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Role of the lipid matrix in the action of local anesthetics☆

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ABSTRACT

The binding of local anesthetics (LAs) to cell membranes is required for LAs to reach the target ion channels, but lipid interactions may also play a role in a purely membrane-mediated mode of activity. Here, we used solid-state NMR and further biophysical techniques to characterize the effect of six LAs covering a wide range of structures and properties (benzocaine, bupivacaine, mepivacaine, lidocaine, procaine, QX-314) on membranes. Membrane partitioning log D values (between 2.1 and 3.7) varied little with pH, in contrast to octanol partitioning. Membrane thinning was induced by most LAs, except for benzocaine. A conformational change in the lipid headgroup was observed, with a pronounced dependence on the protonation state, indicating the importance of the positive charge that is maintained by most membrane-bound LAs. We found stabilization of negative membrane curvature in the case of benzocaine, and of positive curvature in the case of bupivacaine, procaine, mepivacaine and, most pronouncedly, for QX-314. Comparing the LAs with respect of their influence on membranes as observed in the different experiments, benzocaine and QX-314 were always found at either extreme of the scale, with bupivacaine and lidocaine closer in their effect to benzocaine. This order of influence correlates with the depth of membrane insertion and with the protonation state, both of which were identified as key factors for LA behavior. Finally, we found indications that LAs are able to alter the activity of bacterial mechanosensitive channels without any expected LA binding sites, thus supporting a membrane-mediated activity of LAs.

1. Introduction

Local anesthetics (LA) are important drugs routinely used in every day medical care. They share a characteristic amphipathic (amphiphilic) molecular architecture, consisting of two parts, a hydrophobic, mostly aromatic moiety, and a hydrophilic moiety, which often possesses a protonatable amine. Both segments are connected by a polar linker, either a carboxyamide or an ester link, defining two groups of LAs which differ in their metabolic stability. (See Fig. 1 for typical examples as used in this study.) This structure endows LAs with an amphiphilic character, resulting in a relatively high octanol/water partitioning with log P values in the range of 2–3 on one hand, and, at physiological pH, a cationic amine with a pK_a value between 8 and 9 [1,2] (Fig. 1).

Voltage gated ion channels have been established as the major target with respect to the anesthetic and analgesic activity of LAs. Several binding sites for LA molecules have been suggested within the channels, which are accessible from the cytosol or fenestrations within the membrane core [3–5]. Hence, as for many drugs, to reach the target, LAs need to possess a balanced solubility in both aqueous medium as well as in the lipid membrane. Therefore, the interaction of LAs with the lipid bilayer

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has been of interest to understand their pharmacology.

However, the interaction of LAs with membranes has also been discussed to constitute a mode of activity itself [6]. By changing the physical properties of the lipid bilayer, LAs could indirectly influence membrane protein conformation and function, rather than directly by specific ligand-receptor interaction. Such a membrane-mediated mechanism would be able to explain the wide range of activities which has been described for LAs, involving receptors and channels different from voltage gated ion channels (see [6] and references therein).

The change of the lateral pressure profile when amphiphiles insert into the lipid bilayer has been identified as one way of a membrane-mediated action on membrane proteins [7]. Membrane proteins are adapted to the lateral pressure profile across the bilayer by their cross-sectional shape, and a change in the lateral pressure profile can then shift the equilibrium between different conformational states, e.g., between closed and open channel conformations [7,8]. Bacterial mechanosensitive channels, such as MscL and MscS, possess a pronounced response on changes in the lateral pressure in the membrane. E.g., these channels can be opened by the insertion of amphiphiles in one of the two leaflets of the bilayer [9]. Also, the LAs procaine and tetracaine have been found to change the opening threshold of mechanosensitive channels [10]. Accumulating in one leaflet of the bilayer they have induced curvature of the membrane, which has resulted into affecting the mechanosensitive channels without a specific ligand-receptor interaction.

