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Chlorpromazine interaction with glycerophospholipid liposomes studied by magic angle spinning solid state ^{13}C-NMR and differential scanning calorimetry

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Abstract

Phosphatidylserine (PS) extracted from pig brain and synthetic dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were used to make DPPC/DMPC and DPPC/PS large unilamellar liposomes with a diameter of ~1 μm. Chlorpromazine-HCl (CPZ), an amphipathic cationic psychotropic drug of the phenothiazine group, is known to partition into lipid bilayer membranes of liposomes with partition coefficients depending on the acyl chain length and to alter the bilayer structure in a manner depending on the phospholipid headgroups. The effects of adding CPZ to these membranes were studied by differential scanning calorimetry and proton cross polarization solid state magic angle spinning ^{13}C-nuclear magnetic resonance spectroscopy (CP-MAS-^{13}C-NMR). CP-MAS-^{13}C-NMR spectra of the DPPC (60%)/DMPC (40%) and the DPPC (54%)/DMPC (36%)/CPZ (10%) liposomes, show that CPZ has low or no interaction with the phospholipids of this neutral and densely packed bilayer. Conversely, the DPPC (54%)/PS (36%)/CPZ (10%) bilayer at 25°C demonstrates interaction of CPZ with the phospholipid headgroups (PS). This CPZ interaction causes about 30% of the acyl chains to enter the gauche conformation with low or no CPZ interdigitation among the acyl chains at this temperature (25°C). The DPPC (54%)/PS (36%)/CPZ (10%) bilayer at a sample temperature of 37°C (T_C = 31.2°C), shows CPZ interdigitation among the phospholipids as deduced from the finding that ~30% of the phospholipid acyl chains carbon resonances shift low-field by 5–15 ppm.

Keywords: Cross polarization solid state magic angle spinning ^{13}C-nuclear magnetic resonance spectroscopy; Differential scanning calorimetry; DPPC/DMPC liposome; DPPC/PS liposome; Chlorpromazine-HCl interaction

1. Introduction

Biological membranes define the boundaries of the cells and subcellular organelles and create a semi-permeable filter which maintains chemical gradients and provides the structural basis for pumping and signaling devices. Processes like membrane-directed protein self-assembly, regulation of membrane permeability and cell surface recognition are vital in a number of cellular processes, e.g. respiration, neurotransmission and photosynthesis. Membrane biochemistry also forms the basis in the pharmacology of drug delivery and drug design [1]. Consequently, studies of model membrane systems have implications in cellular biochemistry and medicine.

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Chlorpromazine (CPZ), a major antipsychotropic drug of the phenothiazine group, is believed to exert its action by antagonizing neuronal D_2 receptors. However, like other cationic amphipaths, CPZ is incorporated in bilayer membranes with partition coefficients depending on the acyl length of the phospholipids [2] and alters the bilayer structure according to the nature of the phospholipid headgroups [3]. The importance of the headgroup has recently been demonstrated by our studies on phospholipid monolayers where CPZ increased the molecular area of the acid glycerophospholipids phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylglycerol (PG) by more than 100% at as low concentrations as 1 μM (bulk phase) while having no effect on the neutral phospholipids phosphatidylcholine (PC) and phosphatidylenthanolamine (PE) (A. Varnier, et al., submitted to Biochemistry). Numerous effects of CPZ in cellular systems have been reported that most likely originate from its interactions with biological membranes. The well-known protective effect of CPZ on red cell osmolysis ('membrane-stabilizing' effect) clearly involves CPZ-plasma membrane interactions, and depends on the membrane's content of linoleate [4]. Studies with human platelets have shown that CPZ [5,6] and the structurally related trifluoperazine [7] at concentrations (bulk phase) above 40 μM make the cells permeable for small, cytoplasmic components, such as adenine nucleotides, but not for large molecules like lactate dehydrogenase. This effect has not been recognized in most studies of phenothiazines on biological systems, which have employed permeabilizing concentrations leading to gross cellular effects probably due to ATP depletion. However, in non-permeabilizing concentrations, CPZ has profound effects on glycerophospholipid metabolism in hepatocytes by redirecting de novo synthesis from neutral to acidic glycerophospholipids by inhibition of PA phosphatase [8,9]. In resting platelets, CPZ specifically increases the level of phosphatidylinositol 4-phosphate and increases the turnover of the acidic polyphosphoinositides [10] while in thrombin-activated platelets CPZ distorts the tight metabolic coupling between the metabolites in the polyphosphoinositide cycle [11] and inhibits protein phosphorylations and secretion [5]. Thus, in intact cells, CPZ exerts effects on numerous enzymatic conversions of acidic glycerophospholipids which hardly can be explained by direct interactions between CPZ and the enzymes involved. It is more likely that CPZ interferes with the physical positioning of substrate relative to the enzymes in the membranes, a phenomenon referred to as *substrate presentation* in order to explain the inhibition by CPZ of PA phosphohydrolase [12].

