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NMR investigations of interactions between anesthetics and lipid bilayers

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ABSTRACT

Interactions between anesthetics (lidocaine and short chain alcohols) and lipid membranes formed by dimyristoylphosphatidylcholine (DMPC) were studied using NMR spectroscopy. The orientational order of lidocaine was investigated using deuterium NMR on a selectively labelled compound whereas segmental ordering in the lipids was probed by two-dimensional ^{1}H–^{13}C separated local field experiments under magic-angle spinning conditions. In addition, trajectories generated in molecular dynamics (MD) computer simulations were used for interpretation of the experimental results. Separate simulations were carried out with charged and uncharged lidocaine molecules. Reasonable agreement between experimental dipolar interactions and the calculated counterparts was observed. Our results clearly show that charged lidocaine affects significantly the lipid headgroup. In particular the ordering of the lipids is increased accompanied by drastic changes in the orientation of the P–N vector in the choline group.

1. Introduction

Local anesthetics have been used in practical medicine since the beginning of the 20th century. Despite their extensive and important clinical use the mechanism of action still remains, to a large extent, a mystery. Several theories claiming to explain the therapeutic effect have been put forward [1–3] but no complete consensus on the details of the process has yet been reached. About a century ago Meyer and Overton reported that the potency of general anesthetics was correlated with their solubility in olive oil [4,5]. Later, when it was realized that the cell membrane was composed of lipids, it was a natural step to extend Meyer and Overton observations to the lipid membrane, and so the lipid theory [6] of anesthesia was born.

Many studies have been performed to examine the effects anesthetics impose on lipid membranes. Observed changes in bilayer properties such as fluidity, main transition temperature, area per lipid and lipid volume have been reported in several studies [7–15]. These investigations, however were not able to provide a plausible explanation for the anesthetic mechanism because changes of the bilayer structure or dynamics when introducing anesthetics are quite small, compared to changes caused by variations in temperature by one or two degrees [16]. Since an increase in body temperature of this magnitude doesn't cause anesthesia, it appears that these changes cannot be, on their own, responsible for the anesthetic action.

Due to the mentioned shortcomings of lipid related explanations of the anesthetic action, protein oriented theories, where the anesthetic action is explained by the binding to specific protein sites, have grown in popularity during the last decades [15,17,18]. Several investigations have been carried out [13,14] in order to study whether the primary mechanism of action of local anesthetics is a result of changes in the lipid bilayer or due to the binding to proteins. Although still not fully understood, the molecular mechanism of anesthesia is presently considered to be a result of the Na^{+} voltage-gate ion channel being blocked by the anesthetic molecule, thus blocking nerve impulses [19]. Despite the fact that a binding site on the Na^{+} channel has been hypothesized, the influence from the lipid environment on the Na^{+} channel or other membrane bound proteins remains unclear and changes in bilayer structure or dynamics could give a significant contribution to the inactivation of ion channels involved in anesthesia [20–22].

Lidocaine (Fig. 1) is one of the most common locally acting anesthetic molecules. It belongs to the ionisable amines family of local

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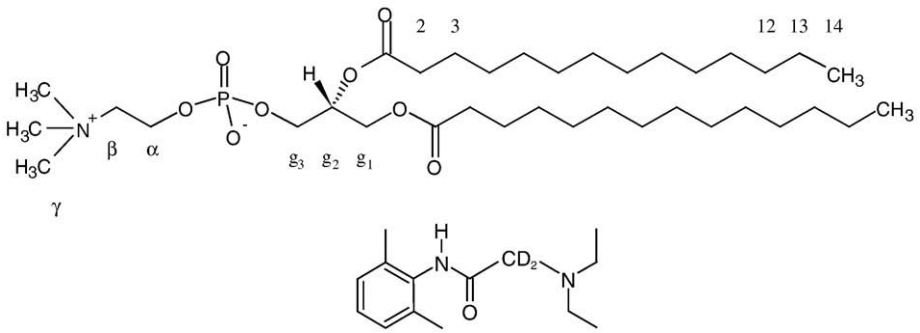

Fig. 1. Schematic structures of dimyristoylphosphatidylcholine (DMPC, top) and lidocaine (bottom).

anesthetics and is present in both charged and uncharged forms, with a pK_a value estimated to be about 6.8 in lipid membranes [23]. Experimental studies [24,25] and computer simulations [26,27] indicate that charged and uncharged forms of lidocaine are located at different depths in the bilayer. In addition the two forms of lidocaine exhibit different orientation in the bilayer [27]. It has been speculated whether the position of the anesthetic could be crucial for the anesthetic effect [28] followed by an assumption that the charged species, which has a preference for the headgroup region of the lipid bilayer, should be responsible for the anesthetic action. On the other hand, the high mobility of uncharged molecules as compared with the charged form [27], may also be an important factor in the anesthetic effect.

