PHOSPHOLIPID PACKING AND CONFORMATION IN SMALL VESICLES REVEALED BY TWO-DIMENSIONAL ^{1}H NUCLEAR MAGNETIC RESONANCE CROSS-RELAXATION SPECTROSCOPY

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ABSTRACT Two-dimensional ^{1}H-NMR spectroscopy has been used to examine cross-relaxation in sonicated phospholipid vesicle systems. The observed pattern of proton cross-relaxation reveals several important features of these vesicle systems. For example, cross-relaxation rates on each monolayer of the vesicle system can be resolved and reflect the expected geometric packing constraints of the vesicle system. Small but significant magnetization-exchange is also seen to develop between the headgroup N-methyl resonance and the terminal methyl resonance. Spectra taken with deuterated lipids indicate that this exchange is not mediated by spin-diffusion down the length of the alkyl chains. Since spin-diffusion is the only process that is expected to facilitate magnetization-exchange over distances of 15–20 Å, a close proximity of headgroup and terminal methyl protons in a fraction of the membrane lipid is indicated by these results. This could occur by events such as lipid interdigitation or alkyl chain bends that terminate lipid alkyl chain ends near the membrane surface.

INTRODUCTION

From both physiochemical and biological points of view, an understanding of the structure and phase behavior of phosphatidylcholine (PC) membranes is extremely important. A variety of techniques have been used to characterize the properties of PC membranes and a number of structures ranging from planar films to small unilamellar vesicles have been examined. In the gel phase of dipalmitoylphosphatidylcholine (DPPC) dispersions, three distinct phases have been observed. In each of these phases, the hydrocarbon chains appear to be in an extended configuration (1–4). The membrane has a width corresponding to a bilayer with the terminal methyl groups located near the membrane center. Recently, it has been shown that the addition of certain surface active molecules can induce the formation of a completely interdigitated phase in gel state DPPC (5–7). Above the main bilayer phase transition, interdigitation has not been observed.

In sonicated vesicle systems, the small radius of curvature results in packing differences between the inner and outer monolayers for both the hydrocarbon and polar headgroup regions of the lipid (8–13). This curvature is believed to have an effect upon the distribution of chain ends in the membrane as well as the order parameter across the membrane (14, 15). These and other statistical mechanical treatments of bilayer dynamics (16) predict a wide distribution for the position of alkyl chain ends. In the present report, we describe the pattern of ^{1}H cross-relaxation seen in small sonicated vesicles of DPPC using two-dimensional methods. In this system, ^{1}H cross-relaxation should be determined by dipolar order parameters and internuclear distances between protons (17, 18, 19). Our results indicate that the relative cross-relaxation rates in DPPC reflect the lipid chain order and headgroup packing in vesicles. This data is consistent with conclusions drawn from previous ^{1}H-NMR measurements (17). Data is also presented that suggests a close proximity between headgroup and terminal methyl protons in a population of the vesicle lipid.

MATERIALS AND METHODS

DPPC was obtained from Sigma Chemical Co. (St. Louis, MO) and was used without further purification. Egg phosphatidylcholine (egg PC) was purified according to the procedure of Singleton et al. (20) and was stored in $CHCl_3$ under argon at −20°C. Chain deuterated DPPC-d_8 was synthesized from glycerophosphorylcholine and hexadecanoic-7,7,8,8-d_4 acid (98.9% atom D, obtained from MDS Isotopes, St. Louis, MO) according to published procedures (21). Headgroup deuterated DPPC-d_9

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was synthesized by methylation of dipalmitoylphosphatidylethanolamine with deuterated methyliodide. Chain perdeuterated DPPC-d_{62} was obtained from Avanti Biochemicals (Birmingham, AL). Phospholipid vesicles were prepared by sonication of lipid dispersions in 100 mM sodium phosphate buffer, pD = 7.0, as previously described (22). Vesicle suspensions were centrifuged at 35,000 *g* for 20 min to remove titanium dust from the sample and float any unsonicated lipid.