Changes in the glycerophospholipid microstructure by CPZ may alter receptor activities and the function of other integral proteins in biological membranes. In attempts to elucidate the nature of possible alterations by CPZ of biological membranes on an atomic level, we describe here two model membrane systems, DPPC/DMPC and DPPC/PS liposomes made with a diameter of $\sim$1 μm and the effects of adding CPZ to these membranes as revealed by solid state magic angle spinning with proton cross polarization [13] ^{13}C-nuclear magnetic resonance spectra (CP-MAS-^{13}C-NMR) and differential scanning calorimetry (DSC). The present study describes the effect of chlorpromazine on the acyl chain mobility with phosphatidylcholine, phosphatidylserine and the amphiphile under one stoichiometric relationship (10 mol% chlorpromazine). The chosen molecular ratio should have a CPZ amount well below the high molar ratios where the bilayer structure is destroyed and also below detection in the CP-MAS-^{13}C-NMR spectra. Thus, only the CPZ interaction effects on the phospholipids will be detected. The molecular ratio (10% CPZ) of this study demonstrated great mobilization of the chains for the bilayer containing phosphatidylserine with *sn*-2-unsaturated acyl chain. Further studies are currently in progress aiming to characterize this mobilization at different mole proportions of lipids and drug.

2. Materials and methods

2.1. Liposome preparation

Phosphatidylserine (PS, pig brain, code A-36) was obtained from Doosan Serdary Research Laboratories (Englewood, NJ). In general, the main fatty acids of brain PS are 18:0 (40%), 18:1 (31%), 20:4 (3%) and 22:6 (9%) (Interim Catalog, Avanti Polar Lipids, Alabaster, AL). Unsaturated fatty acids thus consti-

tute 50% of all fatty acids in PS, which is what one would expect if one of the two acyls in every PS molecule were unsaturated. Synthetic 1,2-dipalmitoylphosphatidylcholine (DPPC) and 1,2-dimyristoylphosphatidylcholine (DMPC) and chlorpromazine-HCl (CPZ) were obtained from Sigma. The DPPC/PS lipid system was made in a molar composition of 60% (DPPC) and 40% (PS) and dissolved in *tert*-butanol and then lyophilized to dryness. The DPPC/PS lipid system was kept under an argon atmosphere and not exposed to air. The DPPC/DMPC lipid system was made similarly to the DPPC/PS lipid system, except for the argon atmosphere during preparation. The two samples of dry powder where then each suspended in 4 ml D_2O (the DPPC/PS and the DPPC/DMPC lipid systems). These suspensions contained multilamellar liposomes, and unilamellar vesicles were obtained by freeze–thawing (seven times). In order to achieve good sample homogeneity, both the DPPC/PS and the DPPC/DMPC lipid systems were then subjected to filtering by a Lipsofast membrane system (Avestin, Ottawa, ON, Canada) through the 1000-nm filter 25 times. Unilamellar liposomes that are large ($\sim$1 μm) and homogeneous in size will have small and equal curvature and should make all of the liposomes' outer monolayer equally accessible to CPZ. At this stage, both the DPPC/PS and the DPPC/DMPC lipid suspensions were divided into two equal parts and to one part of each was added an amount of chlorpromazine-HCl (dissolved in D_2O) that would give a 10% molar ratio. Thus samples of DPPC (60%)/PS (40%), DPPC (54%)/PS (36%)/CPZ (10%), DPPC (60%)/DMPC (40%) and DPPC (54%)/DMPC (36%)/CPZ (10%) were obtained. The two samples with added CPZ were then incubated on a waterbath for 24 h at 44°C.

2.2. Video-enhanced microscopy

Video-enhanced microscopy was employed in the evaluation of lipid sample homogeneity and stability of filtered ($\sim$1 μm) and lyophilized liposomes. The microscopic equipment consisted of a Nikon Optiphot-2 microscope (Tokyo, Japan) fitted with differential interference contrast (DIC) optics. The microscope was connected to a Dage-MTI video-camera system (Model VE-1000) consisting of a black and white television camera with a Pasecon imaging tube and a control unit with monitor for analog adjustment of black level, gain and shading. A Macintosh computer is used for digitizing the pictures, with help of a Perceptics PixelBuffer framegrabber. The image analysis software is a package from Graftek, France consisting of the programs Optilab and Concept V.i for LabView (National Instruments, Austin, TX). This showed that the liposomes had the expected size. The samples were then subjected to 24 h of lyophilization giving partially hydrated liposomes with a hydration level of $\sim$12 water molecules per molecule lipid (determined by ^{1}H-MAS-NMR). Fully hydrated phospholipids have 22 to about 34 water molecules per lipid, depending on the headgroup [14,15].

2.3. Differential scanning calorimetry

DSC thermograms were obtained on a MicroCal VP-DSC instrument from MicroCal (Northampton, MA). All solutions were degassed under vacuum to avoid air bubbles. A scanning rate of 1.5°C/min was used for all samples and thermograms of water served as reference. All data were acquired by computer software developed by MicroCal. The liposome samples subjected to DSC analysis were dissolved in water to give 4 mM suspensions. Experiments on partially hydrated lipid samples (see NMR samples), e.g. 12 water molecules per molecule lipid, could not be performed on the MicroCal VP-DSC instrument.

2.4. CP-MAS-^{13}C-NMR

The MAS-NMR experiments were obtained at 100.6 MHz with a Bruker DMX-400 instrument equipped with magic angle spinning hardware and using ZrO_2 rotors with a diameter of 4 mm. Experiments were done at sample temperatures of 25 and 37°C with sample spinning rates of 5000 and 3000 Hz, respectively. Calibration of the MAS probe temperature was done by the manufacturer (Bruker, Germany) upon delivery of the solid state equipment. Confirmation of the MAS probe temperature calibration in the temperature range with relevance to phospholipid bilayer phase transitions was carried out on a pure DPPC sample. ^{13}C-NMR spectra of the pure DPPC sample was recorded from 20 to 44°C and the DPPC phase transition was found to occur between

40.6 and 42.6°C. The MAS ^{13}C-NMR spectra were obtained with proton cross polarization (contact time of 1 ms) to enhance the carbon signal intensities. All experiments were carried out with a relaxation delay of 5 s between transients. A total of 4000 transients were collected for each experiment carried out at 25°C and a total of 12 000 transients for each of the experiments at 37°C. The spectra were multiplied with an exponential window function increasing the linewidth by 2 Hz to reduce the noise prior to Fourier transformation.

