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The membrane potential has no detectable effect on the phosphocholine headgroup conformation in large unilamellar phosphatidylcholine vesicles as determined by ^{2}H-NMR

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In this study the effect of a transmembrane electrical potential on the phospholipid headgroup conformation was investigated using the ^{2}H-NMR technique. Large unilamellar vesicles were prepared of dioleoylphosphatidylcholine, specifically ^{2}H-labeled at the α- or β-position of the choline group. No conformational change of the phosphocholine headgroup could be detected after induction of a valinomycin-induced K^+-diffusion potential across the bilayer. However, this method could be used to measure the redistribution of tetraphenylphosphonium across the bilayer in response to $\Delta\psi$, which reorients the phosphocholine headgroups in the opposite bilayer–water interfaces.

Introduction

Many biological membranes experience a membrane potential ($\Delta\psi$). Membrane potentials are involved in many processes, such as channel function and insertion and translocation of proteins [1–6]. The way by which the membrane potential acts in these processes is largely unknown, but because of the variety of processes, it probably involves different mechanisms. One possibility is that the membrane potential has a direct effect on the phospholipids in the membrane, and in particular on the headgroup region, which determines the quality of the membrane–water interface. It was demonstrated by several groups that phospholipid headgroups are sensitive to electric charges on the membrane surface [7–10]. In this study we considered the possibility that the headgroup dipoles could be affected by changes in transmembrane potential.

To investigate this possibility, the effect of the membrane potential on the headgroup conformation of phosphatidylcholine, an abundant phospholipid in biological membranes, was studied using ^{2}H-NMR. The quadrupolar splitting ($\Delta\nu_Q$) of specifically headgroup deuterated phospholipids was shown to be sensitive to the conformation of the headgroup [7–10]. Using large unilamellar vesicles (LUVs) consisting of dioleoylphosphatidylcholine (DOPC), specifically deuterated at the α- or at the β-position of the choline headgroup, the conformation of the PC headgroup was studied in the absence and presence of a transmembrane electrical potential generated by valinomycin and a K^+-gradient across the bilayer.

The results presented in this study indicate that there is no measurable conformational change of the PC headgroup when a membrane potential of approx. 200 mV (negative inside) is present across the membrane. In contrast, the method is demonstrated to measure the redistribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the bilayer, in response to the membrane potential.

Materials and Methods

Phospholipids. Choline, specifically deuterated at the α-position, was synthesized by cleavage of *N,N*-dimeth-

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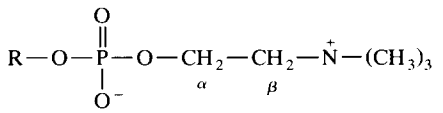
Abbreviations: $\Delta\psi$, membrane potential; NMR, nuclear magnetic resonance; $\Delta\nu_Q$, quadrupolar splitting; LUVs, large unilamellar vesicles; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; $DiSC_2$-(5), 3,3′-diethylthiadicarbocyanine iodide; TPP^+, tetraphenylphosphonium; TPB^-, tetraphenylborate.

ylglycineethylester (Sigma Chemical Co., St. Louis, MO) with $LiAlD_4$ (MSD isotopes, München, Germany), followed by methylation of the obtained *N,N*-dimethylethanolamine with CH_3I (Merck, Darmstadt, Germany), according to Harbison and Griffin [11]. Choline, specifically deuterated at the β-position, was synthesized by the same procedure, only the ester was deuterated by deuterium exchange with methanol-OD (Campro Scientific, Veenendaal, the Netherlands), prior to cleavage with $LiAlH_4$ (Sigma) instead of $LiAlD_4$. The deuterated cholines were identified by ^{1}H-NMR and ^{13}C-NMR, and subsequently coupled to 1,2-dioleoyl-*sn*-glycero-3-phosphate (Avanti Polar Lipids, Birmingham, AL) as described by Harbison and Griffin [11]. The obtained headgroup deuterated 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) was purified by silica column chromatography, with chloroform/methanol (9:1 v/v) as the first and chloroform/methanol/water (65:35:4, v/v/v) as the second eluents. The final products gave only one spot on silicagel thin layer chromatography (chloroform/methanol/water (65:35:4, v/v/v) and were obtained with a 80% overall yield, based on weight. The terms α- and β-deuterated DOPC (α-$^2H_2$- and β-$^2H_2$-DOPC) are used throughout this paper, and are defined as follows:



1,2-Dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) was purchased from Avanti Polar Lipids (Birmingham, AL). Phospholipid concentrations were determined according to Rouser et al. [12].

