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Structure and dynamics of plasmalogen model membranes containing cholesterol: a deuterium NMR study

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Deuterium nuclear magnetic resonance (^{2}H-NMR) was used to investigate the structure and dynamics of the *sn*-2 hydrocarbon chain of semi-synthetical choline and ethanolamine plasmalogens in bilayers containing 0, 30, and 50 mol% cholesterol. The deuterium NMR spectra of the choline plasmalogen yielded well-resolved quadrupolar splittings which could be assigned to the corresponding hydrocarbon chain deuterons. The *sn*-2 acyl chain was found to adopt a similar conformation as observed in the corresponding diacyl phospholipid, however, the flexibility at the level of the C-2 methylene segment of the plasmalogen was increased. Deuterium NMR spectra of bilayers composed of the ethanolamine plasmalogen yielded quadrupolar splittings of the C-2 segment much larger than those of the corresponding diacyl lipids, suggesting that the *sn*-2 chain is oriented perpendicular to the membrane surface at all segments. Cholesterol increased the ordering of the choline plasmalogen acyl chain to the same extent as in diacyl lipid bilayers. T_1 relaxation time measurements demonstrated only minor dynamical differences between choline plasmalogen and diacyl lipids in model membranes.

Introduction

Plasmalogens (1-(*O*-alk-1′-enyl)-2-acyl-glycerophospholipids) are not only major constituents of the membranes of many anaerobic procaryotes [1–6] but also make up a large part of the phospholipids in most mammalian tissues. In the heart and in the white matter of the brain, for instance, they represent approx. 30% of the total phospholipids [7]. The biological role of plasmalogens is essentially unknown. It is noteworthy, however, that a genetic defect in plasmalogen synthesis as observed in the cerebro-hepato-renal (Zellweger) syndrome and in similar peroxisomal disorders, is associated with severe cellular dysfunctions [8].

By studying the radii and packing-characteristics of unilamellar vesicles prepared from choline plasmalogens with defined hydrocarbon chains in the *sn*-2 position of the glycerol moiety, Hermetter et al. found larger vesicle radii for the choline plasmalogen than for the corresponding diacyl lipid, whereas the overall packing density of the bilayers of both types was the same [9]. Upon addition of 30 and 50 mol% cholesterol they observed an increase of the vesicle radii which was most pronounced in the case of the choline plasmalogen samples. For the latter, unilamellar vesicles could no longer be obtained at a cholesterol concentration of 50 mol% whereas under the same conditions the diacyl and alkyl-acyl analogues still formed bilayer vesicles. From these

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and other results the authors were led to conclude that the double bond in the alkenyl moiety had a more pronounced influence on the structural organization of phospholipid/cholesterol membranes than the fact that this chain was linked to the glycerol backbone by an ether linkage.

The purpose of the present study was to characterize by ^{2}H-NMR the structure and dynamics of plasmalogen model membranes containing choline and ethanolamine head groups and to investigate the influence of cholesterol on such membranes. To this end, the choline and ethanolamine plasmalogens were selectively deuterated at the C-2 or the C-9,10 carbon atoms of the *sn*-2 hydrocarbon chain. ^{2}H-NMR spectra of pure plasmalogen membranes and membranes containing admixtures of either cholesterol or diacylglycerophosphocholine were recorded at different temperatures. Furthermore, in order to provide insight into the dynamics of plasmalogen membranes the deuterium T_1 relaxation times were determined as a function of temperature and cholesterol composition.

Materials and Methods

Deuterium-labelled choline plasmalogens. Semisynthetic 1-*O*-alkenyl-2-[2′,2′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine, 1-*O*-alkenyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine and 1-*O*-alkenyl-2-[2′,2′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphoethanolamine were prepared as described earlier [10]. The hydrocarbon chain in position 1 of glycerol consisted of approx. 70% 1′-hexadecenyl moieties. The lipid was thin-layer chromatographically clean.

Cholesterol. Cholesterol was purchased from E. Merck AG, recrystallized four times from absolute ethanol and dried under high vacuum for 48 h before use.

Preparation of multilamellar vesicles. Typically 10 mg of lipid was dissolved in dichloromethane and transferred to an NMR sample tube where the solvent was removed under a stream of nitrogen. The lipid was further dried under high vacuum for at least 48 h until no further change in weight was observed. The dry lipid was resuspended in pure deuterium-depleted water by extensive vortexing. The final water content in all samples was about 66% (w/w). In the case of the cholesterol containing samples, the appropriate amount of cholesterol was dissolved in 0.5 ml dichloromethane and added to a defined amount of dried lipid in the NMR sample tube. The mixture was vortexed to assure a homogeneous lipid/cholesterol solution. The solvent was removed under nitrogen and the sample dried under high vacuum for at least 48 h. The sample tube was weighed again to determine the exact amount of the added cholesterol. Finally, the lipid-cholesterol mixture was resuspended in deuterium depleted water.