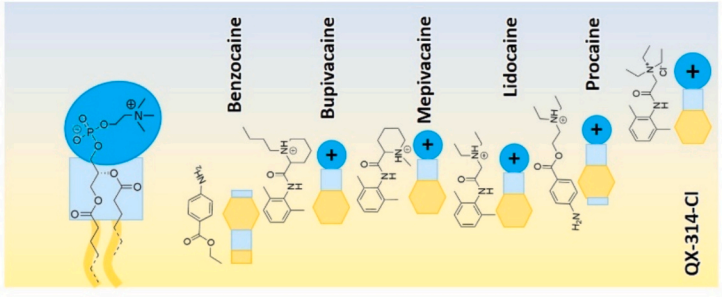
LAs have also been found to dissolve lipid micro domains, also termed lipid rafts, and in this way influence integrity of membrane protein complexes or assemblies which are recruited in such lipid domains [11]. This way, for example, lidocaine has been able to prevent malaria infection by inhibiting Gsα mediated signal transduction [12].

The effect of LAs on lipid bilayers has been addressed by a number of studies. LAs change the physical properties of lipid membranes, such as fluidity and bilayer thickness. For example, they have been found to decrease the fluidity of the membrane core whilst increasing it in the lipid headgroup region [13], and to cause membrane thinning [14]. Furthermore, LAs modify the electric P-N dipole in zwitterionic lipid membranes, and influence the surface charge [15,16].

Solid-state NMR, in particular experiments like the “electrometer” based on solid-state ^{2}H NMR introduced by Seelig [16–19], NMR derived order parameters [15,18,20] and magic angle spinning ^{1}H NMR [21,22] have been particularly useful to determine the localization of LAs within the lipid bilayer. Also, NMR studies on ^{2}H labeled LAs have given insight into the mobility and level of insertion of LAs [15,23]. Altogether, despite their ability to cross the entire membrane, LAs seem to possess a preferred residence region in the bilayer, termed “transient site” [22]. In their protonated form, LAs have been found to reside with the aromatic moiety at or slightly below the glycerol/chain boundary, bringing the positive charge of LAs in proximity of the phosphate of zwitterionic lipids [21,22]. Cholesterol has been observed to influence the position of the two uncharged LAs benzocaine and butamben, which reside near the glycerol in the presence of glycerol and in the upper chain region in the absence of cholesterol [23].

In our present study, we aimed at comparing a representative set of LAs, all studied with a wide range of methods, in order to gain a comprehensive view of the interaction of LAs with lipid membranes. This way, we intended to systematically extend the current knowledge of the behavior of LAs in membranes, which so far is based mostly on studies focusing on a narrow range of LAs, or using a limited set of methods.

As a representative set, covering both ester and carboxyamide LAs, as well as different protonation states and polarities, we chose the LAs benzocaine, bupivacaine, mepivacaine, lidocaine, procaine and QX-314 (Fig. 1). Benzocaine lacks the easily ionizable amine of typical LA molecules, and hence, with a pK_a of 2.65, is uncharged at physiological pH. All other LAs of the studied set possess a protonatable amine, and are predominantly charged under physiological conditions. QX-314, with its quaternary amine, constitutes a permanently charged, membrane-impermeable LA. It has been used as nociceptor-selective blocker, where it was applied together with agonists of TRP channels to enter the nociceptor neuron cytosol [24]. The pK_a values of bupivacaine,



	Benzocaine	Bupivacaine	Mepivacaine	Lidocaine	Procaine	QX-314-Cl
pKa	2.65	8.21	7.92	8.19	9.06	12.14*
logP octanol/water	1.91	3.41	1.95	2.48	1.91	undef.
logD octanol/water, pH7.4	1.91	2.54	1.32	1.63	0.23	-1.47
logD membrane/water, pH 7.4	3.73 ± 0.22	3.07 ± 0.17	2.08 ± 0.11	2.13 ± 0.08	2.44 ± 0.08	2.42 ± 0.08
critical micelle concentration, pH 7.4 / mM	7.9 ± 0.7	51 ± 8	64 ± 8	113 ± 6	85 ± 5	89 ± 8

