# Phospholipid Headgroup–Headgroup Electrostatic Interactions in Mixed Bilayers of Cardiolipin with Phosphatidylcholines Studied by $^2$H NMR†

Teresa J. T. Pinheiro, Andrzej A. Duralski, and Anthony Watts*

*Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.*

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ABSTRACT: The headgroup–headgroup interactions in binary mixed bilayers of diacylphosphatidylcholines (PC) and cardiolipin were analyzed by $^2$H NMR. Specific changes in the quadrupole splittings of the choline headgroup deuterated PC at $\alpha,\beta$-methylenes, and $\gamma$-methyls are observed upon the insertion of the negatively charged tetraacylphospholipid, cardiolipin. The effects are consistent with an electrostatic interaction between PC and cardiolipin headgroups, in which a concerted conformational reorientation of the entire phosphocholine moiety toward the membrane surface is involved. On the basis of the "choline-tilt" model by Macdonald and co-workers (1991) the variations in the quadrupole splittings are consistent with a change in orientation of the choline P–N vector up to 23° for the highest cardiolipin concentrations. Additional information on headgroup conformational changes was obtained through the analysis of the dependence on temperature of the quadrupole splittings for the various deuterium-labeled segments. Evaluation of the deuterium spin-lattice ($T_1$) relaxation times for the deuterons in the various positions of the choline headgroup in mixed bilayers of PC and cardiolipin showed that the internal fast segmental motions were not affected on addition of cardiolipin to PC membranes.

Lipid headgroups are located in the interface between the hydrophobic interior of membranes and the hydrophilic aqueous environment. The chemical structure of the lipid headgroup determines important physical properties of the membrane, such as its surface charge density, which can modulate the molecular interactions with components in the aqueous phase. Diacylphosphatidylcholine (PC)[1] is one the most common phospholipids in membranes. It carries a zwitterionic headgroup, phosphocholine, which has a large electric dipole moment of about 19 D (Shepherd & Büldt, 1978). $^2$H NMR of headgroup deuterated phospholipids provides a very specific approach to investigate the conformational and dynamic properties of the mechanisms underlying the headgroup interactions with other molecules. In particular, $^2$H NMR has been used to study the interaction of deuterated choline headgroups in PC bilayers with metal ions (Hauser *et al.*, 1976; Brown & Seelig, 1977; Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Macdonald & Seelig, 1987a,b), anionic and cationic amphiphiles (Altenbach & Seelig, 1985; Scherer & Seelig, 1989), charged local anaesthetics (Boulanger *et al.*, 1981; Browning & Akutsu, 1982), chaotropic anions (Macdonald & Seelig, 1988), and charged peptides (Sixl & Watts, 1985; Roux *et al.*, 1989; Kuchinka & Seelig, 1989), as well as by mixing phosphatidylcholines with negatively charged lipids (Sixl & Watts, 1983; Scherer & Seelig, 1987).

Biological membranes are usually composed of different phospholipid types, which are determined by the chemical nature of their polar headgroups. It is, therefore, of great importance to understand the characteristics of the lipid–lipid interactions in a bilayer structure, in particular those involving the lipid headgroups. In the present study we have confined our analysis to the headgroup–headgroup interactions in mixed membranes of diacylphosphatidylcholines (PC) with the tetraacylphospholipid cardiolipin, using $^2$H NMR of deuterons specifically placed in the PC choline headgroup. On addition of the negatively charged cardiolipin to electrically neutral PC membranes, a conformational change occurs in the PC choline headgroup, producing dramatic changes in the $^2$H NMR spectra. The changes in the quadrupole splittings provide information on the nature of the headgroup–headgroup interaction, and by application of a suitable model the conformational perturbations in the choline headgroup induced upon the insertion of cardiolipin in PC membranes were evaluated in terms of the tilt angle of the choline headgroup P–N vector with respect to the membrane surface. Analysis of the temperature dependence of the quadrupole splittings provides further insight on the PC choline headgroup conformation in mixed bilayers with cardiolipin, and the dynamic characteristics were investigated with deuterium spin–lattice ($T_1$) relaxation measurements.

*The "Choline-Tilt" Model.* It has been established from deuterium NMR studies that the headgroup of phosphatidylcholine undergoes a conformational change in response to surface charge (Seelig *et al.*, 1987, and references therein). The origin of this charge may be external to the membrane, as in the case of binding of surface ligands, like divalent cations and peptides (Brown & Seelig, 1977; Akutsu & Seelig, 1981; Roux *et al.*, 1988), or internal to the membrane as in the case of mixing charged phospholipids and amphiphiles (Sixl & Watts, 1983,1985; Roux *et al.*, 1989). The data indicate that the $^-$P–N$^+$ electric dipole moment of the choline headgroup is the critical property responsible for the sensitivity to charge. This dipole reorients itself in response to changes in the surface charge density, and in the cases where the headgroup dipole moment is large, such as for the phosphocholine moiety in PC molecules which is about 19 D (Shepherd & Büldt, 1978), headgroup tilts can be treated as dipole tilts (Macdonald *et al.*, 1991). This effect has been referred to as the "molecular voltameter" and proves to be valid in all cases involving

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* To whom correspondence should be addressed.

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[1] Abbreviations: CL, cardiolipin; PC, diacylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine.