Sample preparation. Lipid films of 100 μmol were dried under high vacuum and subsequently hydrated in 1 ml buffer containing 150 mM K_2SO_4, 20 mM Hepes (pH 7.0; K^+-buffer) by manual shaking. From these multilamellar lipid dispersions large unilamellar vesicles (LUVs) were prepared by the extrusion technique [13], after 10 freeze–thaw cycles, using 400 nm polycarbonate filters. The diameter of these LUVs was determined by dynamic light scattering to be on average 244 nm (polydispersity factor 0.31). K^+_{in}/Na^+_{out} ion gradients were applied to the LUVs by replacing the external K^+-buffer by buffer containing 150 mM Na_2SO_4, 20 mM Hepes (pH 7.0; Na^+-buffer) by passing the LUVs through a prepacked Sephadex G25 column (9 ml; Pharmacia LKB, Uppsala, Sweden) equilibrated with Na^+-buffer. After this procedure the volume of the sample was 2.5 ml which was used for two NMR experiments. A membrane potential was generated by addition of valinomycin (Boehringer, Mannheim, Germany; 1 mg/ml in ethanol) to a 1:10^4 molar ratio with respect to phospholipid, to LUVs exhibiting a K^+_{in}/Na^+_{out} ion gradient. The presence of a membrane potential upon addition of valinomycin to LUVs, experiencing a K^+_{in}/Na^+_{out} ion gradient, was established using 3,3′-diethylthiadicarbocyanine iodide ($DiSC_2$-(5), Molecular Probes, Eugene, OR) as a membrane potential-sensitive fluorescent dye [14]. To collapse the membrane potential, gramicidin (Sigma; 2 mM in ethanol) was added to a 1:400 molar ratio with respect to phospholipid.

Titrations with TPP^+ or tetraphenylborate (TPB^-) (Janssen Chimica, Geel, Belgium; TPPCl 400 mM in Na^+-buffer and NaTPB 50 mM in Na^+-buffer) were carried out by adding increasing amounts of the ions to the vesicles up to 63 and 3 mM, respectively.

NMR. ^{2}H-NMR spectra were recorded at 46.1 MHz on a Bruker MSL-300 spectrometer at the indicated temperatures. A quadrupolar echo pulse sequence was used with a 12 μs 90° pulse and 50 μs delay between the pulses. The interpulse time was 100 ms and the spectral width 71.5 kHz. An exponential filtering resulting in a 150 Hz line broadening was applied to the cumulative free induction decays.

The isotropic signal which is present in all ^{2}H-NMR spectra results from the natural occurrence of ^{2}H in water and is not observed in the ^{31}P-NMR spectra of the same samples.

Results and Discussion

Usually, NMR experiments on orientational order in lipid bilayers are done, using aqueous multilamellar dispersions in order to reduce motional averaging due to vesicle tumbling and lateral diffusion of the lipids within the bilayer [15]. For our NMR experiments, the aim was to generate a well-defined and stable valinomycin-induced K^+-diffusion potential across the bilayer. This requires the use of unilamellar vesicles and conditions under which a stable potential and a defined quadrupolar splitting can be observed. We therefore used vesicles made by the extrusion technique [13], using filters with 400 nm pore size, which give rise to mainly unilamellar vesicles, which are large enough to obtain defined NMR spectra.