Two-dimensional cross-relaxation NMR spectra were obtained using a 360 MHz NT-360 spectrometer (Nicolet Magnetics Corp., Fremont, CA) equipped with a 293B pulse programmer and a 1280 data system. The following pulse sequence was used ($\pi/2 - t_1 - \pi/2 - t_1/4 - \tau_m/2 - \pi\text{comp} - \tau_m/2 - \pi/2 - t_2$) and the data was collected and processed using standard Nicolet software. The 90° pulses were phase-shifted to allow quadrature detection in both dimensions and to suppress axial peaks using procedures that are common to two-dimensional homonuclear spectroscopy (23–26). The composite 180° pulse renders *J* cross-peaks (including zero quantum *J* cross-peaks) time-dependent, allowing them to be effectively surpressed by symmetrization of the F_1, F_2 data set (23, 25).

RESULTS

Two-dimensional Spectra of Sonicated DPPC Vesicles

Fig. 1 is a proton cross-relaxation spectrum of sonicated DPPC vesicles at 50°C. Here, the spin-system was allowed to cross-relax for 500 ms (i.e. the mixing-time, τ_m, was 500 ms). Magnetization-exchange, as seen in this spectrum, develops in several regions of the lipid molecule. For example, the headgroup methylenes cross-relax with the *N*-methyl (*N*-Me) protons but not with other regions of the lipid. Relaxation among the glycerol backbone protons is also observed at shorter mixing times (e.g. τ_m = 50–200 ms; data not shown). Of particular interest here are cross-peaks that develop between the *N*-Me resonance and the alkyl chains. Two *N*-Me/methylene cross-peaks are observed, labeled 4 and 5 in Fig. 1. These peaks, as seen in higher resolution spectra, have chemical shifts corresponding to the internal and external *N*-Me resonances on the two-dimensional diagonal. The addition of paramagnetic ions such as Mn^{++} to the vesicle exterior selectively reduces the size of peak 5. Therefore, peaks 4 and 5 apparently correspond to *N*-Me/methylene cross-relaxation developing on the internal and external vesicle surfaces, respectively. Taking into account the magnitudes of the diagonal resonances and the time-dependence of the cross-peak amplitudes (27), *N*-Me/methylene cross-relaxation on the internal vesicle surface is approximately twice as efficient as on the external vesicle surface.

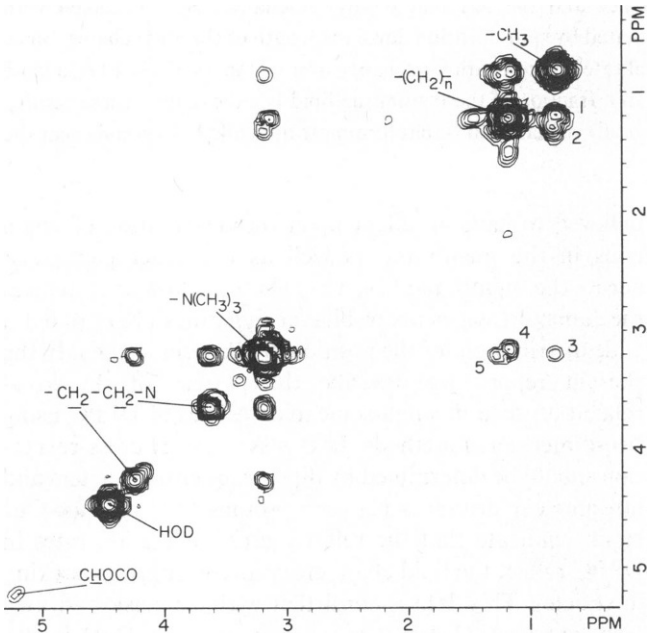

FIGURE 1 A two-dimensional proton cross-relaxation spectrum of sonicated DPPC vesicles (100 mM lipid) at 50°C in 100 mM deuterated sodium phosphate buffer, pD = 7. The mixing time, τ_m, was 500 ms and the data set consisted of 128 blocks with 256 data points per block. Each block represents 32 acquisitions and the data set required a total accumulation time of ~4 h. The data was digitally filtered using a double sine multiplication and zero-filling in the t_1 dimension. The spectrum is plotted in absolute value mode using a magnitude correction following the second fourier transform. Peaks 1 and 2 result from t-Me/methylene cross-relaxation while peaks 4 and 5 are a result of *N*-Me/methylene cross-relaxation. Peak 3 results from t-Me/*N*-Me cross-relaxation. Each contour represents an amplitude factor of $\sqrt{2}$.