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deposition of charges at the membrane surface. Independent of the chemical nature of the compounds involved, the same qualitative effect on the phosphocholine headgroup is observed as long they carry an electric charge of the same sign. In summary, addition of positive charges to the PC membrane surface moves the $N^+$ end of the dipole toward the water phase, changing the orientation of the $^-P–N^+$ vector up to 30° at the highest charge concentrations (Scherer & Seelig, 1989), while negative charges at the membrane surface have the opposite effect, forcing the $N^+$ terminus toward the membrane interior.

The basis of the "choline-tilt" model rests on the sensitivity of the $^2H$ NMR spectrum to the molecular conformation as expressed by the equation (Macdonald *et al.*, 1991)

$$\frac{\Delta\nu_Q^i}{\Delta\nu_Q} = \left|\frac{1}{2}(3\cos^2\phi_i - 1)S_f\right| \quad (1)$$

where $\Delta\nu_Q^i$ is the quadrupole splitting at the choline deuterium labelled position $i$, $\Delta\nu_Q \approx 125$ kHz is the quadrupole splitting associated with a CD bond in the absence of motion, $\phi_i$ is an average angle between the CD bond vector and the axis of motional averaging (normal to the membrane surface), and $S_f$ is an order parameter representing the degree of wobbling about $\phi_i$. Equation 1 is valid for membrane lipids undergoing rotation about their long axis at rates faster than the $^2H$ NMR time scale of $10^{-5}$–$10^{-6}$ s. This is the case for lipids in liquid-crystalline bilayers. At slower motional rates there is an appreciable loss of sensitivity of the $^2H$ NMR spectrum to the molecular conformation (Spiess, 1985). In the "intermediate" time scale regime ($10^{-5}$–$10^{-3}$ s) losses in the intensity and spectral line shape distortions occur in the $^2H$ NMR spectrum, and eq 1 is no longer applicable.

Using the quantitative interpretation for the headgroup quadrupole splittings by Bloom (Roux *et al.*, 1989), a quantitative description of the "choline-tilt" or "molecular voltameter" model has been recently presented by Macdonald and co-workers (1991) that can describe the major features of the response of the quadrupole splittings to surface charge. We will recall here some the fundamental equations of this model which have been used in our calculations. Based on the premise that charges exert a torque on the choline headgroup, and considering the relationships between the electrical field at the membrane surface, the angle of tilt of the choline headgroup, and the geometry of the CD bond vectors in the choline moiety, the dependence of the choline quadrupole splittings on the surface charge density can be summarized in the following expression:

$$\Delta\nu_Q^i = \left|\frac{1}{2}\{3[(1 + C^2)^{-1/2}\cos\varphi_i]^2 - 1\}S_f\right|\Delta\nu_Q \quad (2)$$

The subscripts $i$ refer to the deuterium-labeled position $\alpha$ or $\beta$ of the choline headgroup; $\varphi_i$ is the angle between the CD bond vector and $y$-axis in the coordinate frame presented by Macdonald *et al.* (1991);[2] $S_f$ and $\Delta\nu_Q$ are as defined previously, and $C$ is a function of the membrane surface charge density ($\sigma$) according to

$$C = \frac{\sigma AB^{-1} + \cos 60}{\sin 60} \quad (3)$$

[2] In this paper this angle is referred to as $\beta$, which we have replaced by $\varphi$ to avoid confusion with $\alpha,\beta$ nomenclature for the choline headgroup segments.

where $\sigma A = 1.165 \times 10^{-20}$ J for a 100% charged lipid membrane, and $B$ is taken to be $6.95 \times 10^{-21}$ J. The density of charge at the surface ($\sigma$) of mixed membranes of PC with a negatively charged phospholipid can be estimated according to

$$\sigma = \frac{e_0 z_n X_n}{A(1 + PX_n + X_n)} \quad (4)$$

where $e_0$ is the elementary charge, $A$ is the cross-sectional area for the PC molecule, which is approximately 68 Å$^2$, $P$ is the ratio of the surface areas of negative to neutral lipid, and $X_n$ is the mole fraction of negative charged lipid of charge $z_n$.

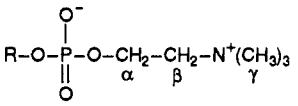
The angles $\varphi_i$ and $\phi_i$ can be obtained by using the approach described in Macdonald *et al.* (1991). Here, as a first approximation it is assumed that the CD bond vector lies perpendicular to the headgroup P–N vector which leads to a simplified relation (eq 5) between the tilt angle for the choline

$$\cos\varphi_i \sin\theta = \cos\phi_i \quad (5)$$

headgroup P–N vector with respect to the membrane surface normal ($\hat{u}$) and the angle $\phi_i$ between the CD bond vector and $\hat{u}$. For a neutral PC membrane the choline headgroup assumes a conformation in which the P–N vector lies nearly parallel (within 30°) to the plane of the membrane (Büldt *et al.*, 1978). This equilibrium position represents the situation at which all forces, including intra- and intermolecular contributions, are averaged to zero. According to conformational calculations by Pullman and Saran (1975) at neutrality ($\sigma = 0$), $\theta$ is found to be 60°.