We were unable to obtain a homogeneous dispersion of the pure ethanolamine plasmalogen in water. However, when the ethanolamine plasmalogen was first mixed with unlabelled 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine in a ratio ehtanolamine plasmalogen : POPC of 1 : 0.85 (w/w) a homogeneous liquid-crystalline bilayer phase could be obtained without difficulties.

^{2}H-NMR spectroscopy. ^{2}H-NMR spectra were recorded on a Bruker CXP 300 FT-NMR spectrometer operating at 46.1 MHz, employing the standard quadrupole-echo sequence with a 90° pulse of 3.5 μs and a waiting period between the two successive 90° pulses of 30 μs [11]. The recycle delay and the spectral width were 250 ms and 62.5 kHz, respectively. The measuring time was of the order of 1 h/spectrum and the temperature controlled within 0.5 °C by a thermostatically regulated gas flow system.

Deuterium T_1 relaxation times of the plasmalogens in multilamellar vesicles were obtained using the standard inversion-recovery pulse sequence (180°-τ-90°) followed by the quadrupole echo observation sequence. For the determination of one T_1 value at least 10 experiments with τ varying from 0.1 to 300 ms were performed. The experiments were evaluated by performing a three parameter non-linear Marquardt fit on the average height of the two 90° orientation peaks of the ^{2}H powder-type spectra according to the equation [12]

$$M(\tau) = P(3)[1 - \{1 + P(2)(1 - \exp[-w/P(1)])\} \times \exp(-\tau/P(1))]$$

$P(1)$, $P(2)$ and $P(3)$ denote the parameters to be fit, i.e. T_1 relaxation time, effective flip-angle and infinity magnetization $M(\infty)$, respectively. $M(\tau)$ denotes the magnitude of magnetization at $t = \tau$ and w stands for the recycle delay time, thereby accounting for saturation effects. By using the above equation the major sources of systematic error, that is errors in the pulse flip-angle and errors in determination of the infinity magnetization, are minimized. The error limits given refer to $\pm$ the standard deviation of the mean.

Results and Discussion

Typical ^{2}H-NMR spectra of liposomes composed of either 1-*O*-alkenyl-2-[2′,2′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine or 1-*O*-alkenyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine in pure form and with varying proportions of cholesterol are shown in Fig. 1. All spectra exhibit the typical line shape of liquid-crystalline bilayers with one or two well resolved quadrupole splittings. A numerical summary of the quadrupoles splittings together with those of a related diacyl lipid, i.e. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), is found in Table I.

TABLE I

COMPARISON OF THE QUADRUPOLAR SPLITTINGS $\Delta\nu_Q$ OF THE *sn*-2 HYDROCARBON CHAIN DEUTERONS OF PLASMALOGENS AND DIACYL PHOSPHOLIPIDS

Segment	Lipid	Temp. (°C)	Quadrupole splitting (kHz)
C-2	choline plasmalogen	0	19.3, 15.1
		30	13.2
	ethanolamine plasmalogen [a]	0	25.6
		30	23.2
	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine [b]	0	18.8, 9.4
		27	16.4, 10.8
C-9/10	choline plasmalogen	0	16.3, 3.8
		30	13.9, 3.4
	ethanolamine plasmalogen [c]		n.d.
	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine [b]	0	15.8, 3.6
		27	13.4, 2.5

[a] This plasmalogen was diluted with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine prior to measurement (ethanolamine plasmalogen : 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (1 : 0.85, w/w)).

[b] Reference [21].

[c] n.d., not determined.

Inspection of Fig. 1 and Table I demonstrates that under most conditions two separate quadrupole splittings were observed for the two deuterons at the C-2 position and those at the C-9,10 carbon atoms in agreement with previous findings [13,14]. As far as the deuterons at the 9′-10′ *cis*-double bond are concerned there is very little difference between the quadrupole splittings of the plasmalogen and the diacyl lipid. In contrast, distinct motional differences are observed between the two lipids at the C-2 position of the *sn*-2 oleic acyl chain. In 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, the two deuterons are easily distinguishable by virtue of their widely different quadrupole splittings of 16.4 and 10.8 kHz (at 27 °C). In contrast, the corresponding choline plasmalogen exhibits just one splitting of 13.2 kHz at the same temperature. The latter value is the average of the two quadrupole doublets observed in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. In the choline plasmalogen, the difference between the two deuterons is thus averaged out suggesting a greater motional freedom of the oleic acyl chain at the C-2 position of the choline plasmalogen compared to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. However, at temperatures lower than 30 °C or in the presence of cholesterol two quadrupole splittings are also detectable in the choline plasmalogen (cf. below).