A transfer of proton magnetization is observed between the *N*-Me and terminal methyl (t-Me) resonances; cross-peak 3, Fig. 1. Slightly more of this peak develops at the position of the external shifted resonances, as seen in higher resolution spectra (data not shown). In addition, two cross-peaks also develop between the t-Me and methylene chain resonances; peaks 1 and 2 in Fig. 1. Thus, there appear to be two types of t-Me/methylene and *N*-Me/methylene interactions. Peaks 1 and 2 also appear to represent cross-relaxation from internal and external populations of lipid, respectively (see Discussion). The protons that result in peak 2 cross-relax approximately three times more efficiently than the protons resulting in peak 1.

Cross-Relaxation in Vesicles Containing Deuterated Lipids

To delineate the pathways of cross-relaxation between the headgroup *N*-Me protons and the alkyl chain protons, spectra were obtained for DPPC vesicles formed from several deuterated lipids. In vesicles formed from deuterated DPPC-d_8, where a gap of deuterated methylenes is placed in the lipid chains, approximately the same rate of t-Me/*N*-Me magnetization-exchange is observed as in vesicles formed from totally protiated DPPC. When *N*-Me/alkyl chain cross-peaks are measured in vesicles formed from a mixture of headgroup labeled DPPC-d_9 and chain perdeuterated DPPC-d_{62}, peaks 3, 4, and 5 in Fig. 1 remain, but are reduced by approximately the product of the mole fractions for each labeled lipid. Cross-peaks that develop in this lipid mixture must be the result of intermolecular interactions.

Hydrophobic Ion, Lipid Cross-Relaxation

A two-dimensional cross-relaxation spectrum of egg PC vesicles containing a high concentration of tetraphenylphosphonium (ϕ_4P^+) is shown in Fig. 2. A striking feature observed in this spectrum is the presence of intermolecular exchange between the ϕ_4P^+ phenyl protons and the t-Me protons of egg PC. This cross-peak develops in less than 100 ms and is not the result of a spin-diffusion mechanism. Under negative stain electron microscopy, egg PC vesicles appear to maintain their normal morphology in the presence of high levels of ϕ_4P^+.[1]

Dependence of the Cross-Relaxation Rate Upon S_{HH}

A theoretical description of cross-relaxation in a system of protons where anisotropic motions as well as intermolecular distance fluctuations occur is clearly complex. However, the following example demonstrates how proton cross-relaxation might be expected to vary with the dipolar order parameter, S_{HH}. We ignore internuclear distance fluctuations and consider the case where dipolar interactions are averaged by both a fast local motion and a slow isotropic tumbling. When the fast local motions are characterized by a correlation time τ_c and an order parameter S_{HH}, and the slow tumbling has a correlation time τ_m, the spectral density function $J(\omega)$ can be given by (28–30)

$$J(\omega) \propto S_{HH}^2 \cdot \{\tau_m/[1 + (\tau_m\omega)^2]\} + (1 - S_{HH}^2) \cdot \{\tau_c/[1 + (\tau_c\omega)^2]\}.$$

Carbon deuterium order parameters are usually small in membrane systems (usually $S_{CD} < 0.2$) (31) and we expect S_{HH}^2 to be $<<1$. Thus, the value of the second set of terms in the above expression should not depend strongly upon S_{HH}. The value of the first set of terms, however, will depend strongly upon S_{HH}. Because $\tau_m >> \tau_c$, this first set makes the biggest contributions to the spectral densities that promote energy-conserving transitions, $J(\omega = 0)$ (17, 31). Since proton cross-relaxation in vesicles is dominated by energy conserving transitions (18, 19), variations in S_{HH} will have a large effect upon cross-relaxation in this system. In regions of the vesicle where S_{HH} is large, cross-relaxation and spin-diffusion will be more efficient (17).