Fig. 1. The studied local anesthetics and their basic physicochemical parameters, depicted in the amphiphilic region (blue-yellow gradient) of a lipid bilayer. The molecular structures are shown as chemical structure and cartoon representing their building blocks. The molecular structures of the LAs are composed of an amine, which is protonated at neutral pH (blue circle), and a hydrophobic aromatic segment (orange hexagon), bridged by a polar carboxyamide or ester linker (light blue square). Benzocaine lacks the protonated group at neutral pH, and possesses a different architecture from the other LAs. QX-314, on the other hand, is permanently protonated. The local anesthetics are depicted at their approximate localization within the bilayer in relation to the indicated lipid molecule on the left, as found in this study. The dissociation constants pK_a of bupivacaine, mepivacaine, lidocaine and procaine are taken from Strichartz et al. [1]. The pK_a value of benzocaine is the average for computed values using chemicalize and pubchem. (* note, that the pK_a value of QX-314 refers to the amide of the linker.) The octanol/water partitioning log P and log D values were obtained from Strichartz et al. [1], except for the value of QX-314 from Butterworth et al. [2]. The membrane partitioning log D values and critical micelle concentrations are from this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mepivacaine, lidocaine and procaine fall in the range of 8.2 to 9.1 (Fig. 1). The octanol/water partitioning of the uncharged species is characterized by log P values of ~1.9 for benzocaine, mepivacaine and procaine (Fig. 1). Lidocaine (log P = 2.48) and bupivacaine (log P = 3.41) are more hydrophobic in their uncharged form. The octanol/water partitioning, taking into account both the charged and uncharged species, is reflected by the log D value, where bupivacaine possesses the highest value of log D = 2.54 at pH 7.4. Benzocaine, mepivacaine and lidocaine are moderately hydrophobic with log D values in the range of 1.6 to 1.9 at physiological conditions, procaine exhibits a log D of 0.23 approaching the polar regime, and the permanently charged QX-314 clearly hydrophilic with a log D of −1.47 at pH 7.4 (Fig. 1).

From the different dissociation propensities and polarities we expect the LAs of this set to insert into the lipid bilayer at different insertion depths, and hence exert a different influence on the bilayer. Particularly, given the postulated membrane-mediated effect on membrane proteins [6], LAs could modulate the lateral pressure profile differently depending on their insertion depth, and hence might cause selective conformational changes in membrane proteins or channels. Insertion depths, as we expected them from pK_a and log D values, are indicated in Fig. 1. The permanently uncharged benzocaine should be able to penetrate most deeply into the bilayer, and the permanently cationic QX-314 is very likely retained near the charges of the lipid headgroup. The other LAs should assume intermediate positions, with the more hydrophobic bupivacaine potentially inserting more and the rather polar procaine inserting less deeply.

To substantiate the expected insertion into bilayers and resulting interactions with membranes, we conducted experiments to examine changes of different membrane properties and lipid segments. At first, we characterized basic parameters, such as the membrane partitioning and critical micelle concentration (CMC). The influence of LAs on the lipid acyl chains and membrane thickness was studied by ^{2}H NMR on chain-deuterated lipids and X-ray scattering. The response of the lipid headgroup to the presence of LAs was evaluated by ^{2}H NMR "electrometer" experiments, and the preference of LAs for positive or negative lipid curvature was evaluated using the lamellar-to-hexagonal transition of DOPE lipids [16,17]. Finally, we probed for a potential membrane-mediated influence of LAs on membrane proteins, by comparing the activity of mechanosensitive channels in the presence of the six different LAs.

2. Materials and methods

2.1. Materials

The lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) sodium salt (DMPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, Alabama). For ^{2}H NMR we used DMPC deuterated in both acyl chains (DMPC-d_{54}) and DMPC deuterated in the choline of the headgroup (DMPC-d_4), purchased from Avanti Polar Lipids. The local anesthetics benzocaine, bupivacaine hydrochloride monohydrate, lidocaine hydrochloride monohydrate, lidocaine, and procaine hydrochloride were purchased from Sigma-Aldrich. Mepivacaine hydrochloride was obtained from European Pharmacopoeia reference standards (Strasbourg, France), and QX-314 chloride from Tocris Bioscience (Wiesbaden-Nordenstadt, Germany). Coumarin-6 and soybean azolectin were obtained from Sigma-Aldrich.

2.2. Membrane partitioning

Membrane partitioning was measured after exposing LAs to lipid vesicles, using ^{1}H solution NMR to quantify the unbound LA fraction. Two different procedures were applied, (A) measurement of the LA signal in presence of large unilamellar vesicles (LUV) and (B) separating the aqueous phase with unbound LAs after reconstitution into multilamellar vesicles (MLV) by ultrafiltration.