A different behavior is observed for 1-*O*-alkenyl-2-[2′,2′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphoethanolamine. In pure form this lipid cannot be dispersed in water to form homogeneous, liquid crystalline bilayer vesicles. Over a large range of temperatures only broad and featureless ^{2}H-NMR spectra were observed. However, when mixed with about equal amounts of non-deuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine a homogeneous dispersion in water was easily obtained. The ^{2}H-NMR spectrum of this mixed bilayer is shown in Fig. 2. It consists of a single quadrupole splitting of 23.2 kHz separation (at 30 °C). The inequivalence of the two deuterons has been removed; more importantly, however,

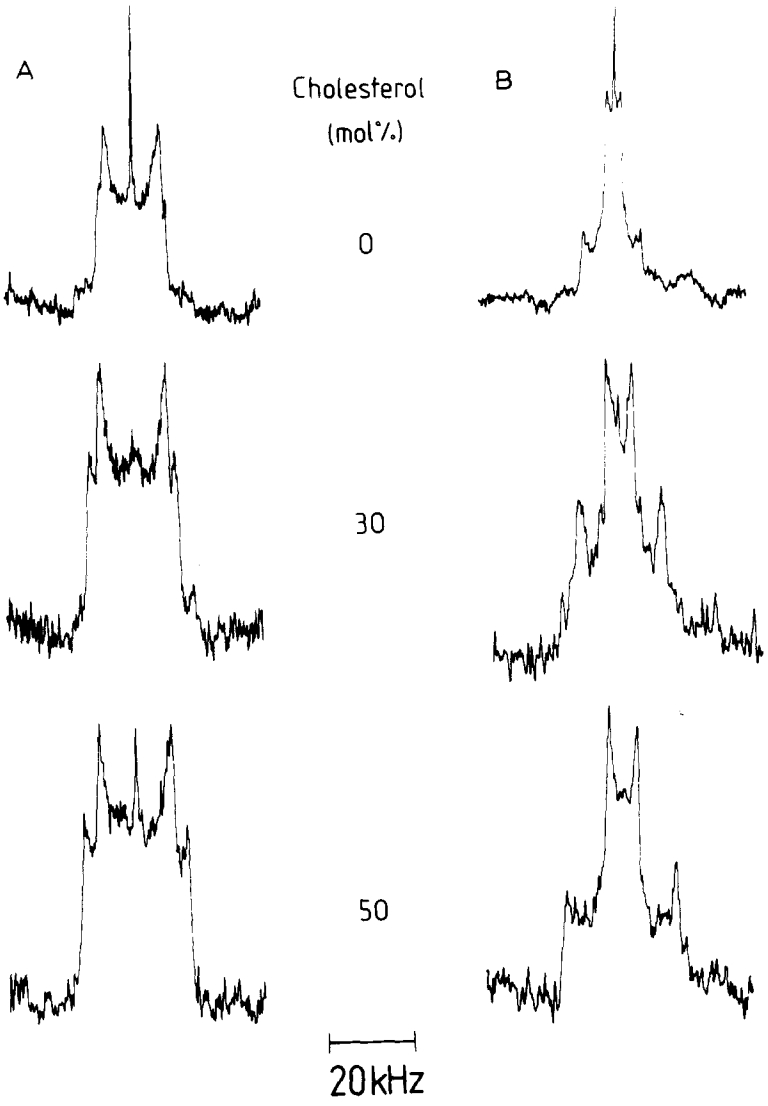

Fig. 1. Deuterium NMR spectra of bilayers composed of plasmalogens and different amounts of cholesterol. Lipids were dispersed in water (66% H_2O, w/w). Measuring temperature 20 °C, 15000 scans, 62.5 kHz spectral width, 150 Hz linebroadening. (A) 1-*O*-Alkenyl-2-[2′,2′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine. (B) 1-*O*-Alkenyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine.

the quadrupole splitting is quite large approaching the separation found typcially in the *sn*-1 chain of diacyl lipids. A similar result has also been obtained for the PE fraction isolated from *Clostridium butyricum* and *Clostridium beijerinkii* which also contain a high fraction of plasmalogen [15].