DISCUSSION

The two-dimensional ^1H cross-relaxation spectrum of DPPC vesicles shown above clearly reveals interactions between *N*-Me and methylene resonances occurring on each vesicle interface. Cross-peaks 1 and 2 in Fig. 1 also appear to be due to internal and external populations of lipid. The chemical shift difference between these peaks is identical to that for peaks 4 and 5, and this assignment is consistent with that proposed for the broad and sharp methylene resonances that appear in 500 MHz ^1H spectra (17). From this assignment, a network mapping out cross-relaxation on both internal and external vesicle monolayers can be drawn (see Fig. 3). From the assignments given in Fig. 3, the efficiency of cross-relaxation on each vesicle surface can be compared. Cross-relaxation is more efficient for *N*-Me/methylene chain interactions on the internal vesicle surface but more efficient for t-Me/methylene chain interactions on the external surface. As noted above, we expect cross-relaxation to be more efficient in regions where the dipolar order parameter is larger. The variation in S_{HH} across the vesicle bilayer inferred from our cross-relaxation data is consistent with the expected packing asymmetry for lipids in small vesicles (13, 15). That is, when internal and external vesicle surfaces are compared, S_{HH} is larger on the internal surface at the level of the lipid headgroup and larger on the external surface at the alkyl chain ends.

The t-Me/*N*-Me cross-peak (peak 3 in Fig. 3.) is not necessarily unexpected since spin-diffusion along the alkyl chains could facilitate this magnetization transfer. However, measurements of this cross-peak in deuterated lipids suggest that this is not the case. When protons in the seven

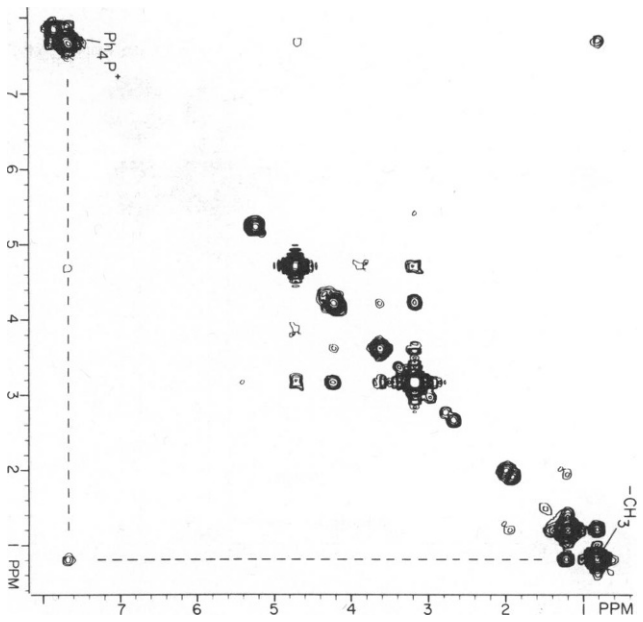

FIGURE 2 A two-dimensional cross-relaxation spectrum of sonicated egg PC vesicles (130 mM lipid) containing 26 mM tetraphenylphosphonium at 25° C. The mixing time shown here is 50 ms and the data set was identical in size to that used for the spectrum in Fig. 1. Total accumulation time was ~4 h. The cross-peak that is indicated here results from ϕ_4P^+/t-Me proton cross-relaxation and is well above the 't_1-noise' level as previously determined (19).

[1] We have examined the ^{31}P-NMR spectra of phospholipid dispersions containing high levels of phosphonium ions. Broad asymmetric spectra characteristic of a bilayer phase are seen with no indication of 'unusual' phases in the presence of high concentrations of this ion.

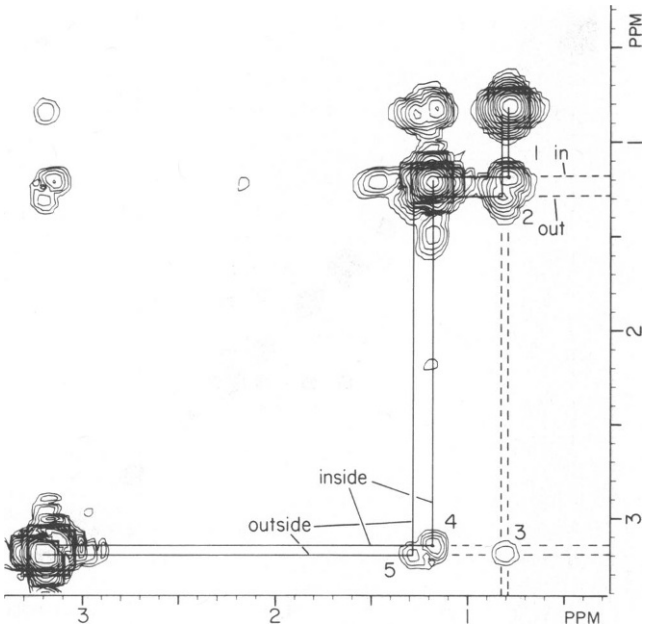

FIGURE 3 An expansion of the region in Fig. 1 from 3.5 to 0.3 ppm. Also shown are the cross-relaxation networks for interactions on both vesicle surfaces.