Alcohols can act as general anesthetics and their anesthetic potency was observed to be related to the increase of the hydrocarbon chain up to 1-dodecanol. In analogy with other local anesthetics, the alcohols are claimed to change membrane properties [29]. A recent computational study focused on the interaction between membranes and alcohols (methanol and ethanol) indicated that the location of ethanol was close to the glycerol backbone, whereas methanol is closer to the water membrane interface [30,31]. The effect of alcohol on the membrane structure was also investigated using NMR spectroscopy [32,33].

NMR is a powerful experimental method for studies of molecular order, structure, and dynamics in soft matter such as lipid bilayers. Deuterium NMR has proven particularly useful for estimations of order parameter profiles of hydrocarbon chain segments in unoriented lipid systems [34–39]. Deuterium solid-state NMR was also employed in previous investigations of local anesthetic interactions with membranes [40–42]. Deuterium NMR experiments are usually reasonably simple to carry out, and produce uncomplicated spectra. A limitation, however is that these spectra reflect only the motional behavior of the C–D vector. A major shortcoming of ^{2}H NMR is, however, that isotopic labeling is required. This can be both complicated and expensive.

An alternative technique for investigations of fluid phase lipids, which does not require isotopic enrichment or tedious sample preparation, is to carry out measurements of ^{1}H–^{13}C dipolar couplings by two-dimensional (2D) separated local field (SLF) spectroscopy under magic-angle spinning (MAS) conditions [43,44]. Recently, we have introduced several efficient SLF pulse schemes for measurements of dipolar couplings in solid and liquid-crystalline systems, such as lipid bilayers [45–49].

In the present study we use NMR spectroscopy and molecular dynamics (MD) computer simulations for investigations of the effect of local anesthetic lidocaine and alcohols (ethanol and methanol) on the dimyristoylphosphatidylcholine (DMPC, Fig. 1) bilayer. Using deuterium NMR we investigate concentration and pH dependence of the orientational order of selectively deuterium labeled lidocaine-d$_2$. The ^{1}H–^{13}C dipolar couplings in DMPC are employed to investigate the segmental order of the lipids. These couplings determined for several concentrations of lidocaine are compared with the results from MD simulations of similar systems.

2. Materials and methods

2.1. Sample preparation and characterization

Unlabelled dimyristoylphosphatidylcholine DMPC and lidocaine, methanol and ethanol were purchased from Sigma-Aldrich and used without further purification. Lidocaine-d$_2$ labelled in the acidic methylene group (between the carbonyl and amine), was prepared according to the previously published procedure [50]. Introduction of lidocaine in the multilamellar vesicles (MLVs) was performed by dissolving DMPC and a desired amount of lidocaine in a mixture of chloroform and methanol (2:1, v/v). The solutions were dried under a stream of nitrogen followed by vacuum pumping overnight. The lipid films were thereafter placed in a chamber with a humidity of 96% at 40 °C, created by a saturated solution of potassium sulphate, which resulted in hydration levels of approximately 15 water molecules per lipid (n_w=15). The pH was adjusted using the phosphate buffer with the concentration 100 mM. The water content in the pH adjusted samples was the same as in the samples where no buffer was added.

In contrast to lidocaine, the alcohols were added only after the hydration level of 15 water molecules per lipid was reached. Subsequently, the sample was subject to several freeze–thaw–vortexing cycles. The reason for this was to prevent evaporation which occurs since the alcohols are very easily mixed into membranes.

2.2. NMR experiments

All NMR experiments carried out on MLVs were performed at a magnetic field of 9.4 T on a Chemagnetics Infinity-400 spectrometer equipped with a 6 mm triple-resonance MAS probe and a static probe with a 5 mm horizontal solenoid coil for the ^{2}H NMR experiments. The typical mass of the sample for the MAS experiments was around 250 mg whereas for the ^{2}H NMR was about 100 mg. The set temperature was 40 °C which, due to the sample heating originating from the MAS, resulted in a temperature of 42 °C [51]. Heteronuclear ^{1}H–^{13}C dipolar couplings were measured using a 2D MAS technique denoted R-PDLF spectroscopy [46], which incorporates R-type recoupling [52] into the proton-detected local field (PDLF) protocol [53]. We used refocused INEPT [54] for the ^{1}H to ^{13}C polarization transfer since this scheme provides high spin-pair selectivity. The spinning frequency was 5.15 kHz in the 2D SLF experiments, and the ^{1}H field strengths during dipolar recoupling and heteronuclear decoupling were 46 and 27 kHz, respectively. The 2D spectra were acquired using typically 112 scans and 90 increments in the t_1-dimension. A recycling delay of 6 s was employed. Further details of the R-PDLF method can be found elsewhere [46,55].