In order to find the optimal conditions for the experiments, the temperature dependence of the ^{2}H-NMR spectra of aqueous multilamellar dispersions and of LUVs, consisting of either α- or β-$^2H_2$-DOPC, was studied. The results are shown in Fig. 1. The spectra of the dispersions show the characteristic lineshapes with $\Delta\nu_Q$ values (distance between the two peaks) which decrease with increasing temperature. This effect is most pronounced for β-$^2H_2$-DOPC ($\Delta\nu_Q$ decreased from 6.2 to 4.1 kHz at 5 and 40°C respectively) comparable to previous observations by Altenbach and Seelig [16]. The main difference between the spectra of the LUVs and those of the dispersions is that the LUV spectra are broadened and especially at higher temper-

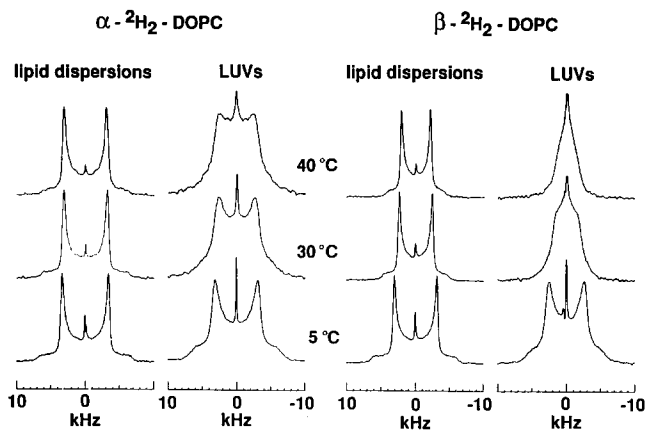


Fig. 1. Temperature dependency of the ^{2}H-NMR spectra of lipid dispersions and LUVs, prepared from α- and β-$^2H_2$-DOPC.

atures lose their defined doublet structure. This effect can be explained to be caused by an increased motional averaging of the quadrupolar interaction due to the lateral diffusion of the lipid molecules over the more curved bilayer of the LUVs [15]. In agreement with the data on the dispersions, the temperature effects are more pronounced for β-$^2H_2$-DOPC. Because at higher temperatures the quadrupolar splittings of the LUVs are only poorly defined, we selected 5°C as the experimental temperature for subsequent studies. At this temperature the bilayer is still liquid-crystalline and in addition has a relatively low permeability, allowing the generation of a more stable membrane potential.

The sensitivity of the LUV system at 5°C for changes in the quadrupolar splitting was studied by recording ^{2}H-NMR spectra of α- or β-$^2H_2$-DOPC LUVs with different surface charges, generated by incorporation of positively and negatively charged lipophilic ions [8]. Fig. 2 shows the effects of the addition of TPP^+ (upper 2 spectra) and TPB^- (lower 2 spectra) to the LUVs on the quadrupolar splittings of the ^{2}H-NMR spectra (neutral membrane surface: middle 2 spectra). The $\Delta\nu_Q$ of α-$^2H_2$-DOPC LUVs decreases with positive surface charge and increases with negative surface charge, while the $\Delta\nu_Q$ of β-$^2H_2$-DOPC LUVs shows the opposite behaviour; i.e., it increases with positive surface charge and decreases with negative surface charge. The results for TPP^+ are quantified in Fig. 3. For TPB^- the changes were for α-$^2H_2$-DOPC from 6.3 to 7.8 kHz (3 mM TPB^-), and for β-$^2H_2$-DOPC from 4.8 kHz to 3.1 kHz (3 mM TPB^-). The counterdirectional changes of the quadrupolar splittings for α- and β-deuterated DOPC in response to the surface charge indicate conformational changes of the PC headgroup as a result of the surface charges, as was described by Seelig and co-workers for aqueous lipid dispersions [8]. The extent of the changes is of the same magnitude for the LUV system as it was described for the lipid dispersions [8], indicating a comparable sensitivity for the two systems.

