+}, was proposed to originate from the C—O stretching modes.

Although a partial loss of symmetry is to be expected upon binding of divalent cations to the phosphate group, the changes observed in this region of the spectral traces for SM and DHSM do not resemble the splitting pattern seen for either the PS-Ca^{2+} or the PG-Ca^{2+} complex. The band observed at 1120 cm^{-1} for SM and at 1115 cm^{-1} for DHSM in the presence of Ca^{2+} was not as sharply defined and/or as intense as that reported for the PS-Ca^{2+} or PG-Ca^{2+} complexes. Therefore, one cannot exclude the possibility that this band results from a population of Ca^{2+}-bound phosphate groups in which conformational changes in the phosphodiester backbone shift the frequency of the C—O and P—O stretches. The nature of these changes is unclear at this time but differs from that caused by dehydration alone, because the band under consideration was not detected in the spectra of the partially dehydrated SPLs.

The region between 1000 and 1070 cm^{-1} was affected significantly upon addition of Ca^{2+} (Fig. 2). The complexity of this region due to the overlap of C—O and P—O stretching bands leads to difficulties in proper assignment. Furthermore, although previous studies on other phospholipids have reported the dehydration of the phosphate group, there are no details on the phosphate asymmetric or symmetric stretching bands corresponding to the population of phosphate groups bound to Ca^{2+}. Experimental and theoretical evidence (Murashov and Leszczynski, 1999; Schneider et al., 1996) indicated that the most likely complex formed between Ca^{2+} and dimethyl phosphate (DMP) is that in which one of the anionic oxygens is bound to the metal and the other to water. The changes in frequency for the phosphate symmetric and asymmetric stretches have been calculated theoretically for this likely complex and other complexes of various cations and water (Murashov and Leszczynski, 1999). As intuitively expected, the presence of the doubly charged cations leads to a significant weakening of the P—O bonds involving the anionic oxygens. This is reflected in a large red shift for the asymmetric stretch, from 1229 cm^{-1} for the (H_2O, H_2O) DMP complex to 1068 cm^{-1} for the (H_2O, Ca^{2+}) DMP complex (Murashov and Leszczynski, 1999). In addition, the frequency of the Ca^{2+}-bound symmetric phosphate stretch was also predicted to decrease from 1068 cm^{-1} for the (H_2O, H_2O) DMP complex to 1037 cm^{-1} for the (H_2O, Ca^{2+}) DMP complex (Murashov and Leszczynski, 1999). These shifts would place the asymmetric and symmetric stretches of the Ca^{2+}-bound phosphate groups near the region where a large increase in intensity was observed in our spectra as well as those reported for the PS-Ca^{2+} complex (Dluhy et al., 1983). Upon partial removal of water from our Ca^{2+}-containing samples, the features of this spectral region remained (data not shown).

It is therefore proposed that the strong band(s) centered at 1048 cm^{-1} may result from the overlap of both the phosphate asymmetric and symmetric stretching bands of phosphate groups bound to Ca^{2+} with the phosphodiester stretching bands.

Effect of Ca^{2+} on DHSM. The phosphate asymmetric stretch of DHSM shifted from 1223 to 1233 cm^{-1} as the Ca^{2+} concentration was raised to 2.0 M (Fig. 3 *b*). This shift suggests that, as in SM, Ca^{2+} induced the dehydration of some phosphate moieties. The extent of this effect, however, was less pronounced than for SM. It is likely that the strength with which water molecules are attracted to the phosphate groups is greater in DHSM, because the absence of the double bond between C4 and C5 allows for tighter packing of DHSM molecules compared with SM. The reduced extent of dehydration effected by Ca^{2+} on phosphate groups is also supported by the smaller shift, from 1085 to 1090 cm^{-1} of the DHSM phosphate symmetric stretching band as compared with that of SM.

The band at 1115 cm^{-1} only appeared when Ca^{2+} was added to the DHSM samples, and its assignment is unclear, as addressed previously. As for SM, the addition of Ca^{2+} led to an increase in the intensity of the region between 1040 and 1060 cm^{-1} with a maximum at 1046 cm^{-1}. These changes may be interpreted as the overlap of both the phosphate asymmetric and symmetric stretching bands of phosphate groups bound to Ca^{2+} with the phosphodiester stretching bands.