For (A), a 2 mg/ml lipid suspension of DMPC was converted into LUVs by extrusion using 100 nm polycarbonate membranes. LAs were added as hydrochloride (or with additional equimolar HCl) in a 1:20 LA: lipid ratio. The suspensions were prepared in three different pH buffers: pH 5.0 (phosphate buffer, 10 mM), pH 7.4 (phosphate buffer, 10 mM) and pH 10.0 (borate buffer, 10 mM). Pairs of samples, with and without lipids, were prepared for each LA. The samples were examined using a 300 MHz ^{1}H NMR spectrometer (Avance I, Bruker) employing a water gate sequence, and selected signals of the LA, mostly of the aromatic moiety, were quantified. The integrals of the sample without and with lipid were used to obtain the ratio x of the unbound fraction of LA molecules and total number of LA molecules. The log D value is then given by

$$\log D = \log[x/(1-x)\ m_W/m_L\ \rho_L/\rho_W]$$

with the ratio of water and lipid weight of the sample, m_W/m_L, and the water and lipid mass densities ρ_W and ρ_L. The lipid density was calculated as $\rho_L = 1.043$ g/ml, assuming a lipid molecule volume of 18 Å × 60 $Å^2$ = 1080 $Å^3$.

In approach (B), multilamellar vesicles were prepared from 10 mg DMPC or 5 mg DMPC + 5 mg DMPG. The lipids with LA were mixed in methanol in a molar ratio of LA:lipid of 1:20, the solvent was removed with nitrogen gas and vacuum overnight, and the resulting lipid film was hydrated with buffer to yield a 10 mg/ml suspension. Samples with pH 5.0 and 7.4 were hydrated with 100 mM phosphate buffer, samples with pH 10.0 were prepared with 10 mM borate buffer. All LAs were used as hydrochlorides, or with additional equimolar HCl. The samples were vortexed and freeze/thawed 5 times, ultrasonicated until they became clear, and incubated for 2 h at 40 °C. To obtain the aqueous phase with unbound LA, the samples were filtered using a commercial ultrafiltration setup (Amicon Ultra-0.5 Centrifugal Filter Device), employing spinning for 30 min at 14000 g. The flow-through was collected and 400 µl of it were mixed with 50 µl D_2O and 50 µl TSP (3-(trimethylsilyl) propionic acid) solution (0.1 mg/ml) as internal standard for ^{1}H NMR analysis. The unbound LA was quantified using the signals of the aromatic region of ^{1}H NMR spectra acquired using water suppression (WATERGATE), employing a 600 MHz Bruker Avance III spectrometer. Log D values were calculated as described above.

2.3. Critical micelle concentration

To determine the CMC of the examined LAs, coumarin-6 was used as fluorescent probe that is recruited by micelles due to its hydrophobicity and exhibits an augmented fluorescence inside the more hydrophobic environment of a micelle [25]. The fluorescent dye was dissolved in dichloromethane at a concentration of 6 µM, and 50 µl from this stock were transferred to an empty vial for evaporation of the solvent. Solutions containing different concentrations of LAs were then added to the wells of a 96-well-plate, and left on a shaker overnight. Emission of the fluorescent probe was read out by a Tecan Spark plate reader. The CMC was determined from the fluorescence intensity (logarithmic values) as a function of LA concentration as the point where the fluorescence intensity started to rise with concentration. This point was determined as the intersection of two linear fits, fitting the data of concentrations below and above the beginning of the intensity rise.

2.4. Solid-state NMR

The oriented samples for solid-state ^{2}H NMR on chain deuterated lipids were prepared from multilamellar vesicles, containing LA at LA: lipid molar ratio of 1:0.5, 1:1, 1:2, 1:3, 1:5, 1:10, 1:20 and 1:40. First, 4.5 mg DMPC, 0.5 mg DMPC-d_{54} and the amount of LA needed for the desired LA:lipid ratio were co-dissolved in methanol. The solvent was removed under nitrogen gas and under vacuum, and 250 µl of 2 mM

HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate) buffer (pH 7.2) was added to the lipid film. The suspension was vortexed and homogenized by 5 freezing/thawing cycles, and spread on 5 glass slides (7.5 mm × 12 mm × 0.06 mm, Marienfeld). After drying the slides were stacked, covered with an additional glass slide and incubated in 100% relative humidity overnight at 48 °C. The hydrated sample was wrapped in parafilm and cling foil to prevent dehydration.