The addition of cholesterol to plasmalogen membranes considerably increases the quadrupole splitting as demonstrated in the spectra of Fig. 1. Fig. 3 then displays the variation of the quadrupole splittings as a function of the cholesterol concentration in membranes composed of the choline plasmalogen. At the highest cholesterol concentration measured (50 mol% cholesterol) the quadrupole splittings of all segments were twice as large than those of observed for cholesterol-free membranes. This is in quantitative agreement with similar measurements on diacyl lipid-cholesterol membranes [16–18].

Information about the rate of segment reorientation can be obtained by measuring the T_1 relaxation rates. The T_1 relaxation times of the choline plasmalogen membranes encompass a range of 8 to 30 ms depending on the temperature and membrane composition (cf. Table II). The T_1 times increase with increasing temperature indicat-

TABLE II

SPIN-LATTICE RELAXATION TIME (T_1), CORRELATION TIME (τ_c) AND ACTIVATION ENERGIES E_a OF BILAYER MEMBRANES COMPOSED OF DEUTERATED CHOLINE PLASMALOGEN AND CHOLESTEROL

Labelled segment	mol% chol.	Temp. (K)	T_1 (ms) [a]	τ_c (ps) [b]	E_a (kJ/mol) [c]
C-2	0	278	8.2±0.9	274±30	12.4±2.3
		288	10.5±1.4	214±30	
		298	11.6±0.9	194±15	
		308	14.7±1.5	153±15	
		318	16.2±1.2	138±10	
C-2	50	288	8.9±0.7	252±20	14.4±2.6
		298	10.5±0.5	214±10	
		308	13.5±0.5	166±10	
		318	15.7±0.7	142±10	
C-9, C-10	0	278	9.1±0.6	247±16	19.3±1.6 (16.2±1.8) [d]
		288	11.1±0.4	202± 8	
		298	13.7±0.3	164± 4	
		308	18.3±0.8	123± 6	
		318	27.4±1.4	82± 5	
C-9, C-10	50	278	8.9±0.5	261±15	13.9±1.9
		288	11.6±0.7	200±12	
		298	13.0±0.9	178±12	
		308	16.2±0.9	142± 8	

[a] T_1 measurements were made using the Inversion-Recovery Method with a solenoid probe head and 3.5 μs 90° pulses.
[b] correlation times were evaluated according to Brown et al., 1979 [19].
[c] Activation energies were determined from a non-linear two-parameter Marquardt fit of the correlation times.
[d] When the 318 K data point is omitted from evaluation.

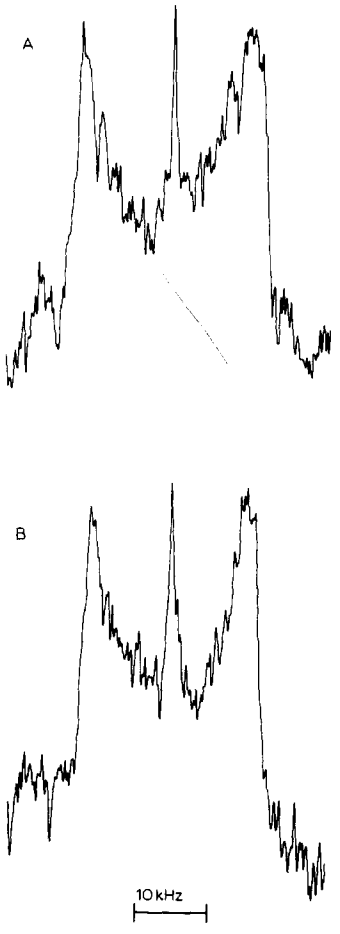

Fig. 2. Deuterium NMR spectra of bilayers composed of 1-*O*-alkenyl-2-[2′,2′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphoethanolamine and POPC (1:0.85, w/w) in water (66% H_2O, w/w). Measuring temperature: (A) 0°C, (B) 30°C. 20000 scans, 62.5 kHz spectral width, 150 Hz linebroadening. The quadrupolar splittings amount to 25.6 kHz (A) and 23.2 kHz (B).

ing that the motions fall into the so-called fast correlation time regime. Fig. 4 shows Arrhenius plots of the relaxation times. From the slope of the straight lines the activation energies can be evaluated (cf. Table II) which are about 12.4–19.3 kJ/mol. Again, these data are similar to that observed for diacyl lipids [19]. The T_1 relaxation data can be further analyzed to provide the correlation time τ_c of the segmental motions [19]. The results of this evaluation are also listed in Table II and are in broad agreement with those observed with other cholesterol containing membranes [20].