3. Results

The dynamic and thermotropic properties of bilayers have been studied by methods like DSC and NMR for more than two decades [16–22]. When a phospholipid bilayer goes from the gel phase to the liquid crystalline phase upon increase in temperature, the transition is accompanied by several structural changes in the lipid molecules. Of these changes, the *trans*-gauche isomerization in the acyl chains is the principal change and the average number of gauche conformers can be related to the bilayer fluidity. Furthermore, a drug molecule interdigitating among the phospholipid molecules causes perturbations that contribute to the bilayer fluidity. Fig. 1 presents the relatively sharp gel to liquid crystalline phase transition of the DPPC/DMPC bilayer and the corresponding somewhat broader DPPC/DMPC/CPZ transition. The influence of the unsaturated acyl chains in PS can be seen in the broad gel to liquid crystalline phase transition of the DPPC/PS bilayer in Fig. 2. Fig. 2 also demonstrates that the presence of CPZ increases the temperature range of the DPPC/PS bilayer gel to liquid crystalline phase transition (see the DPPC/PS/CPZ curve in Fig. 2). Table 1 lists quantitative data from the thermograms of the two phospholipid bilayers studied, in the absence and presence of CPZ. Both the DPPC/DMPC and the DPPC/PS bilayers experience a 1.2°C decrease in the transition temperature when CPZ is present (Table 1). The CP-MAS-^{13}C-NMR spectrum of the partially hydrated (~1 μm) DPPC (60%)/DMPC (40%) liposomes is shown in Fig. 3, top. The corresponding spectrum of the DPPC (54%)/DMPC (36%)/CPZ (10%) liposomes is displayed in Fig. 3, bottom. The assigned resonances [23] labeled in the top spectrum of Fig. 3 are carbonyl (172.95 ppm), the nitrogen-bonded methyl groups (54.27

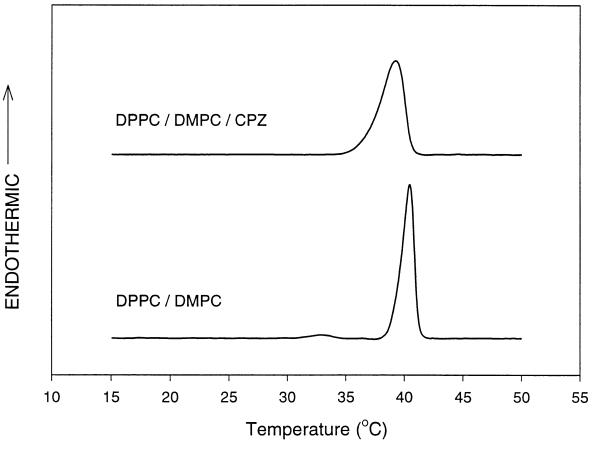

Fig. 1. Differential scanning calorimetry thermograms obtained from aqueous LUV suspensions of DPPC (60%)/DMPC (40%) and DPPC (54%)/DMPC (36%)/CPZ (10%).

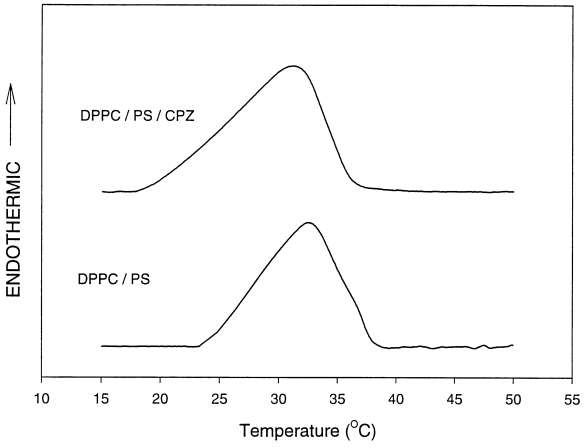

Fig. 2. Differential scanning calorimetry thermograms obtained from aqueous LUV suspensions of DPPC (60%)/PS (40%) and DPPC (54%)/PS (36%)/CPZ (10%).

Table 1
Differential scanning calorimetry values of transition temperature (T_C), half-width ($T_{C_{1/2}}$), and enthalpy-change (ΔH) of the four LUV preparations studied

Sample	T_C (°C)	$T_{C_{1/2}}$ (°C)	ΔH (kJ/mol)
DPPC/DMPC	40.4, 32.9[a]	1.2	11.7
DPPC/DMPC/CPZ	39.2	2.4	13.3
DPPC/PS	32.4	8.1	14.1
DPPC/PS/CPZ	31.2	9.1	18.1

[a]Pretransition temperature.

ppm), the large $(CH_2)_n$ peak (32.54 ppm) and the terminal acyl methyl group (14.10 ppm). The carbonyl peak (Fig. 3, top) is broad and does not allow distinction between *sn*-1 and *sn*-2 carbonyl carbons. However, it has been demonstrated [24] that ^{13}C-enriched *sn*-1 and *sn*-2 carbonyl carbons in DPPC that *sn*-1 is found slightly upfield from *sn*-2 carbonyl resonances. As can be seen in the top spectrum (Fig. 3), the resonance at 54.27 ppm is the nitrogen-bonded methyl groups of DPPC (60%)/DMPC (40%). The chemical shifts of all spectra are referred to the acyl end methyl group chemical shift of 14.10 ppm [25]. This chemical shift is believed not to vary with temperature in the range 15–55°C. The lack of fine structure in the DPPC (54%)/DMPC (36%)/CPZ (10%) $(CH_2)_n$ chemical shift region upon addition of CPZ can be seen expanded in Fig. 4, bottom.