Overall, these experimental data indicate that Ca^{2+} ions are capable of dehydrating and binding to phosphate moieties in both SPLs. Furthermore, the effectiveness of these alterations is more pronounced for SM than for DHSM. This could be attributed to the less tight packing of SM molecules that allows for an easier approach and interaction of the metal ions with the phosphate groups.

Interface region

Conformational changes in the interfacial region can be analyzed through the amide I′ band, which reflects primarily changes in the C═O stretching component.

Effect of Ca^{2+} on SM. The amide I′ band of SM changed from a broad band centered ~1632 cm^{-1} (no Ca^{2+}) into one with two major components, a fairly sharp one at 1620 cm^{-1}, and a broader one at ~1640 cm^{-1} in the presence of 2.0 M Ca^{2+} (Fig. 4). To check if these changes resulted

from dehydration alone, we monitored this band as water was removed from lipid samples prepared in D_2O in the absence of Ca^{2+}. As D_2O was removed, the single band centered at 1632 cm^{-1} evolved into a two-component profile with maxima at 1650 and 1628 cm^{-1} (Fig. 4 *a*). The relative contribution of the 1628 cm^{-1} band increased as the sample became more dehydrated. This trend suggests that this low-frequency component is associated with strongly bound amide groups involved in lipid-lipid interactions through carbonyl groups. The higher-frequency component at 1650 cm^{-1} may be attributed to dehydrated (unbound) moieties.

The frequency of the sharper amide I′ band component in the presence of Ca^{2+}, 1620 cm^{-1}, is lower than 1628 cm^{-1}. Moreover, the experimental data showed that the relative contribution of the 1620 cm^{-1} component was Ca^{2+} dependent. Therefore, it is proposed that this band may represent amide groups bound directly to Ca^{2+} or that the extensive dehydration of the phosphate groups leads to the formation of inter-lipid H-bonds of greater strength than those seen in the partially dehydrated samples of SM only. The contribution of the higher-frequency component ($\sim$1640 cm^{-1}) was increasingly smaller as the Ca^{2+} concentration increased. This band may be attributed to amide groups that are dehydrated due to the presence of Ca^{2+} ions that compete for the binding of water molecules.

Effect of Ca^{2+} on DHSM. Ca^{2+} affected the amide I′ band of DHSM (Fig. 5) differently and to a lesser extent than that of SM. The two main components were centered at 1638 and 1623 cm^{-1} and their contributions did not change significantly with increasing Ca^{2+} levels. The band at 1623 cm^{-1} might reflect an enhancement of lipid-lipid interactions through amide groups as dehydration ensues. However, one cannot rule out the possible direct binding of Ca^{2+} to a very small fraction of amide groups. The amide I′ higher frequency component at 1638 cm^{-1} is attributed to the population of DHSM molecules affected by dehydration. As it was previously described, Ca^{2+} induces the partial removal of water molecules surrounding phosphate groups. It is therefore likely that this effect may propagate to water molecules in the vicinity of the amide groups. As a result, water molecules are pulled away from the interface region, and the amide I′ frequency increases.

In conclusion, Ca^{2+} has important, yet different influences on each of the described SPLs. In contrast to SM, the changes in the amide I′ band of DHSM were relatively minor, suggesting that the tight H-bonding network established among DHSM molecules presents a stronger barrier that diminishes the impact of Ca^{2+} on the interfacial regions of this lipid.

Temperature-dependent studies

Head group region

Significant changes were observed in the phosphate stretch region of both SPLs as temperature was increased (Figs. 6 and 7), and the SPLs underwent the transition from the gel state to liquid-crystalline state without Ca^{2+} and in the presence of Ca^{2+}.