The deuterium spectra were acquired at 61.4 MHz employing the conventional quadrupole echo sequence [56] ($90°_x$–t_1–$90°_{\pm y}$–t_2–acq) with a 90° pulse length of 3 μs and $t_1 = t_2 = 30$ μs. A recycle delay of at least five times the spin-lattice relaxation time was used. All the experiments were carried out at 40 °C and the temperature gradient across the sample was estimated to be less than 1 °C.

2.3. Molecular dynamics computer simulations

Details of the computer simulation, the parameters of the force fields and the properties of the bilayer are reported in previous publications [26,27] here we only summarize essential technical aspects. Five different lipid bilayer systems, each consisting of 128 (64×2) DMPC lipids and 3655 water molecules, were simulated. In two of the systems 12 charged/uncharged lidocaine molecules were dissolved, whereas two other systems contained 36 charged/uncharged lidocaines. To keep the electroneutrality, 12 and 36 Cl^- ions were added to the systems with charged lidocaine. One system containing a pure fully hydrated DMPC bilayer was simulated as a reference. The lipid force field parameters for bonded and non-bonded interactions and atomic partial charges are based on the GROMOS force field [57,58]. The united atom model was used for the CH, CH_2, and CH_3 groups in DMPC lipids and in lidocaine, except for the polar hydrogen atom on the charged lidocaine, which was described explicitly. The temperature was set to 313 K and the pressure to 1 bar. The systems were simulated for 100 ns using a time step of 2 fs.

3. Results and discussion

Anesthetics have a tendency to partition between the hydrophilic and hydrophobic regions of the membrane. Although the distribution between these regions is often ruled by the hydrophobic properties of the anesthetic itself, lidocaine exists in charged and uncharged forms, which influences the partition coefficient between the different regions of the lipid membrane as a function of the pH.

We start the analysis by considering the orientation order of lidocaine in the DMPC bilayer. In Fig. 2 typical deuterium NMR spectra for the selectively labelled molecule are displayed. The spectra do not show the characteristic Pake features, which may indicate that the sample is not completely homogenous. In Table 1 the deuterium quadrupolar splittings are collected for lidocaine at different concentrations and pHs. The experiments where the concentration of lidocaine was varied were carried out without the buffer. Comparison with the pH dependence, assuming no major interactions with the buffer [59], indicates that the quadrupolar coupling for pH 6.7 is similar to that with the same lipid-anesthetic composition in the absence of buffer. Since the pK_a for lidocaine in a phosphatidylcholine lipid was determined to be ~6.8 [23], it can be assumed that both charged and uncharged forms of lidocaine are present in membranes with no buffer.

Table 1
Experimental deuterium quadrupolar splittings (kHz) for lidocaine-d_2 in the DMPC bilayer

x_{LID}	$\Delta\nu_Q$	$\lvert S_{CD}\rvert$	pH	$\Delta\nu_Q$	$\lvert S_{CD}\rvert$
0.05	1.8	0.014	4.3	2.1	0.017
0.15	2.0	0.016	5.5	1.9	0.015
0.25	1.6	0.013	6.7	1.5	0.012
0.33	1.5	0.012	7.9	1.2	0.010
0.50	1.1	0.009	8.8	1.2	0.010

The concentration dependence was measured in absence of buffer, and the pH dependence is studied on the $x_{LID} = 0.33$ sample, where $x_{LID} = n_{LID}/n_{DMPC}$.

A decrease of the deuterium quadrupolar couplings with increased pH is also observed. In principle, such decrease can be explained by three possible situations: a) molecular orientation of lidocaine in the membrane, b) motional averaging and c) combination of a) and b). At this point we can use the trajectories generated in the MD simulations. In Fig. 3 the probability distribution functions, $P(\beta)$ are displayed, where β is the angle between the C–D vector in the acidic methylene in the lidocaine molecule and the normal to the bilayer. The distributions for the charged and uncharged lidocaine molecules are indeed very different, while there is only a minor effect of the concentration ($x_{LID} = 0.09$ and $x_{LID} = 0.28$). The distribution corresponding to the charged form exhibits a clear maximum at $\beta = 90°$ which is consistent with the molecular orientation (assuming that the long axis is defined by the amine nitrogen and one of the aromatic carbons) parallel to the bilayer normal. In contrast, the distribution of uncharged molecules is much flatter with a weak maximum at $\beta = 0°$, which indicates the orientation perpendicular to the director. In principle, the two CD vectors in a CD_2-group may be different, but it turns out that the distribution functions are essentially equivalent. Yet another way to demonstrate the location and orientation of lidocaine molecules is the density of the nitrogen and aromatic carbon atoms calculated as a function of distance from the bilayer center, $P(r)$. These distributions obtained from the trajectories are displayed in Fig. 4. Nitrogen atoms in charged lidocaine molecules are located closest to the bilayer surface, at a distance of 1.4 nm from the membrane center. This distance corresponds approximately to the location of the carbonyl oxygen atoms in DMPC molecules [60]. The binding of ions to carbonyl rather than to the phosphate group has been previously observed in computer simulations [61–63]. The aromatic carbons in charged molecules are located closer to the center, indicating a