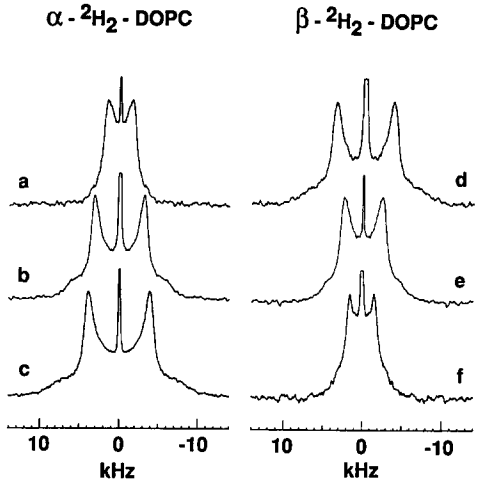


Fig. 2. Effect of the surface charge on the ^{2}H-NMR spectra of α-$^2H_2$-DOPC (a–c) and β-$^2H_2$-DOPC LUVs (d–f). Spectra are in the absence of lipophilic ions (b, e), or in the presence of 63 mM TPP^+ (a, d) or 3 mM TPB^- (c, f). The spectra were recorded at 5°C.

Next, the effect of a membrane potential on the PC headgroup conformation was investigated. NMR spectra were recorded of LUVs, consisting of either α- or β-$^2H_2$-DOPC, exhibiting a K^+_{in}/Na^+_{out} ion gradient, in the absence and in the presence of a valinomycin-induced membrane potential. Both for the α- and for the β-$^2H_2$-DOPC LUVs there was no difference in the obtained ^{2}H-NMR spectra before and after addition of valinomycin. Fig. 4 (a–c) illustrates this for the LUVs consisting of β-$^2H_2$-DOPC. The difference spectrum of the situation with and without valinomycin shows that both situations give rise to identical ^{2}H-NMR spectra. The presence of the membrane potential, which can be expected to be approximately 200 mV [17], was confirmed employing the potential sensitive fluorescent dye $DiSC_2$-(5) and by measuring the

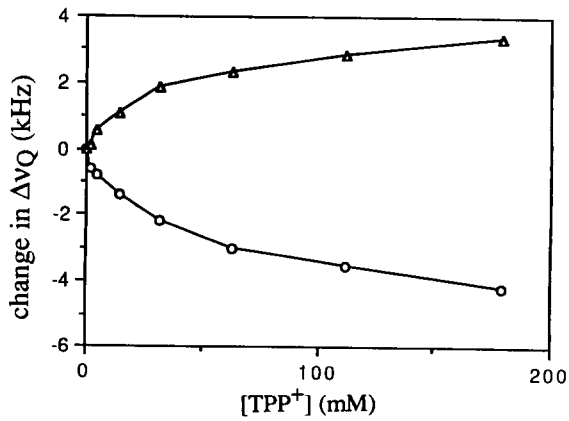


Fig. 3. Variation of the quadrupolar splitting ($\Delta\nu_Q$) of α-$^2H_2$-DOPC LUVs (circles) or β-$^2H_2$-DOPC LUVs (triangles) in TPP^+ titration experiments.

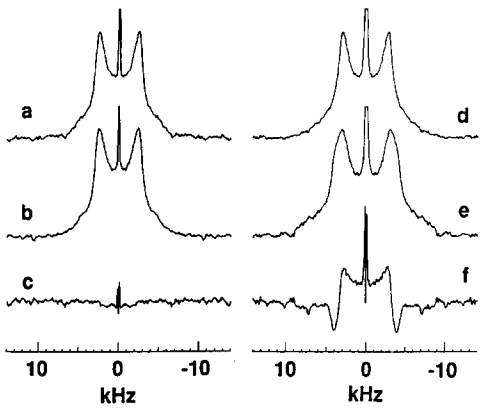


Fig. 4. Effect of a membrane potential on the ^{2}H-NMR spectrum of β-$^2H_2$-DOPC LUVs in the absence (a, b) or presence (d, e) of 10 mM TPP^+. LUVs, exhibiting a K^+_{in}/Na^+_{out} ion gradient, before (a and d) and after (b and e) addition of valinomycin. (c) difference spectrum of a and b; (f) difference spectrum of d and e. The spectra were recorded at 5°C.

changes in ^{31}P-NMR chemical shift of P_i enclosed in the LUVs, due to the generation of a ΔpH as a result of the $\Delta\psi$ [17] (data not shown).