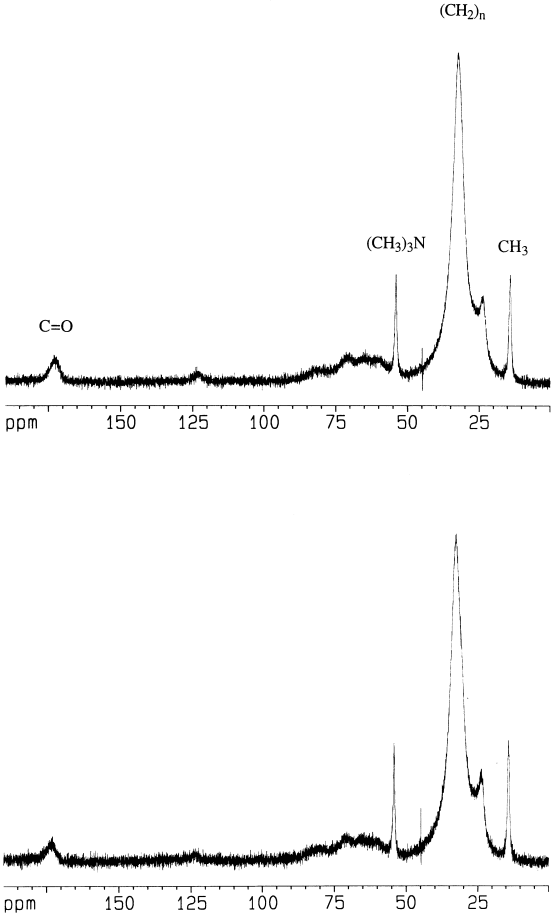

Fig. 3. Top: CP-MAS-^{13}C-NMR spectrum of the partially hydrated DPPC (60%)/DMPC (40%) liposomes acquired at a sample temperature of 25°C. The resonances are assigned and labeled as: carbonyl (172.95 ppm), the nitrogen-bonded methyl groups (54.27 ppm), the $(CH_2)_n$ peak (32.54 ppm) and the acyl terminal methyl group (14.10 ppm). Bottom: the corresponding spectrum of the DPPC (54%)/DMPC (36%)/CPZ (10%) liposomes is displayed. See the text for further details.

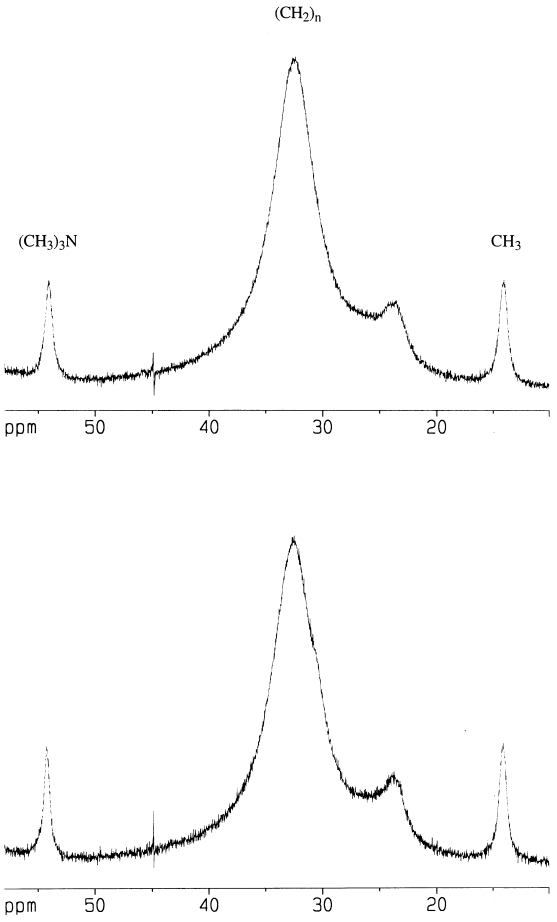

Fig. 4. Top: expansion of the $(CH_2)_n$ spectral region and the two methyl resonances of the DPPC (60%)/DMPC (40%) liposomes shown in Fig. 3, top. Bottom: expansion of the $(CH_2)_n$ spectral region and the two methyl resonances of the DPPC (54%)/DMPC (36%)/CPZ (10%) liposomes shown in Fig. 3, bottom. The effect on the DPPC (60%)/DMPC (40%) membrane structure of 10 mol% CPZ addition is barely visible at about 30 ppm.