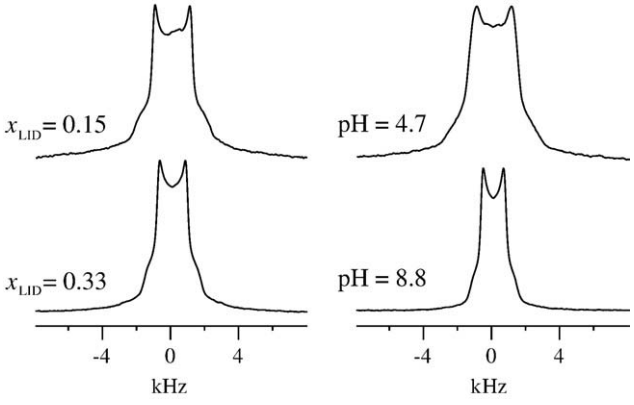

Fig. 2. Typical ^{2}H-NMR spectra of unoriented and hydrated bilayers (at 40 °C) as a function of lidocaine concentration and pH. The traces to the left were collected in samples with no buffer, whereas the spectra to the right were obtained for the $x_{LID} = 0.33$ sample.

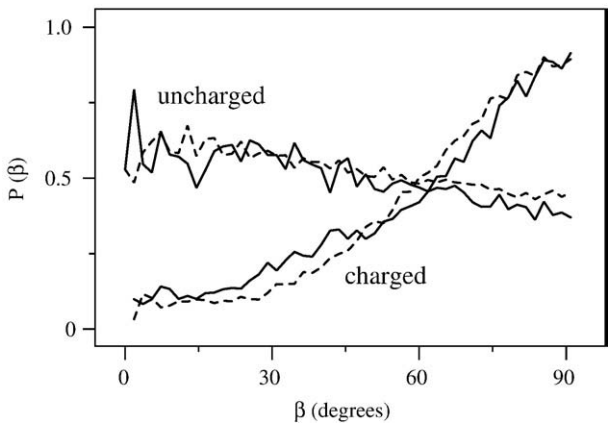

Fig. 3. Normalized orientational distribution functions $P(\beta)$ for the C–D vector of lidocaine, for $x_{LID} = 0.09$ (solid), and $x_{LID} = 0.28$ (dashed) systems.

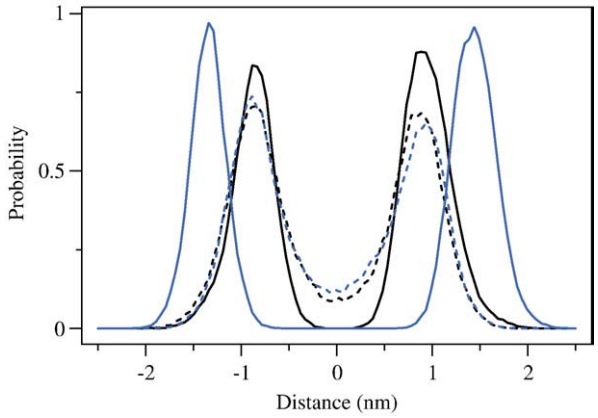

Fig. 4. The probability of finding atoms in lidocaine as a function of distance from bilayer center: nitrogen (blue) and carbon (black) in the charged (solid) and uncharged (dashed) systems. The distributions are calculated for the x_{LID}=0.28 system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecular orientation parallel to the bilayer normal. For uncharged molecules the two distributions essentially coincide, which is consistent with the orientation perpendicular to the normal. Note that in contrast to charged lidocaine molecules there is a probability of finding uncharged species in the center of the membrane, indicating that lidocaine can pass through the bilayer [26]. Thus, we can conclude that the decrease of the quadrupolar splitting with increased pH is a result of the orientation of the lidocaine in the DMPC bilayer. Furthermore, lidocaine molecules in the MD trajectory never leave the bilayer surface, i.e. they are excluded from the water phase. This conclusion is experimentally supported by absence of an isotropic signal in deuterium NMR spectra. There is, however, possibility of a fast exchange between the water and lipid phases and computer simulations are known to overestimate the partition coefficient [64].