In summary, four conclusions can be derived from a comparison of the ^{2}H-NMR data of the plasmalogens and the corresponding diacyllipids: (i) Both lipid classes have a rather similar conformation of the hydrocarbon chains when the head group is phosphocholine. Since it is well-established that the *sn*-2 chain of diacyl lipids is bent at the C-2 position it follows from the similarity of the ^{2}H-NMR splittings that the *sn*-2 chain is also bent in the choline plasmalogen. However, the inequivalence of the two C-2 deuterons is removed in the latter indicating a somewhat enhanced flexibility of the *sn*-2 chain of choline plasmalogens. (ii) A different behavior is observed for the ethanolamine plasmalogen dispersed in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. Here the size of the quadrupole splitting argues

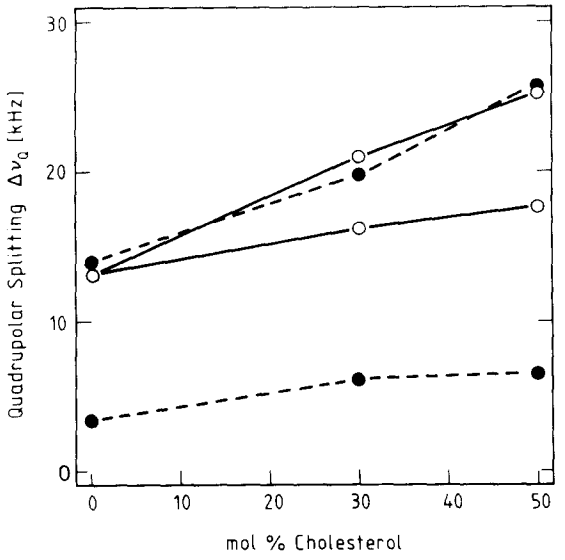

Fig. 3. Variation of the quadrupolar splittings with increasing amounts of cholesterol at 30 °C. ○, [2′,2′-$^2H_2$]choline plasmalogen; ●, [9′,10′-$^2H_2$]choline plasmalogen.

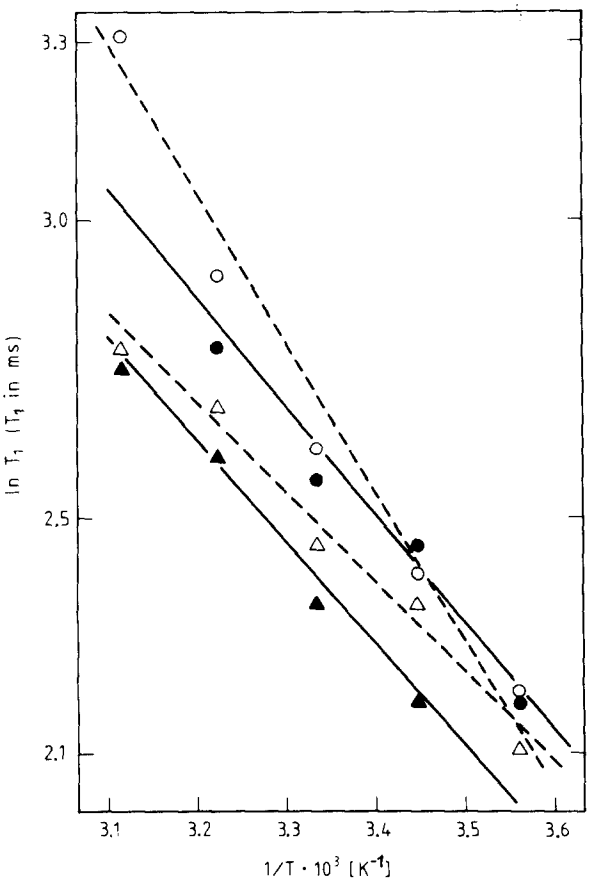

Fig. 4. Arrhenius plots of the T_1 relaxation times. The spin-lattice relaxation times (T_1) were measured using the saturation recovery method. ○ (dashed line), [9′,10′-$^2H_2$]choline plasmalogen; ● (solid line), [9′,10′-$^2H_2$]choline plasmalogen + 50 mol% cholesterol; △ (dashed line), [2′,2′-$^2H_2$]choline plasmalogen; ▲ (solid line), [2′,2′-$^2H_2$]choline plasmalogen + 50 mol% cholesterol.

against a bending of the *sn*-2 chain but suggests that this chain is perpendicular to the membrane surface at all segments. (iii) Cholesterol increases the ordering of the *sn*-2 fatty acyl chain in plasmalogens to the same extent as it does in diacyllipids. (iv) Dynamic differences between the two lipid classes are small, at least as judged from the ^2H-T_1 relaxation times.

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