## EXPERIMENTAL PROCEDURES

*Materials.* Beef heart cardiolipin and diacylphosphatidylcholines were purchased from Sigma Chemical Co., St. Louis, MO, and used without further purification. The following nomenclature is employed for the headgroup segments in phosphatidylcholines:




1,2-Dimyristoyl- or 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine (DMPC or DOPC, respectively) was specifically deuterated at the $\alpha$- and $\beta$-methylenes (DMPC-$d_4$ or DOPC-$d_4$) or at the $\gamma$-methyls (DOPC-$d_9$) in the choline headgroup. DOPC-$d_9$ was synthesized by methylation of DOPE with deuterated methyliodide as described by Eibl (1978). DMPC-$d_4$ were synthesized similarly by methylation with methyliodide of DMPE-$d_4$ which was synthesized as described in Sixl and Watts (1982). DOPC-$d_9$ was purified by crystallization from acetone at –20 °C. DMPC-$d_4$ and DOPC-$d_4$ were purified by HPLC on a LiChroprep Si 60 column which was eluted with chloroform–methanol–ammonia solvent of successively increasing polarity and then by crystallization as for DOPC-$d_9$.

*Sample Preparation.* A solution containing 10–25 mg of deuterated phosphatidylcholine alone or in binary mixtures with cardiolipin at the desired molar stoichiometry was prepared in chloroform/methanol (1:1, v/v). A lipid film was then formed by rotatory evaporation and left under high vacuum for a minimum of 8 h, to remove all traces of the organic solvent. Multilamellar liposomes were prepared by hydration with equivalent total lipid weight (10–25 $\mu$L) of 20 mM cacodilate buffer at pH 6.0 containing 0.1 M NaCl and

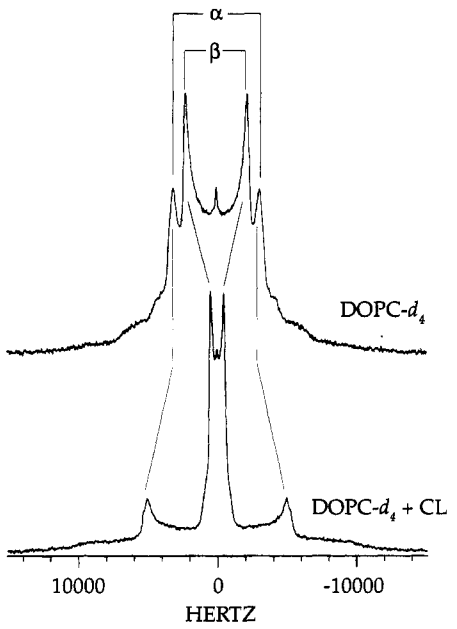



FIGURE 1: 55.3-MHz deuterium NMR spectra of DOPC-$d_4$ in hydrated bilayers alone and in mixed bilayers with cardiolipin (CL) in the molar ratio DOPC-$d_4$/CL of 4:1; temperature 35 °C.

5 mM EDTA, followed by gentle vortexing and six cycles of freeze-thawing. Lipid hydration was carried out under a nitrogen atmosphere, using predeoxygenated buffer to prevent oxidation of the unsaturated fatty acyl chains within the lipids.

*NMR Measurements.* $^2$H NMR spectra were recorded on a home built 360 MHz ($H_0$ = 8.4 T) spectrometer, equipped with a Nicolet pulse programmer and operating at 55.3 MHz for the $^2$H nucleus. Single pulse modes were used for the $^2$H NMR spectra with $\pi/2$ pulse widths varying from 7 to 9 $\mu$s. Deuterium spin–lattice ($T_1$) relaxation times were measured using a standard inversion–recovery pulse sequence ($\pi_x$–$\tau$–$\pi/2_y$–*acquisition*–$D_0$), where the recycle time ($D_0$) was at least $5T_1$. The NMR spectrometer was equipped with a nitrogen gas flow variable temperature unit. On the variable temperature measurements the samples were equilibrated for periods of 20 min prior to spectral acquisition, and the temperature was stable to an accuracy of ±0.5°.

## RESULTS AND DISCUSSION

*Headgroup–Headgroup Electrostatic Interactions.* The deuterium NMR spectrum of DOPC-$d_4$ in fully hydrated bilayers at 35 °C is shown in Figure 1 (upper spectrum). This line shape is a characteristic powder pattern spectrum for a spin-1 nucleus and typically observed with headgroup deuterated phospholipids in randomly oriented bilayers in the liquid-crystalline ($L_\alpha$) phase. The spectral line shape is dominated by an axially symmetric electric field gradient tensor (EFG), where the intense resonance lines are originated from the molecules whose $^2$H EFG tensors have their unique axes oriented at 90° relative to the applied magnetic field (Seelig, 1977). The separation between these maxima provides a direct measure of the quadrupole splitting ($\Delta\nu_Q$). At 25 °C the $\alpha$ and $\beta$ deuterons in the choline headgroup of DOPC-$d_4$ in $L_\alpha$ bilayers exhibit a quadrupole splitting of 6.4 and 4.9 kHz, respectively. The assignment of the quadrupole splittings has been previously reported by Gally *et al.* (1975) for hydrated bilayers of DPPC. The $^2$H NMR spectrum of DOPC-$d_4$ is substantially altered in mixed bilayers with cardiolipin (CL), as shown in Figure 1 for a binary mixture in the molar ratio CL:DOPC-$d_4$ of 1:4. The $\alpha$-$CD_2$ quadrupole splitting