The orientational order is conveniently quantified using the order parameters, S_{CD}, which in turn are easily calculated from the trajectory. For the charged/uncharged form we obtain S_{CD}=−0.20/0.09 and S_{CD}=−0.21/0.05 for x_{LID}=0.09 and x_{LID}=0.28, respectively. The negative sign of S_{CD} shows that the average orientation of the C–D vector in charged molecules is perpendicular to the bilayer normal. The order parameter can also be derived from the quadrupolar splitting in the deuterium NMR spectrum using the following relation: $\Delta\nu_Q=3/4q_{CD}|S_{CD}|$, where q_{CD} is the static nuclear quadrupole coupling constant ($q_{CD}\approx$167 kHz for a CD_2 group). The values of the order parameter $|S_{CD}|$ derived from the experimental splittings are included in Table 1. Clearly, the orientational order of lidocaine in the MD simulation is higher than that observed in the experiments. On the other hand, the error in S_{CD} obtained from the analyses of the trajectories is $\sim 1/\sqrt{N}$, where N is the number of lidocaine molecules in the simulation box. Thus, for 36 molecules the error for S_{CD} becomes ~±0.2, indicating that the experimental results are within these limits.

An analysis of the quadrupolar couplings for different sample compositions shows a decrease of the splitting as the lidocaine concentration is increased. This effect is consistent with the general picture that the orientational order in the bilayer decreases upon addition of anesthetics such as tetracaine or lidocaine [24,25,40]. An exception from this trend is the concentration x_{LID}=0.15; we will return to this anomalous behaviour in the discussion of the dipolar couplings.

We now turn to the analysis of ^1H–^{13}C dipolar interactions. In order to investigate the effect of lidocaine on the bilayer, the couplings were measured for different DMPC:lidocaine compositions. Typical slices extracted from a 2D R-PDLF experiment carried out in the x_{LID}=0.15 mixture are displayed in Fig. 5. The slices from the 2D spectra can be extracted provided that the chemical shifts of the sites in DMPC are known [55]. The motionally averaged heteronuclear dipolar couplings, d_{CH}, collected in Table 2, were calculated from the spectral splittings $\Delta\nu$ using the relationship $\Delta\nu$=0.315 d_{CH}, where the coefficient 0.315 is the effective scaling factor for the R-PDLF pulse sequence [46]. We start the analysis of the dipolar couplings by considering the ^1H–^{13}C interaction in the acidic methylene of lidocaine. This interaction reflects the same motion as the quadrupolar splitting determined in a deuterium NMR experiment. The dipolar splitting for lidocaine (Fig. 5) is 83 Hz which corresponds to the d_{CH} coupling of 263 Hz. Assuming that a rigid C–H vector measured in the solid-state corresponds to $d_{CH}^{max}=-22.5$ kHz, an order parameter ($|S_{CH}|=d_{CH}/d_{CH}^{max}$) of $|S_{CH}|$=0.012 is obtained, which is in good agreement with the value of $|S_{CD}|$ in Table 1. We have also extracted the splitting corresponding to the C–H interaction for the *para*-site in the phenyl ring of lidocaine. This splitting is 0.1 kHz, which assuming $d_{CH}^{max}=-23.0$ kHz for an aromatic C–H vector [65] gives $|S_{CH}|$=0.015. In order to unambiguously determine molecular orientation of lidocaine in the bilayer several dipolar couplings i.e. order parameters are required. It is so, because the principal frame of the ordering tensor is not know. An additional complication is the fact that lidocaine is a flexible molecule.

The ^1H–^{13}C dipolar interactions determined in absence of lidocaine are in good agreement with these previously reported for pure DMPC [55]. Recently, the effect of uncharged lidocaine on the segmental order in DMPC was investigated employing deuterium NMR [66]. Using the order parameters determined in that study we can calculate the corresponding dipolar couplings: 5.1, 3.1, 2.6 kHz in pure DMPC, and 4.8, 2.5, 2.0 kHz in x_{LID}=0.30 for sites 2, 12 and 13, respectively. These values can be compared with our dipolar couplings collected in Table 2.

For all sites (except g_2) in the DMPC molecule several C–H vectors exist and can give rise to dipolar interactions, usually however not all couplings can be determined experimentally. A general trend for most fragments of DMPC is an increase at the lowest lidocaine content followed by a decrease of couplings at the higher concentration. The same trend is observed for the quadrupolar splitting derived from deuterium NMR spectra (see Table 1). The decrease of the dipolar