Fig. 4, top, displays the corresponding spectral region of the DPPC (60%)/DMPC (40%) liposomes. The effect of addition of 10% CPZ on the DPPC (60%)/DMPC (40%) membrane structure is barely visible at about 30 ppm in Fig. 4, bottom. Fig. 5, top, shows the CP-MAS-^{13}C-NMR spectrum of the partially hydrated ($\sim$1 μm) DPPC (60%)/PS (40%) liposomes and in Fig. 5, bottom, the corresponding spectrum of the DPPC(54%)/PS(36%)/CPZ(10%)

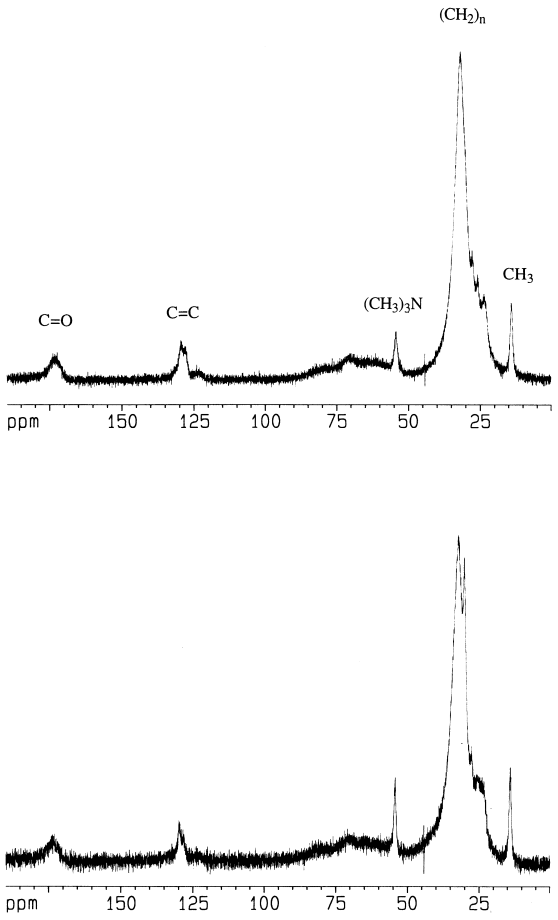

Fig. 5. Top: CP-MAS-^{13}C-NMR spectrum of the partially hydrated DPPC (60%)/PS (40%) liposomes acquired at a sample temperature of 25°C. The resonances are assigned and labeled as: carbonyl (172.90 ppm), the nitrogen-bonded methyl groups (54.36 ppm), the C═C groups (129.51 and 128.19 ppm), the $(CH_2)_n$ peak (32.15 ppm) and the acyl terminal methyl group (14.10 ppm). Bottom: the corresponding spectrum of the DPPC (54%)/PS (36%)/CPZ (10%) liposomes is displayed. The high-field shifted $(CH_2)_n$ peak is found at 30.29 ppm. See the text for further details.

liposomes is displayed. The assignments of the resonances are labeled in the top spectrum of Fig. 5 as carbonyl (172.90 ppm), C═C groups (129.51 and 128.19 ppm), the nitrogen-bonded methyl groups (54.36 ppm), the large $(CH_2)_n$ peak (32.15 ppm) and the acyl terminal methyl group (14.10 ppm) [23]. The carbonyl peak (Fig. 3, top) is broad and does not allow distinction between the carbonyl carbons esterified in the *sn*-1 and *sn*-2 positions.

It is worth noting that a CP-MAS-^{13}C-NMR spectrum of pure CPZ showed that the largest resonances in that spectrum appeared at 45 and 133 ppm at a sample temperature of 25°C (data not shown). At a 10 mol% level and with natural ^{13}C abundance, individual CPZ peaks are not present in the spectrum of DPPC (54%)/PS (36%)/CPZ (10%) displayed in Fig. 5, bottom. For instance, the two resonances at 45 and 133 ppm in the pure CPZ spectrum cannot be detected in the DPPC (54%)/PS (36%)/CPZ (10%) spectrum shown in Fig. 5, bottom and Fig. 4, bottom. The DPPC (54%)/PS (36%)/CPZ (10%) CP-MAS-^{13}C-NMR spectrum (Fig. 5, bottom) shows an additional large $(CH_2)_n$ peak at 30.29 ppm in

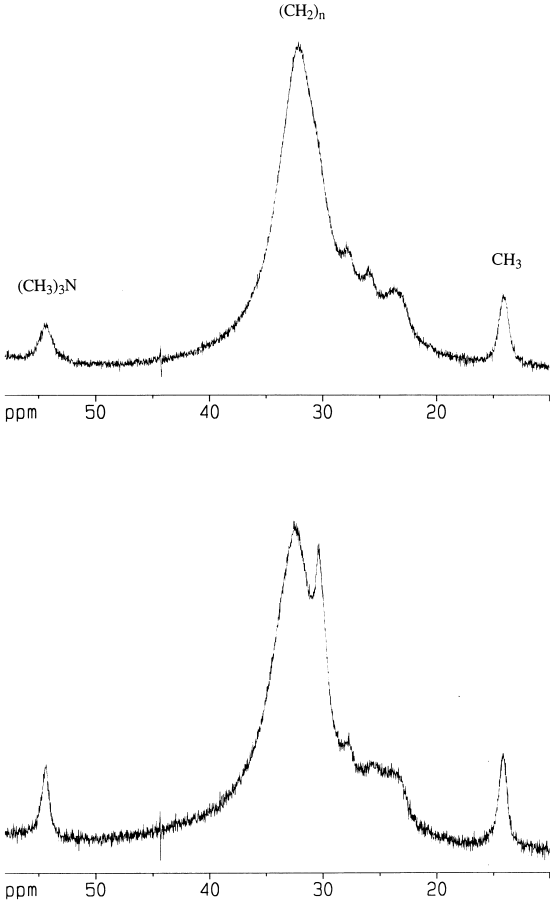

Fig. 6. Top: expansion of the $(CH_2)_n$ spectral region and the two methyl resonances of the DPPC (60%)/PS (40%) liposomes shown in Fig. 5, top. Bottom: expansion of the $(CH_2)_n$ spectral region and the two methyl resonances of the DPPC (54%)/PS (36%)/CPZ (10%) liposomes shown in Fig. 5, bottom. The new $(CH_2)_n$ peak at 30.29 ppm amounts to ~30% of the two $(CH_2)_n$ peaks (32.15 and 30.29 ppm) combined. Note also the reduced linewidth of the nitrogen-bound methyl resonance upon addition of CPZ.