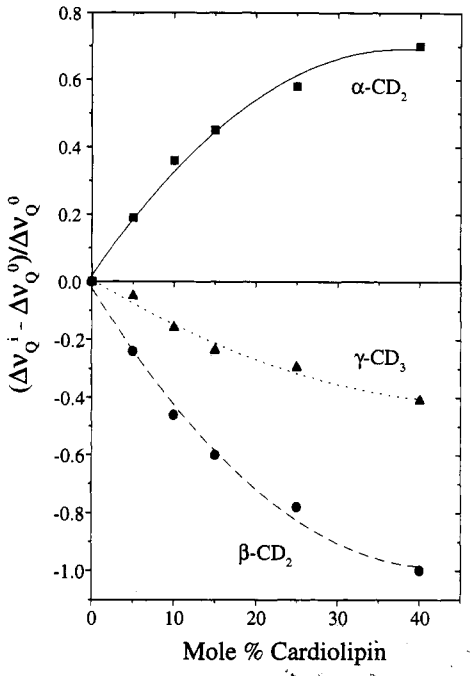



FIGURE 2: Counter-directional charge effect exerted by cardiolipin on the quadrupole splittings ($\Delta\nu_Q$) of headgroup-deuterated phosphatidylcholine in hydrated bilayers at 20 °C. $\alpha$-$CD_2$ and $\beta$-$CD_2$ in DOPC-$d_4$, and $\gamma$-$(CD_3)_3$ in DOPC-$d_9$. The quadrupole splittings for the various concentrations of cardiolipin in binary mixtures with DOPC ($\Delta\nu_Q^i$) are normalized with respect to the value of the same deuterated segment in pure DOPC bilayers, i.e., 0% cardiolipin ($\Delta\nu_Q^0$).

increases from 6.4 kHz in the pure DOPC-$d_4$ bilayers to 9.7 kHz in the mixed bilayers with cardiolipin. In contrast, $\Delta\nu_Q$ for the $CD_2$ segment decreases from 4.9 to 1.0 kHz. The effects on the quadrupole splitting for the $\gamma$-methyls were analyzed here in mixtures of DOPC-$d_9$ with cardiolipin. The $\gamma$ splitting also decreases with increasing concentrations of cardiolipin. This counter directional change in the size of the quadrupole splittings from $\alpha$-methylene to $\beta$-methylene and $\gamma$-methyls in the choline headgroup is clearly illustrated in Figure 2. Here the quadrupole splittings have been normalized with respect to the value for the pure PC membrane (0% cardiolipin) for each deuterium-labeled position. A progressive variation in $\Delta\nu_Q$ for all the deuterated segments is observed with increasing negative surface charge density, i.e., increasing concentration of cardiolipin. This variation is close to linear up to cardiolipin concentrations in the order of 15 mol %, and at higher concentrations seems to approach a limiting value characteristic of the particular deuterium-labeled segment (Figure 2). In the approximately linear concentration range, the data can be summarized by the relation

$$\Delta\nu_Q^i(X_{CL}) = \Delta\nu_Q^i(0) - mX_{CL}\ \text{kHz} \qquad (6)$$

where $\Delta\nu_Q^i(X_{CL})$ is the quadrupole splitting for the choline headgroup segment $i$ at a given mole fraction of cardiolipin, $X_{CL}$, and $\Delta\nu_Q^i(0)$ is the quadrupole splitting for the corresponding segment at $X_{CL} = 0$. The $m$ values for each choline segment, as derived from a linear regression analysis of the experimental data plots $\Delta\nu_Q^i$ ($X_{CL}$) *versus* $X_{CL}$ in the linear concentration range, were found to be $m_\alpha$ = +20.2 kHz, $m_\beta$ = –20.2 kHz, and $m_\gamma$ = –2.2 kHz. Scherer and Seelig (1989) have reported values of $m_\alpha$ = +15.1 kHz and $m_\beta$ = –13.9 kHz for mixtures of a negatively charged amphiphile (didodecylphosphate) to POPC bilayers, which are also similar to those found in mixed bilayers of phosphatidylcholine with phosphatidylglycerol (Macdonald & Seelig, 1987). The slopes

$m$ encountered here for PC/CL mixtures are higher, which may suggest a higher sensitivity for the response of the choline headgroup to the introduction of cardiolipin into PC membranes. Additionally, the symmetry between $m_\alpha$ and $m_\beta$ values is noteworthy. The different steric hindrances exerted at the different headgroup positions in the choline moiety in a membrane structure may contribute for the relation $m_\beta \neq m_\alpha$. If so, these steric hindrances appear to be absent in the mixed PC/CL bilayers. The glycerotetraacyllipid, cardiolipin, has a very small headgroup and a cross-sectional molecular area twice as larger than that of the glycerodiacyllipid PC molecule. Therefore, the insertion of cardiolipin molecules in PC membranes increases the free volume for the choline headgroup. This may be transmitted into small steric hindrances to the phosphocholine headgroup reorientation upon the charge effects imposed by cardiolipin.