From the observation that the membrane potential does not influence the ^{2}H-NMR and ^{31}P-NMR spectra (not shown) of the DOPC LUVs, we can conclude that there is no direct effect of the membrane potential on the phosphatidylcholine headgroup conformation in pure lipid bilayers. This suggests that the potential difference between the two bulk phases on either side of the bilayer is experienced predominantly in the hydrophobic core of the bilayer and not in the headgroup region. This is consistent with theory. Assuming a potential difference between the two bulk phases on either side of the bilayer of 200 mV and dividing this region in three parts with different dielectric constants (ϵ), namely a hydrophobic core of the bilayer of 30 Å thickness ($\epsilon = 2$), headgroup regions of 7 Å thickness each ($\epsilon = 30$) and diffuse double layers of 10 Å thickness ($\epsilon = 80$), it can be calculated, using Eqns. 1 and 2, where ϵ_i (ϵ_j) is the dielectric constant of part i (j) of the bilayer; $d\psi_i$ ($d\psi_j$) is the potential difference across part i (j) of the bilayer; and dx_i (dx_j) the thickness of part i (j) [18], that the electric field is predominantly experienced in the hydrophobic core of the membrane (192 mV) and only for about 3 mV in the headgroup regions.

$$\epsilon_i(d\psi_i/dx_i) = \epsilon_j(d\psi_j/dx_j) \quad (1)$$

$$\Delta\psi = \sum_i d\psi_i \quad (2)$$

It can be expected that in the experimental system there will be an accumulation of the valinomycin–K^+ complex at the interface of the inner monolayer. That this is not sensed by the PC headgroups is most likely due to the very low concentration of this complex (maximally $1:10^4$ with respect to phospholipid). Whether the membrane potential affects the dipoles in the ester bond region of the bilayer is not known, but if so this does not result in a change in conformation of the PC headgroup.

Since we have shown that the membrane potential has no direct effect on the PC headgroup conformation, it seems unlikely that membrane potentials exert their action on membrane processes by directly changing the conformation of lipid headgroups at the membrane-water interface.

To demonstrate the sensitivity of our experimental system to the membrane potential we made use of TPP^+ which will migrate to the vesicle interior upon generation of the membrane potential [19], while the net amount of bound TPP^+ will also increase [20]. Fig. 4d–f shows that upon addition of valinomycin to β-$^2H_2$-DOPC LUVs with a K^+_{in}/Na^+_{out} ion gradient, in the presence of 10 mM TPP^+, a broadening of the spectrum occurs due to the appearance of a second spectral component with a larger quadrupolar splitting, visible as a shoulder in the spectrum. This component, which is accurately revealed in the difference spectrum (f), must originate from the lipids in the inner leaflet of the bilayer, due to TPP^+ accumulation in response to $\Delta\psi$. This conclusion is further confirmed by the results shown in Fig. 5 for LUVs prepared from α-$^2H_2$-DOPC. With this lipid, increasing the TPP^+ concentration decreases the quadrupolar splitting (spectra a–d, compare with Fig. 2), which effect is greatly enhanced by the generation of a membrane potential after which TPP^+ accumulates inside the vesicles