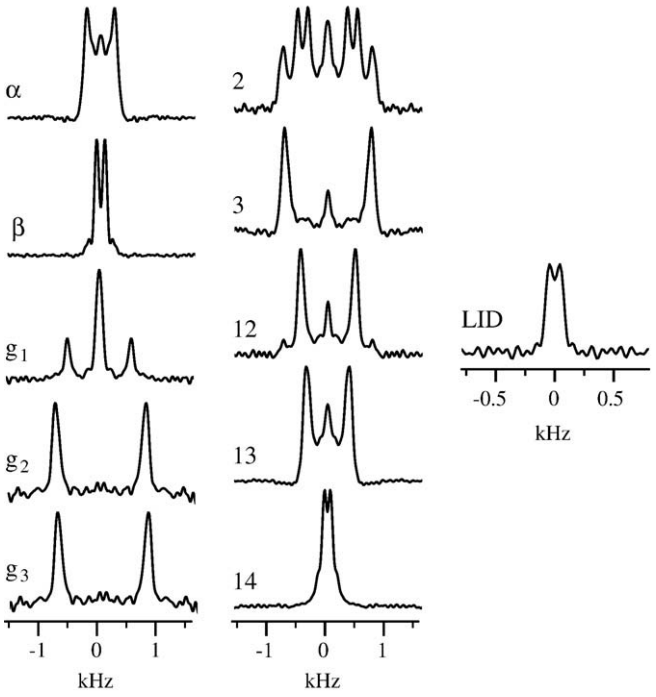

Fig. 5. Selected ^1H–^{13}C dipolar cross-sections through a 2D R-PDLF spectrum of an unoriented DMPC/lidocaine mixture x_{LID}=0.15 acquired at 40 °C.

Table 2
Experimental and calculated from the MD trajectory $^{1}H–^{13}C$ dipolar couplings (kHz) for hydrated DMPC:lidocaine mixtures

Site (DMPC)	Experimental			MD simulations				
	DMPC	x_{LID}=0.15	x_{LID}=0.33	DMPC	Charged		Uncharged	
					x_{LID}=0.09	x_{LID}=0.28	x_{LID}=0.09	x_{LID}=0.28
α	1.3	1.5	1.2	−1.1	0.2	2.0	−0.9	−0.5
				−1.2	−0.2	1.6	−1.1	−1.2
β	0.9	0.5	0.5	−2.1	−1.8	−0.9	−2.0	−1.9
γ	0	0	0	0	0	0	0	0
g_1	0	0	0	−0.2	−0.1	0.2	−0.4	−0.6
	3.4	3.5	3.2	−5.2	−5.0	−4.6	−5.2	−5.0
g_2	4.4	4.3	4.1	4.2	5.0	4.7	4.4	4.4
g_3	5.1	5.0	4.6	4.5	4.0	3.7	4.2	3.7
				6.1	6.2	5.7	6.1	6.0
2	2.0	2.2	2.0	3.4	3.7	3.8	3.6	3.8
	3.1	3.3	3.1	4.0	4.2	4.4	4.0	4.2
	4.6	5.0	4.6	4.0	4.4	4.5	4.3	4.5
3	4.1	4.5	4.1	4.1	4.5	4.7	4.3	4.7
	4.4	4.8	4.4	4.4	4.7	5.0	4.6	5.0
12	2.8	2.9	2.1	2.3	2.6	2.4	2.7	2.9
	2.8	3.0	2.2	2.6	3.0	2.8	2.9	3.0
13	2.2	2.1	1.6	1.9	2.1	1.9	2.1	2.4
	2.2	2.4	1.6	2.2	2.5	2.3	2.4	2.7
14	0.3	0.7	0	0.4	0.4	0.4	0.4	0.4

couplings in the x_{LID}=0.30 sample can be explained by a decreased order in the membrane. Similar observations were reported based on experimental results [25,40,41,67] and computer simulations [26,68]. The initial increase of dipolar interactions upon addition of lidocaine is however more difficult to rationalize. A possible explanation may be an ordering of the membrane created by strong electrostatic interactions where the charged form of lidocaine molecules and several lipids are involved. The effect of increased order when salt is added to bilayers has been previously observed [63]. Yet another possibility may be that lidocaine increases the order in a similar way as the well known effect of cholesterol [69].

The $^{1}H–^{13}C$ dipolar couplings (in Hz) were calculated from the MD trajectory using

$$d_{CH} = -\frac{\mu_0}{16\pi^2}\frac{\gamma_C\gamma_H\hbar}{r_{CH}^3}\langle(3\cos^2\phi_{CH}-1)\rangle$$

where ϕ_{CH} is the angle between the spin–spin vector and the bilayer normal, r^3 is the C–H bond length, and the angular bracket denotes an average over all molecular motions. We assume that the bilayer normal and the z-coordinate of the simulation box coincide. The potential model for DMPC employed in the MD simulation is based on united atoms, which means that no hydrogen atoms were included. Instead, the hydrogen positions were calculated from the carbon coordinates. A consequence of not explicitly including protons in the MD simulation is that vibrational contributions to the $^{1}H–^{13}C$ couplings are to a large extent neglected. The calculated couplings derived from trajectories are included in Table 2. Having access to the coordinates for all atoms we are in position to calculate, from the trajectory, every dipolar interaction. Considering all approximations introduced in computer simulations the general agreement between observed and calculated couplings is satisfactory. In particular regarding the fact that the experimental couplings are determined for a system where both charged and uncharged lidocaine molecules are present. In the couplings calculated from the trajectory we observe again an increase and decrease of the interactions upon increasing the lidocaine content. This trend is particularly pronounced for the charged form of lidocaine. The decrease of the order along the lipid chains is also observed.