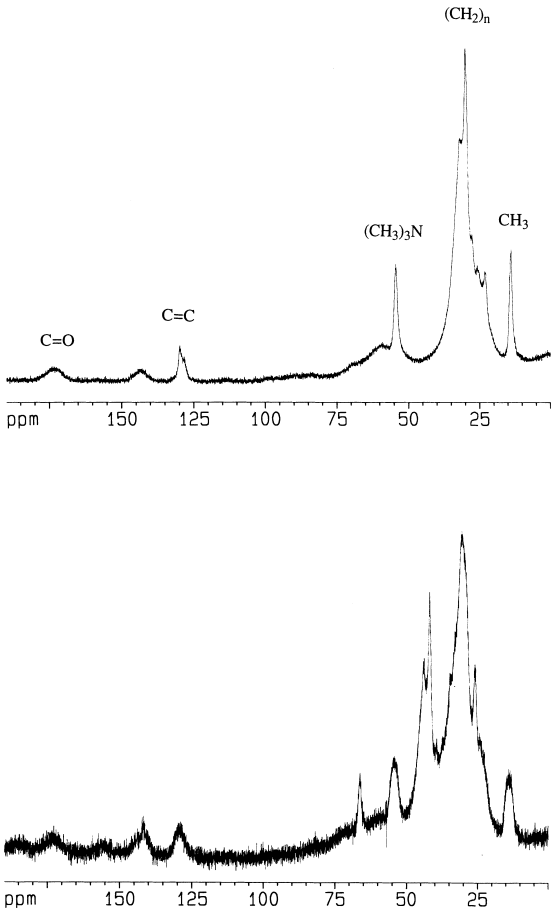

Fig. 7. Top: CP-MAS-^{13}C-NMR spectrum of the partially hydrated DPPC (60%)/PS (40%) liposomes acquired at a sample temperature of 37°C. The resonances are assigned and labeled as: carbonyl (172.90 ppm), the nitrogen-bonded methyl groups (54.36 ppm), the C═C groups (129.51 and 128.19 ppm), the $(CH_2)_n$ peak (32.15 ppm) and the acyl terminal methyl group (14.10 ppm). Bottom: the corresponding spectrum of the DPPC (54%)/PS (36%)/CPZ (10%) liposomes is displayed. The low-field shifted $(CH_2)_n$ peaks are found at 35–45 ppm and the low-field shifted C═C resonances at 142.0 ppm. See the text for further details.

addition to the one at 32.15 ppm found in the DPPC (60%)/PS (40%) spectrum (Fig. 5, top). Thus, this large alteration in the $(CH_2)_n$ region of the spectrum is due to CPZ interaction with the phospholipids. However, this amount of CPZ is not detectable at all as CPZ resonances in the spectrum. Also the C═C resonance at 128.19 ppm is affected by the presence of CPZ – see the reduced size of this peak in Fig. 5, bottom versus the corresponding peak in Fig. 5, top. The observed fine structure in the DPPC (54%)/PS (36%)/CPZ (10%) $(CH_2)_n$ region is expanded in Fig. 6, bottom. Fig. 6, top displays the corresponding spectral region of the DPPC (60%)/PS (40%) liposomes. As can be seen in Fig. 6, top, the NMR peak belonging to nitrogen-bound methyl groups sharpens up from a linewidth (at half-height) of 141 to 86 Hz upon addition of CPZ (see Fig. 6, bottom). The observed change of the nitrogen-bound methyl resonance presumably is due to increased mobility of these methyl groups. This effect and the major changes in the acyl chain $(CH_2)_n$ peak at 32.15 ppm with the appearance of a large peak at