At pH 6.0 cardiolipin carries two negative charges per molecule in two ionized phosphate groups. When cardiolipin is inserted into the zwitterionic PC bilayers (an electrically neutral membrane), the surface charge density is altered becoming a negatively charged membrane. The ionised phosphate groups in the cardiolipin headgroup attract (repel) the $N^+$ ($PO_4^-$) terminus of the phosphocholine moiety in the PC molecules, which results in a concerted conformational reorientation of the entire phosphocholine moiety, thereby inducing the dramatic changes in the deuterium quadrupole splittings observed here (Figures 1 and 2). At a first approximation, the amplitude of the torque exerted in the P–N vector depends on the balance between the repulsive and attractive forces above described. However, other steric factors may also contribute to the final extent of headgroup conformational perturbation, including intra- and intermolecular constraints imposed by the packing arrangement in a bilayer structure.

The direction of the changes observed in the quadrupole splittings of the choline headgroup segments upon the insertion of cardiolipin ($\alpha$-$\Delta\nu_Q$ increases, while $\beta$- and $\gamma$-$\Delta\nu_Q$ decrease; Figure 2) is opposite to that observed with metal ions or positively charged amphiphiles (Seelig *et al.*, 1987). A further demonstration of the response of the choline headgroup to surface charges is shown in Figure 3, where correlation plots of the quadrupole splittings from each of the choline headgroup deuterium-labeled position as a function of the quadrupole splitting from the other labeled positions are presented. In all cases a linear correlation was observed, and in particular the $\alpha$–$\beta$ correlation plot shows a slope of –1.05 in excellent agreement with reported values for deposition of negative charges at the PC membrane surface (Macdonald & Seelig, 1987). The $\alpha$–$\gamma$ and $\beta$–$\gamma$ correlation plots have similar slopes on the order of 0.1, but of opposite sign (Figure 3).

We will apply the "choline-tilt" model of the "molecular voltameter" briefly introduced earlier for the dependence of the $\alpha$ and $\beta$ quadrupole splittings on surface charge density to analyze the charge effects on phosphocholine headgroup upon the insertion of CL into PC membranes. The surface charge density ($\sigma$) of mixed PC/CL bilayers for the various cardiolipin concentrations ($X_{CL}$) was calculated using eq 4, where the ratio $P$ of the surface areas of negative (cardiolipin) to neutral (PC) lipid was taken to be approximately 2; the cross-sectional area for the PC molecule $A$ = 68 Å$^2$; and $z_n$ = $z_{CL}$ = –2. The $\sigma$ values are listed in Table 1 for the corresponding mole fractions of cardiolipin ($X_{CL}$). The $\phi_i$ angles for the $\alpha$ and $\beta$ choline segments (Table 1) were estimated from eq 1 by using the experimental values $\Delta\nu_Q^i$ obtained at various cardiolipin concentrations ($X_{CL}$) in the mixed PC/CL membranes, $\Delta\nu_Q$ = 125 kHz, and $S_f$ was taken to be 0.25 for both $\alpha$ and $\beta$ positions of the choline headgroup, according to Macdonald *et al.* (1991). The values for cos $\varphi_i$, also shown in Table 1, have been determined applying eq 2, in which $C$ was calculated for the various $\sigma$ values according

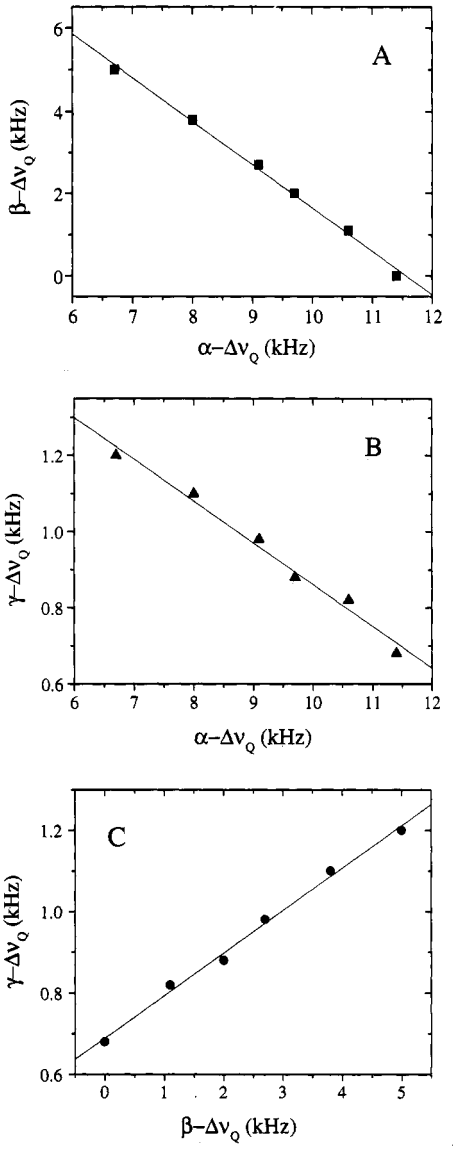



FIGURE 3: Correlation plots for the quadrupole splittings ($\Delta\nu_Q$) of choline headgroup segments of DOPC in mixed bilayers with cardiolipin at various proportions. The quadrupole splitting from a particular deuterium-labeled position for each cardiolipin concentration is plotted as function of the value measured for another labeled position in the same cardiolipin mixture. (A) $\alpha$–$\beta$, (B) $\alpha$–$\gamma$, and (C) $\beta$–$\gamma$ correlations.