In contrast to experimental values, the calculated couplings contain the information about the sign. In fact, at least one coupling changes sign (α) upon addition of lidocaine, which clearly indicates a

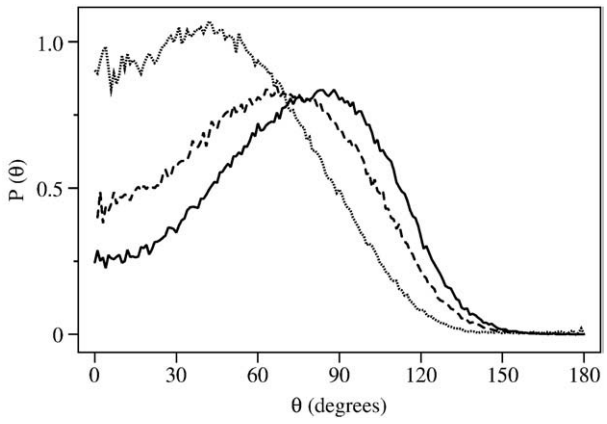

Fig. 6. Normalized distribution functions for the orientation of the P–N vector in DMPC molecule relative the bilayer normal calculated from the trajectories with charged lidocaine: DMPC (solid), x_{LID}=0.09 (dashed), and x_{LID}=0.28 (dotted). Note that the distributions are symmetrized, i.e. they were calculated for both leaflets in the bilayer.

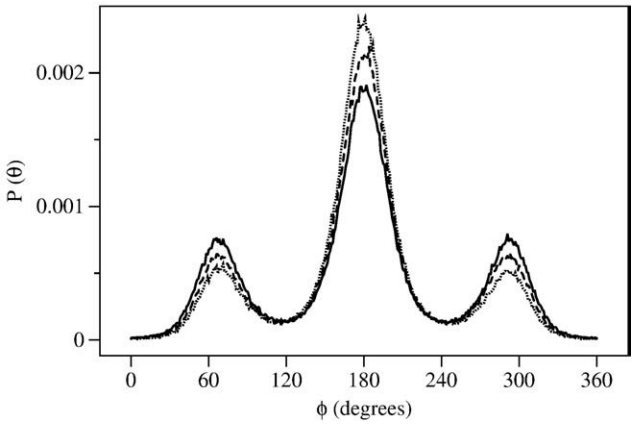

Fig. 7. Normalized distribution functions for the torsion angle $O_{g3}–P–O–C_{\alpha}$: DMPC (solid), x_{LID}=0.09 (dashed), and x_{LID}=0.28 (dotted).

Table 3
Experimental ^1H–^{13}C dipolar couplings (kHz) and order parameters for hydrated DMPC–alcohol mixtures $x_{AlOH}=n_{AlOH}/n_{DMPC}$

Site (DMPC)	DMPC		$x_{MeOH}=0.33$		$x_{EtOH}=0.33$	
	$\|d_{CH}\|$	$\|S_{CH}\|$	$\|d_{CH}\|$	$\|S_{CH}\|$	$\|d_{CH}\|$	$\|S_{CH}\|$
α	1.3	0.06	1.2	0.05	1.2	0.05
β	0.9	0.04	0.8	0.04	0.8	0.04
γ	0	0	0	0	0	0
g_1	3.4	0.15	3.3	0.15	3.3	0.15
g_2	4.4	0.20	4.3	0.19	4.3	0.19
g_3	5.1	0.23	4.9	0.23	4.9	0.23
2	2.0	0.09	2.0	0.09	2.0	0.09
	3.1	0.14	3.0	0.13	3.0	0.13
	4.6	0.20	4.5	0.20	4.5	0.20
3	4.1	0.18	3.8	0.17	3.8	0.17
	4.4	0.20	4.2	0.19	4.2	0.19
12	2.8	0.12	2.4	0.11	2.4	0.11
	2.8	0.12	2.6	0.12	2.5	0.11
13	2.2	0.10	1.9	0.08	1.8	0.08
	2.2	0.10	1.9	0.08	1.9	0.08
14	0.3	0.01	0	0	0	0