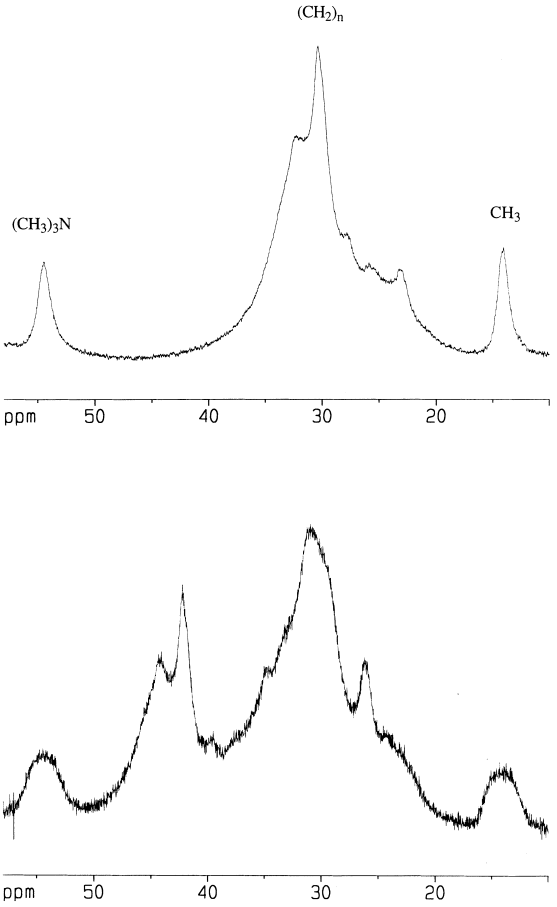

Fig. 8. Top: expansion of the $(CH_2)_n$ spectral region and the two methyl resonances of the DPPC (60%)/PS (40%) liposomes shown in Fig. 7, top. Bottom: expansion of the $(CH_2)_n$ spectral region and the two methyl resonances of the DPPC (54%)/PS (36%)/CPZ (10%) liposomes shown in Fig. 7, bottom. The low-field shifted $(CH_2)_n$ peaks found at 35–45 ppm integrate to ~30% of the $(CH_2)_n$ total peak area. Note also the overall increased linewidth of the resonances (compare bottom and top spectrum).

30.29 ppm with CPZ added (compare Fig. 6, top and bottom) suggest an inclusion of CPZ in the DPPC/PS membrane.

Assignments of partially resolved resonances (Figs. 5 and 6), especially in the $(CH_2)_n$ region are difficult. However, three partially resolved resonances can be seen in the high-field part of the large $(CH_2)_n$ peak (Fig. 5, top). The $n-1$ acyl-chain carbon resonances are found in this chemical shift region [23]. The acyl carbon 15 in DPPC is found at the highest field i.e. at 23.18 ppm. The $n-1$ acyl chain carbon of PS, i.e. acyl chain carbon 17 can then tentatively be assigned to the resonance in the $(CH_2)_n$ region at 25.92 ppm. The third visible peak in this region at 27.85 ppm can then be attributed to carbon 3 of the acyl chain. The glycerol backbone-carbon resonances, g_1, g_2 and g_3 are broadened, see the broad peaks in the 55–85 ppm region of Fig. 5, top. Similarly, the phospholipid headgroup carbons have mobilities that give rise to broadened peaks in this chemical shift region.

The effects of CPZ on the DPPC/PS membrane were further investigated by acquiring CP-MAS-^{13}CNMR spectra on both the DPPC (60%)/PS (40%) liposomes and the DPPC (54%)/PS (36%)/CPZ (10%) liposomes at the more biologically interesting temperature of 37°C, see Fig. 7, top and bottom, respectively. The $(CH_2)_n$ and methyl resonances of Fig. 7, top and bottom, are expanded in Fig. 8, top and bottom, respectively. In Fig. 7, top, an acyl chain carbon resonance in the low-field region can be seen partially resolved at a chemical shift of 32.10 ppm. In this region, both the acyl chain carbon 2 and the $n-2$ carbon are expected [21]. The effect of the CPZ addition to the DPPC/PS membrane is substantial at the sample temperature of 37°C, see Fig. 7, bottom. The acyl chain carbon resonances in the $(CH_2)_n$ region is divided into two major parts with new peaks appearing at 35–45 ppm and the main acyl chain carbon peak is found at 31.0 ppm. Judging from the peak areas in the $(CH_2)_n$ region at 37°C, $\sim$30% of the acyl-chain carbons move low-field, into the 35–45 ppm chemical shift region, upon incorporation of CPZ in the liposomes. Both the terminal methyl group of the acyls (14.10 ppm) and the choline nitrogen-bound methyl groups (54.5 ppm) are broadened with 200 Hz compared to the corresponding linewidths (at half-height) of the pure DPPC/PS sample shown in Fig. 7, top. This broadening effect could be explained by increased mobility of these methyl groups. An additional new carbon resonance (Fig. 7, bottom) has appeared in the glycerophospholipid headgroup chemical shift region at 66.5 ppm. This chemical shift is in good agreement with the earlier observed [23,26] chemical shift of carbon-2 in the choline head group of DPPC.

In comparing the DPPC/PS/CPZ MAS-NMR spectra at the two temperatures investigated (25 and 37°C), one discovers a significant difference. This bilayer has $T_C = 31.2$°C (Table 1) and the effects of the 10 mol% CPZ on the DPPC/PS membrane at 25°C is such that a large portion ($\sim$30%) of the main $(CH_2)_n$ peak at 32.10 ppm shifts high-field to 30.29 ppm. This may be explained as pointed out by Small and Hanahan [27] by CPZ interaction with the phospholipid headgroups (PS) when the acyl chains are in the *trans* conformation (25°C). The observed effect of the interaction causes about 30% of the acyl chains to enter the gauche conformation and to experience a high-field shift [26] with low or no CPZ interdigitation among the acyl chains (see below).

Conversely, above the T_c of 31.2°C, at a sample temperature of 37°C, the acyl chains are all in the gauche conformation and allow interdigitation of CPZ in the bilayer. This leads to a low-field shift of the CPZ affected ($\sim$30%) acyl chain carbon resonances [23] by 5–15 ppm at a sample temperature of 37°C. The corresponding deshielding of these acyl chain carbons are most likely caused by interdigitation of CPZ. The CPZ molecule has chlorine and sulfur atoms, both with a partially negative charge. Thus, an interdigitized CPZ molecule presents partially negative charges to the surrounding phospholipid acyl chain carbons. This will produce an orbital contraction of these acyl chain carbon π-orbitals and deshielding of the corresponding carbon nuclei to give the observed 5–15 ppm downfield shifts.