and eight positions of the alkyl chains are replaced with deuteriums, spin-diffusion should be dramatically reduced (32); yet no significant reduction in the t-Me/*N*-Me cross-peak is detected.[2] Spectra of mixed lipids with protiated headgroup and protiated alkyl chains should eliminate all intramolecular headgroup/alkyl chain cross-relaxation. The magnitude of the cross-peaks produced by this mixed lipid system suggests that a large portion of the magnetization-exchange is due to an intermolecular mechanism. A spin-diffusion mechanism seems less likely in this case. Thus, t-Me/*N*-Me cross-relaxation appears to occur by a more direct mechanism. At present, we cannot distinguish between a direct t-Me/*N*-Me exchange or an exchange mediated by limited spin-diffusion along the upper portion of the alkyl chains (i.e., on the polar side of carbons 7 and 8). In either case a portion of the t-Me and *N*-Me protons must spend time in relatively close proximity.

A close proximity between alkyl chain ends and the membrane-solution interface is suggested in other cross-relaxation spectra we have taken. Fig. 2 is shown as an example. Here ϕ_4P^+/t-Me proton cross-relaxation develops within 100 ms without any indication that spin-diffusion has occurred. Because the free-energy for moving this ion into the bilayer center is ~21 kcal/mole, membrane-bound ϕ_4P^+ must be localized near the membrane interface (33). Thus, a close proximity of some t-Me protons with the membrane interface, in the presence of ϕ_4P^+, is strongly indicated in this spectrum.

Lipid interdigitation and alkyl chain bends are two events that terminate chain ends near the vesicle surface. These configurations of lipid could account for the data seen here. They are not necessarily unexpected since they would reduce the packing constraints placed on lipids inserted into membranes with a small radius of curvature. Because it is difficult to predict the cross-relaxation efficiency for these head-tail associations, it is not possible to accurately estimate the fraction of t-Me groups that might be present in interdigitated or bent chain configurations from our data. If we assume a similar cross-relaxation efficiency for the t-Me/*N*-Me groups, as is seen for protons at the ends of the alkyl chains, then the magnitude of peak 3 could be accounted for by a few percent of the alkyl chain ends.

While the interpretations given here must certainly be viewed as preliminary, they are nonetheless extremely intriguing. The packing asymmetry, suggested by the efficiency of proton cross-relaxation, and the t-Me/*N*-Me exchange are consistent with predictions made by statistical-mechanical treatments of lipid dynamics and conformation (14–16).

One final aspect of the data that deserves comment here is simply the appearance of the strong *N*-Me/methylene cross-peaks, peaks 4 and 5. They apparently do not result from spin-diffusion through the lipid headgroup. This implies a close proximity between the *N*-Me headgroup and some portion of the methylene chains.

In conclusion, two-dimensional cross-relaxation spectroscopy provides a means to examine lipid packing asymmetry in small lipid vesicles. Differences in lipid packing both at the membrane-solution interface and in the membrane interior are apparent. While information on proximity must be viewed with caution due to the possibility of spin-diffusion in lipid systems, a close proximity of headgroup and chain ends in a population of phospholipid is indicated in our experimental observations on DPPC vesicles. This suggests that cross-relaxation may also provide information on lipid interdigitation or alkyl chain bending events in fluid phase vesicle systems. We are currently carrying out experiments to investigate these possibilities.

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[2]The deuterated 7 and 8 carbons of each lipid chain may not lie at the same level in the bilayer; therefore, some cross-relaxation between protons on either side of this deuterated region is possible. In any case, this deuterated region should significantly reduce magnetization transfer via spin-diffusion along the alkyl chain.

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