Table 1: Parameters for the Choline Headgroup in Mixed PC/CL Membranes at Various Cardiolipin Mole Ratios ($X_{CL}$) Assuming the "Choline-Tilt" Model of the "Molecular Voltameter"[a]

| $X_{CL}$ | $\sigma$ (C/m$^2$) | $C$ | cos $\phi_{\alpha(\beta)}$ | cos $\varphi_{\alpha(\beta)}$ | $\theta$ (deg) |
|---|---|---|---|---|---|
| 0.05 | $-20.5 \times 10^{-3}$ | 0.409 | 0.710 (0.643) | 0.767 (0.695) | 67.8 (67.7) |
| 0.10 | $-36.2 \times 10^{-3}$ | 0.280 | 0.726 (0.625) | 0.754 (0.649) | 74.3 (74.4) |
| 0.15 | $-48.7 \times 10^{-3}$ | 0.177 | 0.735 (0.613) | 0.747 (0.622) | 79.7 (80.2) |
| 0.25 | $-67.3 \times 10^{-3}$ | 0.024 | 0.747 (0.598) | 0.748 (0.602) | 84.0 (83.4) |
| 0.40 | $-85.7 \times 10^{-3}$ | –0.127 | 0.760 (–)[b] | 0.766 (–)[b] | 82.8 (–)[b] |

[a] Full descriptions of the calculations are given in the text. In double columns the first value refers to the segment $\alpha$, and the values in parentheses are for the $\beta$ segment. For the tilt angle $\theta$ of the choline headgroup P–N vector with respect to the bilayer normal, also two values are listed on the basis of the calculations from the $\alpha$ and $\beta$ segments, accordingly. [b] At this cardiolipin concentration $\beta$–$\Delta\nu_Q$ = 0.

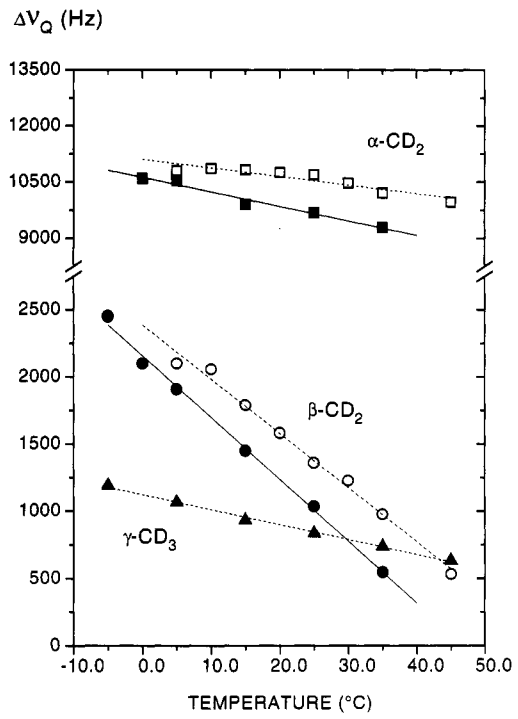



FIGURE 4: Temperature dependence of the quadrupole splitting ($\Delta\nu_Q$) for the headgroup-deuterated segments of phosphatidylcholines (PC) in mixed bilayers with cardiolipin (CL) in the molar ratio PC/CL = 4:1. (Squares) $\alpha$-methylene deuterons in PC-$d_4$; (circles) $\beta$-methylene deuterons in PC-$d_4$; (triangles) $\gamma$-methyls in PC-$d_9$; (open symbols) saturated acyl chains lipid, DMPC; and (filled symbols) unsaturated analogue, DOPC.

to eq 3. The tilt angle $\theta$ for the choline P–N vector induced upon the insertion of cardiolipin into PC membranes was then calculated using eq 5. The conformational reorientation of the choline headgroup is consistent with the predicted effects of deposition of negative charges at the PC membrane surface. The tilt angles $\theta$ of the choline P–N vector with respect to the bilayer normal increased gradually from $\theta = 60°$ for neutrality ($X_{CL} = 0$) with the increase in negative surface charge density, i.e., increasing amounts of cardiolipin (Table 1). The response of the quadrupole splittings for the choline headgroup segments upon the insertion of cardiolipin into PC membranes has been found to follow the "choline-tilt" model, whereby a gradual reorientation of the choline P–N vector up to 23° toward the membrane surface is induced by cardiolipin at the highest concentrations analyzed here ($X_{CL} = 0.40$).