In the presence of Ca^{2+} ions, the band at 1251 cm^{-1} attributed to the asymmetric stretching of dehydrated phosphates shifted back to 1221 cm^{-1} once the temperature was raised above T_m. This change was reversible and indicates that as the temperature increases, rehydration of phosphate moieties takes place. However, even at elevated temperatures, SM samples containing Ca^{2+} (dotted line) did not exhibit exactly the same spectral contours as those of SM alone (solid line). Although the bands observed at 1121 and 1048 cm^{-1} below T_m became broader above T_m, their continued presence suggests that Ca^{2+} ions remain in the vicinity of the phosphate groups even at higher temperatures.

The phosphate symmetric stretching band of SM shifted to a small extent toward lower frequencies with increasing temperature for both samples, with and without Ca^{2+}. This indicates that at higher temperatures Ca^{2+} ions are less bound to the phosphates groups and water molecules can rehydrate these moieties.

The temperature-induced spectral changes in the phosphate and phosphodiester stretching region of DHSM (Fig. 7) were similar to those already discussed for SM. Spectral features reconfirm the rehydration of phosphate groups above T_m as well as the continued binding of Ca^{2+} to some phosphate sites.

Interface region

Figs. 8 and 9 show that as the temperature was increased, the amide I′ band became broad and centered around 1630 cm^{-1} for both SM and DHSM. Temperature studies of SM samples containing Ca^{2+} ions (Fig. 8) showed that the 1620 cm^{-1} component seen at low temperatures moved toward higher frequencies, 1630 cm^{-1}, when the temperature was increased above T_m. This can be interpreted as the weakening of either Ca^{2+}-SM associations or tight H-bonds established among neighboring lipids at low temperatures. These changes could allow water molecules to come closer to the amide groups, and this rehydration would lead to the change in frequency from 1620 to 1630 cm^{-1}. The effect of Ca^{2+}, although diminished at high temperatures, appears not to be eliminated, because the amide I′ bands of SM samples with and without Ca^{2+} did not coincide.

As indicated previously, the amide I′ of DHSM exhibited two major components at 1638 and 1623 cm^{-1} at low temperatures and in the presence of Ca^{2+}. Once the temperature was increased above T_m, both components were shifted, and a broad, single band centered at $\sim$1630 cm^{-1} was observed. The 1623 cm^{-1} band, related primarily to amide moieties involved in lipid-lipid interactions, shifted to a higher frequency. This suggests the weakening of direct lipid-lipid interactions and the intercalation of water mole-

cules among them. The band at 1638 cm^{-1} attributed to freer amide groups shifted to lower frequencies. This implies that, as temperature increases, water molecules are able to rehydrate those amide environments.

In conclusion, the temperature analysis of the amide I′ band showed that, above T_m, the Ca^{2+}-lipid or tight lipid-lipid associations established among amide groups of SM are weakened, and water molecules are able to approach these moieties, rehydrating them. For DHSM, the impact of Ca^{2+} to the interface region is not as significant as in SM.

Hydrophobic region

For both SPLs, 0.2 M Ca^{2+} levels ordered the packing of the hydrophobic chains and led to an increase in T_m. The greater change induced by Ca^{2+} in the value of T_m for SM as compared with DHSM may reflect the more dramatic impact of Ca^{2+} on the interfacial interactions of SM. It is postulated that either the formation of Ca^{2+} bridges among SM molecules or the strong tightening of inter-lipid H-bonds that results from the dehydration of the head group and interfacial region serves to enhance hydrophobic interactions by bringing the lipids closer to one another. The effect of partial dehydration in the case of DHSM also leads to closer proximity, but the extent of the change is not as pronounced.

CONCLUSION

The distinct ways in which Ca^{2+} affects SM and DHSM emphasize the crucial role of the 4,5 *trans* double bond present in SM and not in DHSM. Ca^{2+} ions not only bind and dehydrate the phosphate moieties of SM to greater extent than in DHSM but also strengthen the interfacial network of H-bonds that link amide groups of neighboring SM molecules more significantly than for DHSM. The abstraction of water molecules from the head group and interface brings the lipids closer to each other and thus increases the gel to liquid-crystalline phase transition temperature. These results indicate that Ca^{2+} enhances the differences between SM and DHSM. It is therefore hypothesized that the presence of Ca^{2+} in the vicinity of the bilayer may allow for a better differentiation of these two SPLs, a crucial step in enzyme recognition.

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