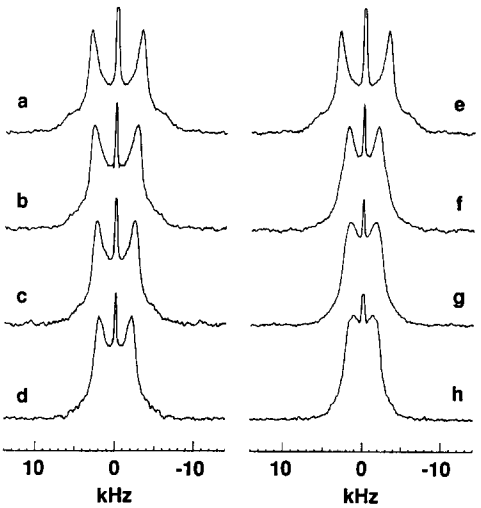


Fig. 5. Effect of $\Delta\psi$ on TPP^+ binding to α-$^2H_2$-DOPC LUVs. ^{2}H-NMR spectra of LUVs, exhibiting a K^+_{in}/Na^+_{out} ion gradient, in the presence of different concentrations of TPP^+, before (a–d) and after (e–h) the addition of valinomycin. (a, e) 0, (b, f) 5, (c, g) 15, and (d, h) 32 mM TPP^+. The spectra were recorded at 5°C.

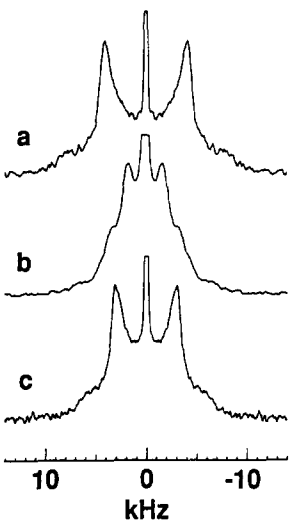


Fig. 6. Effect of $\Delta\psi$ on the TPP^+ distribution across the bilayer of LUVs, consisting of 15 mol% DOPG and 85 mol% α-$^2H_2$-DOPC. ^{2}H-NMR spectra of the LUVs, exhibiting a valinomycin-induced K^+-diffusion potential (a, b), without (a) or with (b) 10 mM TPP^+ present; and after the addition of gramicidin, in the presence of 10 mM TPP^+ (c). Spectra were recorded at 5°C.

(spectra e–h). The spectra did not change during at least 24 h at 5°C, indicating the stability of the membrane potential. Dissipation of $\Delta\psi$ by the addition of gramicidin lead to the immediate redistribution of the TPP^+, resulting in the original spectrum (data not shown), demonstrating the reversibility of the TPP^+ and $\Delta\psi$-induced changes in the quadrupolar splitting.

In the presence of TPP^+ and a membrane potential, two spectral components were often visible, most likely originating from PC headgroups on the two leaflets of the bilayer. This would be the expected pattern for vesicles in which the inner leaflet contains more and the outer leaflet less TPP^+ molecules, and it would suggest that the method can be used to study the response of the individual monolayers on the applied potential. However, the two components were often poorly resolved (see for instance spectrum e in Fig. 4 and spectrum f in Fig. 5). One reason for this might be a fast exchange of TPP^+ molecules between the two leaflets of the bilayer. To increase the sensitivity of the method and in that way verifying the individual responses of the two monolayers, 15 mol% negatively charged DOPG was incorporated in α-$^2H_2$-DOPC LUVs (Fig. 6, spectrum a). TPP^+ will bind more strongly to these negatively charged LUVs and the incorporated negative surface charge increases the quadrupolar splitting from 6.3 to 8.2 kHz. Therefore this system provides a larger spectral window to observe asymmetrical effects on the bilayer. Indeed, now in the presence of 10 mM TPP^+ and a membrane potential, two doublets are observed with $\Delta\nu_Q$ values of 6.7 and 3.4 kHz respectively (Fig. 6, spectrum b), reflecting the asymmetrical distribution of TPP^+ with a higher concentration in the inner leaflet (smallest quadrupolar splitting). They merge into a single doublet with a $\Delta\nu_Q$ of 6.2 kHz upon collapsing the membrane potential by the addition of gramicidin (Fig. 6, spectrum c) indicating the redistribution of the TPP^+ molecules. ^{2}H-NMR of headgroup-labeled lipids in large unilamellar vesicles thus provides a method to monitor the individual behavior of the two lipid interfaces responding to ion gradient-induced redistributions of molecules.

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