*Conformation and Dynamics of the Phosphocholine Headgroup.* The quadrupole splitting of headgroup deuterated phosphatidylcholines in fully hydrated membranes decreases from the $\alpha$-methylene to the $\gamma$-methyls. At 35 °C, $\alpha$-$\Delta\nu_Q$ = 6.2 kHz, $\beta$-$\Delta\nu_Q$ = 4.7 kHz, and $\gamma$-$\Delta\nu_Q$ = 1.0 kHz for the choline headgroup segments of DOPC in hydrated bilayers. $\Delta\nu_Q$ reflects the order and/or conformation of the CD segment, i.e., the amplitude of motion of the deuterated group, which is successively larger toward the outer most segments. The temperature dependence of the quadrupole splittings of $\alpha$-$CD_2$ and $\beta$-$CD_2$ segments of DMPC or DOPC in mixed bilayers with cardiolipin are presented in Figure 4. The $\beta$-quadrupole splitting shows the largest variation with temperature, while $\alpha$ and $\gamma$ splittings vary little over the temperature range analyzed here. A temperature coefficient ($\mathcal{T}$) may be defined as $\mathcal{T} = \Delta(\Delta\nu_Q)/\Delta T$, and, for DMPC and DOPC bilayers alone, $\mathcal{T}$ for the $\beta$ splitting was found to be 78 and 60 Hz/deg, respectively (Pinheiro, 1993). Similar values have been reported for DPPC bilayers (Gally *et al.*, 1975; Akutsu & Seelig, 1981) and POPC bilayers (Altenbach & Seelig, 1984), and it has been suggested that the structural reorganization of the choline headgroup due to temperature may only involve a change in the average torsion angles of the $C_\alpha$–$C_\beta$ bond. On insertion of cardiolipin to DOPC or DMPC bilayers a 2-fold increase is observed for the temperature coefficient $\mathcal{T}$ of the $\alpha$-quadrupole splitting, when compared with the values in the pure PC bilayers; whereas for the $\beta$ splitting a reduction in $\mathcal{T}$ of 25 and 45% is observed for the DOPC and DMPC bilayers, respectively. Those changes suggest that on insertion of cardiolipin into PC bilayers the temperature effects may also involve changes in the torsion angles of the O–$C_\alpha$ bond. Additionally, the different $\mathcal{T}$ values for the $\beta$ splitting in the two analogues (DMPC and DOPC) may arise from possible differences in the packing properties and free volume for these two saturated and unsaturated lipid analogues. Nevertheless, in both cases a common effect is the enhanced sensitivity of the $\alpha$-quadrupole splitting to temperature. This suggests that in addition to the conformational changes induced by the electrostatic interaction between PC and cardiolipin headgroups, dynamic changes also occur possibly driven by steric factors. In particular, the insertion of cardiolipin in PC bilayers can be visualized as a spacer for the choline headgroup. While a PC molecule occupies a cross-sectional area of approximately 68 Å, cardiolipin takes twice as much this area, and due to its small headgroup additional free volume at the bilayer surface is available for the choline headgroup.

The spin–lattice ($T_1$) relaxation for all the deuterated segments in the choline headgroup of PC in the mixed bilayers with cardiolipin decreases with decreasing temperature, approaching a $T_1$ minimum at about 0 °C for the $\alpha$ and $\beta$ positions of the choline headgroup (Figure 5A), and at about −15 °C for the $\gamma$ position (Figure 5B). This indicates that for the higher temperatures the local molecular motions are in the fast correlation time limit ($\omega_0\tau_c \ll 1$) (Abragam, 1961). In this regime, assuming that the fast motions provide the dominant mechanism for the deuterium $T_1$ relaxation, a single effective motional correlation time ($\tau_c$) for the CD fast segmental motions can be calculated according to

$$\frac{1}{T_1} = \frac{3\pi^2}{2}\left(\frac{e^2qQ}{h}\right)^2\tau_c \qquad (7)$$

where $e^2qQ/h$ is the static quadrupole coupling constant, which has a value of 170 kHz for an $n$-$sp^3$ hybridized CD bond (Davis, 1983). At 35 °C, $\tau_c$ for the fast motions of $\alpha$-, $\beta$-, and $\gamma$-CD segments in the choline headgroup of DOPC in the mixed bilayers with cardiolipin is $9 \times 10^{-11}$, $7 \times 10^{-11}$, and $3 \times 10^{-11}$ s, respectively. These values are equivalent of those observed for the same segments in the pure bilayers of DOPC alone (Pinheiro, 1993), which indicates that the rates ($\tau_c^{-1}$) of the internal conformational changes around the –$C_\alpha$–$C_\beta$–N–$C_\gamma$ bonds in the choline headgroup are not affected upon the insertion of cardiolipin into PC bilayers. Identical behavior has been reported for the DMPC-$d_4$ in mixtures with DMPG (Sixl & Watts, 1982), where less than 10% decrease in the correlation rates for the choline fast segmental motions was observed upon the addition of 50 mol % of DMPG to DMPC bilayers. From the gradients of Arrhenius plots of ln $T_1$ *versus* 1/temperature (K) the activation energies ($E_A$) for the motion of each headgroup segment were evaluated. $E_A$ values in the range of 26 ± 4 kJ mol$^{-1}$ were found for all the deuterated segments in DOPC or DMPC in mixed bilayers with cardiolipin, which were identical to the values for the pure PC membranes (Pinheiro, 1993).

Alternatively, at around the temperature of the $T_1$ minimum ($\omega_0\tau_c \approx 1$) a motional correlation time ($\tau_c$) can be estimated