This can be corroborated by the effects of CPZ on the C=C resonances at 129.97 and 128.43 ppm (Fig. 7, top). About half of these (partially overlapping) C=C resonances move low-field by 10–11 ppm (to 142.0 ppm), see Fig. 7, bottom. Unfortunately, peak area quantification of these broad peaks (142.0 and 129.1 ppm in Fig. 7, bottom) is somewhat compromised by the presence of a small broad peak at 143.6 ppm (not assigned) in the DPPC/PS spectrum (Fig. 7, top). However, the conclusion that CPZ causes

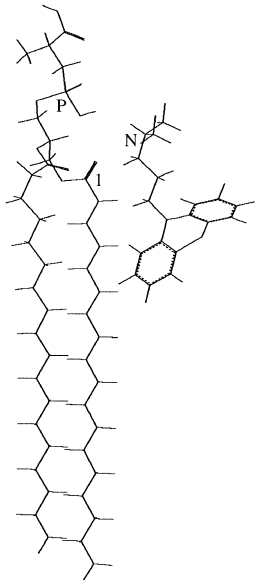

Fig. 9. Molecular model of CPZ interaction with a 1,2-distearoylphosphatidylserine molecule. The CPZ molecule (right) is positioned with its positive charge on the nitrogen atom (labeled N) on the end of the CPZ acyl chain. This positive charge is in the vicinity of the phosphate's (labeled P) negative charge in the PS molecule (left). The PS molecule in the displayed model has the *sn*-1 carbonyl labeled with the number 1. The molecular model suggests that the phospholipid acyl chains are affected by the presence of CPZ. The molecular model was generated by the MacSpartan Plus software (Wavefunction, Irvine, CA).

C═C resonances to move low-field (by 10–11 ppm) seems reasonable.

The lack of CPZ-induced down-field shifts for the phospholipid acyl chain carbon resonances of the DPPC/PS/CPZ sample at 25°C (see Figs. 5 and 6) suggests little or no CPZ interdigitation at this temperature (well below the main phase transition temperature of 31.2°C).

4. Discussion

The PS used in our studies was from pig brain and thus composed of many molecular species, in which the major species containing unsaturated acyl groups were oleoyl-containing species with a double bond at C9. Furthermore, the unsaturated fatty acids in our PS preparation were 22:6($n-3$), 20:4($n-6$) and 18:1($n-9$). Thus, the observed CPZ effects on the C═C resonances in the DPPC/PS spectra at 37°C described above support interdigitation of CPZ among the phospholipids above the main phase transition temperature. Furthermore, at the sample temperature of 37°C the effects of CPZ on the DPPC/PS membrane causes an overall line broadening effect can be seen in all carbon resonances (see Figs. 7 and 8). On the other hand, at 25°C the linewidth of the terminal methyl resonance at 14.10 ppm is markedly reduced due to increased phospholipid mobility by interaction of CPZ with the phospholipid headgroups (PS). This increase in mobility also causes about 30% of the acyl chains to enter the gauche conformation and thus, an expansion of the molecular area per phospholipid with low or no CPZ interdigitation among the acyl chains at this temperature (25°C).

In order to visualize CPZ interaction with the bilayer, a model of CPZ located with its positive charge (acyl chain nitrogen) in the vicinity of a PS (18:0) phosphate group negative charge was made (see Fig. 9). Furthermore, the acyl chain of CPZ and the phenyl rings have been positioned along the PS acyl chain direction as supported by the MAS-NMR experiments. The molecular model was generated by the MacSpartan Plus software (Wavefunction, Irvine, CA). First of all, this model demonstrates the dimensions of the two types of molecules involved (a glycerophospholipid and a chlorpromazine-HCl molecule). The molecular model suggests that phospholipid acyl chains are affected by the presence of CPZ. Furthermore, the molecular model presented in Fig. 9 also suggests that the molecular area of phosphatidylserine is approximately doubled when CPZ enters the membrane. Such an expansion of the phospholipid molecular area upon CPZ inclusion is in fact found from Langmuir–Blodgett experiments (fully hydrated phospholipids) with this type of membrane (A. Varnier, L.M. Tungodden, D. Cejka, E. Bakstad, L. Sydnes, and H. Holmsen, personal communication).

Effects of CPZ on DPPC/DMPC liposomes which carry no net charge, on the other hand, are minute (Figs. 3 and 4) and this suggests a small degree of CPZ interaction with these uncharged glycerophospholipid molecules. A more relevant model membrane for future studies could be a phospholipid mixture like DPPC/EYPC. This chemical system can give information on CPZ interaction differences with the

DPPC/PS system that mainly depend on the headgroups and thus, be a refinement in the determination of the importance of acyl chain unsaturation and headgroup charge.

The finding that at 37°C about 30% of the phospholipid acyl chain carbon resonances shift low-field by 5–15 ppm when CPZ is added to the DPPC/PS membrane has been described above (see also Figs. 7 and 8). When this low field shift and a probable positioning of CPZ in proximity of a PS molecule as pictured in Fig. 9 are judged together with a phospholipid's five 'nearest neighbor' molecules [27] (in the liquid crystalline phase) and the molecular mobility, such as rotations of phospholipid molecules around the bilayer normal in the 10^8–10^{11} Hz frequency range [28] one finds that about six phospholipids are affected by the interdigitized CPZ molecule. Thus, the PS in a PS/CPZ complex and about five surrounding phospholipids (nearest neighbors) have acyl chain carbons that experience a low-field shift (5–15 ppm) when CPZ interdigitates the bilayer. Further carbon-13 as well as phosphorus-31 NMR studies on CPZ interaction with such bilayer systems as described in this work are under way in our laboratory.

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