conformational transition. This effect is only observed for the charged form of lidocaine. In addition poor agreement is observed between experimental and calculated dipolar couplings for the β-site. The changes of the dipolar couplings for the α and β-sites reflect the orientation of the P–N vector in the DMPC molecule. In Fig. 6 we display the distribution functions for the orientation of the P–N vector with respect to the bilayer normal. These distributions are calculated from the trajectories with the charged lidocaine whereas the three corresponding distributions for the uncharged systems are identical (not shown). The distributions in Fig. 6 result in order parameters, S_{PN}, −0.202, −0.137, and 0.003 for pure DMPC, $x_{LID}=0.09$ and $x_{LID}=0.28$, respectively. Note that the detailed information about the orientation of the P–N vector is lost in the experimental dipolar couplings, simply because the sign of the interaction is not known. The sensitivity of the P–N vector to the charge effects has been previously investigated using computer simulations [63,70,71] and NMR spectroscopy [72–74]. In these investigations it was concluded that the average angle of the P–N vector decreases upon addition of salt, which is consistent with our results: 79, 72 and 69° for the systems with 0, 12, and 36 charged lidocaine molecules respectively. In the three systems with uncharged lidocaine the average angle between the P–N vector and the bilayer normal is 79°. We also find that the only conformational parameter in the choline group of DMPC that exhibits lidocaine concentration dependence is the torsion angle O_{g3}–P–O–C_α The distribution for this angle is displayed in Fig. 7; clearly, the *trans* conformation, $\phi=180°$, is enhanced upon increasing the concentration of charged lidocaine. Again, no lidocaine concentration dependence is observed in the uncharged system (not shown).

We have also collected ^{31}P NMR spectra (not shown), but the line shapes were not affected by the presence of lidocaine. The chemical shift tensor of ^{31}P in DMPC is of the order of 50 ppm [75] and remained essentially constant (within 5 ppm) upon increased concentration of lidocaine. It may be so that even if the PN vector is reasonably sensitive to the lidocaine content, the O_{g3}–P–O–C_α torsion angle (see Fig. 7) and thus the CSA tensor of ^{31}P are not.

In addition to lidocaine we have experimentally investigated the effect of two alcohols, methanol and ethanol, on the DMPC bilayer. The experimental ^1H–^{13}C dipolar couplings for mixtures of DMPC and the two alcohols are collected in Table 3. The addition of small alcohols has a limited influence on experimental dipolar interactions, in fact the reductions are similar to these determined for DMPC:lidocaine at the same molar ratio. Ethanol binding to lipids has been investigated using deuterium, phosphorus an proton NMR [32,33,42,76]. In particular, the effect of ethanol addition on the local order in the hydrocarbon chains was investigated using deuterium NMR in DMPC [32,76] and in POPC [42]. There the trends of the order parameter are similar to our observations. The order parameters for α and β carbons in Table 3 are in perfect agreement with those determined using deuterium NMR [76]. The ordering in the glycerol fragment exhibits a similar decrease upon addition of alcohols as the other fragments of the lipids. An analysis of a trajectory generated in computer simulations of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and dipalmitoyl phosphatidylcholine (DPPC) bilayers with methanol and ethanol [30] indicated an enhanced local order for both alcohols. These studies were, however carried out on significantly more concentrated systems $x_{AlOH}\sim 0.7$.

4. Conclusions

In this paper we present NMR investigation of the effect of anesthetics (lidocaine, ethanol and methanol) on the DMPC bilayer. Deuterium NMR was employed to study the orientational order of selectively labelled lidocaine-d_2 and ^1H–^{13}C dipolar couplings were measured using 2D local field spectroscopy under sample spinning conditions. In addition, previously generated molecular dynamics trajectory was analyzed with focus on NMR parameters. The computer simulations were carried out with charged and uncharged forms of lidocaine in the bilayer so to reflect the different pH conditions. The experimental quadrupolar splitting in the acidic methylene group of lidocaine exhibited a clear pH and concentration dependence. At the low pH, the charged lidocaine molecules are oriented parallel to the bilayer normal whereas the uncharged form (at high pH) is on average oriented perpendicular to the normal. A clear effect of anesthetics was also reflected in the dipolar couplings in DMPC. In particular, the addition of the charged form of lidocaine increased the local order and resulted in orientational changes of the P–N vector in the choline fragment of DMPC. Reasonable agreement between experimental dipolar interactions and the calculated counterparts was observed. The experimental system contains both charged and uncharged lidocaine, whereas in the computer simulations we can separate the effect from charged and uncharged forms of lidocaine on the bilayer structure.

Before closing this paper we wish to comment on how our experimental and computational observations may be related to the mechanism of lidocaine action as an anesthetic. Clearly, the lidocaine–membrane interaction perturbs the bilayer structure. We can only speculate that this change in the local order will also affect the lipid–protein (ion channel) interaction which is claimed to be essential for the anesthetic activity. Furthermore, we note that the uncharged form of lidocaine can be transported across the membrane, which is not possible for the charged form. Thus the pH plays most likely a significant role for the activity of lidocaine as an anesthetic.

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