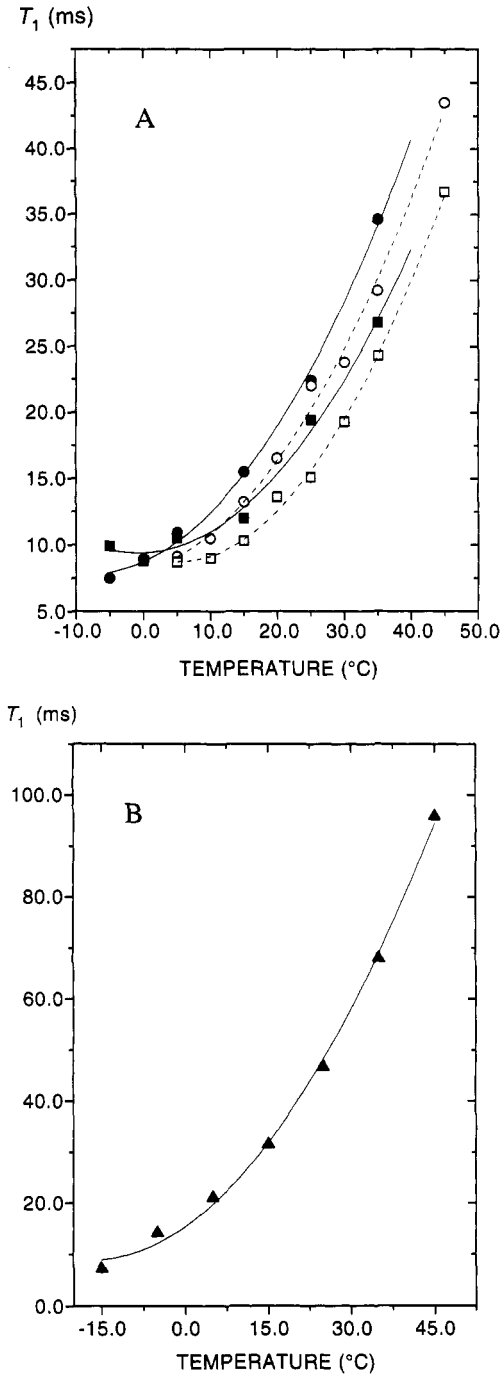



FIGURE 5: Temperature dependence of the deuterium spin–lattice ($T_1$) relaxation times for the various segments in the headgroup of phosphatidylcholines (PC) in mixed bilayers with cardiolipin (CL) at the molar ratio PC/CL = 4:1. (A) $\alpha$-methylene deuterons (squares) and $\beta$-methylene deuterons (circles) in DMPC-$d_4$ (open symbols) or in DOPC-$d_4$ (filled symbols), and (B) $\gamma$-methyls in DOPC-$d_9$.

by $\tau_c \approx \omega_0^{-1}$. At the $^2H$ resonance frequency of the present study, $\omega_0 = 2\pi \times 55.3$ MHz, a single effective motional correlation time for the CD segments at the temperature of the minimum is of the order of a few nanoseconds ($\sim$2.88 ns). In this regime, contributions from the overall molecular reorientations, $10^{-9} \leq \tau_c \leq 10^{-7}$ s (Mayer *et al.*, 1988), are more likely to become predominant over the faster intramolecular motions ($10^{-12} \leq \tau_c \leq 10^{-10}$ s). Unfortunately, completely well defined $T_1$ minima for the various systems studied here are not available (Figure 5), as at lower temperatures freezing of the water at the membrane surface and the proximity of the lipid phase transition would invalidate the $T_1$ analysis as a function of temperature. Nevertheless, it is noteworthy the rare observation of approaching a deuterium $T_1$ minimum for the headgroup deuterated phospholipid membranes.

## CONCLUSIONS

The counter-directional response of the quadrupole splittings of the deuterium-labeled choline headgroup segments to the insertion of cardiolipin into PC membranes is consistent with an headgroup–headgroup electrostatic interaction between cardiolipin and phosphatidylcholine. The negative charges in the cardiolipin headgroup attract (repel) the $N^+$ ($PO_4^-$) terminus of the phosphocholine headgroup, which induces a concerted conformational reorientation of the entire phosphocholine segment. Application of the "choline-tilt" model of the "molecular voltameter" proposed by Macdonald *et al.* (1991) revealed that on addition of 40 mol % of cardiolipin the headgroup choline P–N vector moves toward the membrane surface by about 23°. This value should be interpreted as an average deviation from the average orientation at around 60° with respect to the bilayer normal in a neutral membrane surface (100% PC membrane).

The analysis of the temperature dependence of the quadrupole splittings of the various choline headgroup segments provided some additional information on the average headgroup conformation. While for pure PC membranes the temperature profile of the $\Delta\nu_Q^i$ has been suggested to involve changes in the average torsion angles of the $C_\alpha$–$C_\beta$ bond, on mixed membranes with cardiolipin the temperature seems to involve also changes on the average torsion angles of the O–$C_\alpha$ bond. This is consistent with the reorientation of the entire headgroup choline P–N vector induced upon the electrostatic interaction with the negative charged cardiolipin headgroup as described above. Alternatively, arguments based on the larger free volume available for the phosphocholine moiety in the mixed PC/CL membranes compared with that in the pure PC bilayers, may as well explain the changes in the averaged conformation of the innermost segments of the choline headgroup around the O–$C_\alpha$ bond.

On the basis of the assumption that the fast molecular motions are the dominant mechanism for the deuterium spin–lattice ($T_1$) relaxation of phospholipids in hydrated membranes, it was found that the fast internal segmental choline headgroup motions are all identical from the $\alpha$- to $\gamma$-segments, and those are not affected by the insertion of cardiolipin into PC membranes. Furthermore, the activation energies ($E_A$) for the internal headgroup rotations were also unchanged on addition of cardiolipin. In summary, the physical electrostatic headgroup–headgroup interactions between cardiolipin and PC is reflected into a molecular conformational reorientation of the entire choline moiety, and the fast segmental motions within the choline headgroup are not affected.

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