Oriented samples for ^{2}H NMR on headgroup ^{2}H-labeled lipids were prepared for LA:lipid ratios of 1:0.5, 1:1, 1:2, 1:3, 1:5, 1:10, 1:20 and 1:40. As first step, 4.75 mg DMPC and 0.25 mg DMPC-d_4 were co-dissolved with the desired amount of LA in methanol. The solution was spread directly onto 5 glass slides (7.5 mm × 12 mm × 0.06 mm, Marienfeld), which were then further processed as described above.

To determine the lamellar-to-hexagonal phase transition temperature, oriented samples with DOPE were prepared containing LA at LA:lipid molar ratios of 1:3, 1:10, and 1:30. The samples were prepared as described above for the samples containing DMPC-d_4.

Solid-state NMR experiments were performed on a Bruker Avance III HD 500 MHz spectrometer using a double-tuned HX static probe tunable to ^{2}H and ^{31}P resonance frequencies. The solid-state ^{2}H NMR spectra were recorded using a solid-echo pulse sequence (90° pulse: 6 μs, second 90° pulse: 6 μs, inter-pulse delay: 30 μs, repetition delay: 0.5 s, number of scans: ~8000).

Solid-state ^{31}P NMR measurements were conducted employing a Hahn-echo pulse sequence (^{31}P-90° pulse: 6 μs, 180° pulse: 12 μs, inter-pulse delay: 20 μs, repetition delay: 2 s, ^{1}H-decoupling: 10 kHz, number of scans: ~300). Spectra were recorded for temperatures in the range from 272 to 327 K in steps of 3 K.

2.5. Grazing incidence small angle X-ray scattering (GISAXS) experiments

The GISAXS samples were prepared each on one glass slide with a size of 12 × 18 × 1 mm. For the sample preparation, 1.5 mg DMPC dissolved in methanol together with the respective amount of LA to achieve the molar ratios LA:lipid of 1:1, 1:3, 1:5, 1:10 and 1:20. The mixture was spotted on the glass slides, and the solvent was evaporated on air and under vacuum. The samples were hydrated in 100% relative humidity for 12 h at 48 °C.

The GISAXS experiments were performed at the KIT light source (Karlsruhe Institute of Technology) using the SCD beamline, operating with a wavelength of 1.39 Å. The sample was placed in a closed chamber (using Kapton™ foil) with controlled humidity and temperature (via Peltier element), and the incident beam was adjusted to hit the covered glass surface at grazing incidence. A linear detector of 8 × 64 mm was placed at a distance of 70 cm from the sample, and the direct beam was shielded using a beam stop. The spot size was 0.5 × 0.5 mm. The Bragg reflections were evaluated to obtain the bilayer-to-bilayer repeat distances.

2.6. Patch clamp experiments on mechanosensitive channels

For patch clamp experiments, mechanosensitive channels of small conductance (MscS) were overexpressed in *E. coli*, and extracted from the membrane fraction using Triton X-100 detergent. The channel protein was reconstituted into soybean azolectin multilamellar vesicles at a protein:lipid ratio of 1:50 w/w. An amount of 10 mg azolectin was suspended in buffer (200 mM KCl, 5 mM HEPES, pH 7.2) and mixed with MscS protein in detergent. After incubation for 1 h at 4 °C, the detergent was removed by incubating with ~10 mg adsorbent (Biobeads SM-2, BioRad) for 3 h at room temperature. After removing the adsorbent from the suspension, the membranes with the reconstituted protein were obtained by ultracentrifugation. The pellet was spotted on a glass slide and dried. By rehydrating with 100 μl buffer, vesicles were created.

To determine the influence of the LA on the channel opening, a membrane patch was isolated at the tip of the patch clamp pipette and the current recorded, whilst applying a suction (negative pressure) ramp of −50 mmHg over 2 min, reaching current saturation. The experiment was repeated after adding 7.5 μl of a 50 mM LA solution to the bath and incubating for 1 min. This process was repeated with successive additions of LAs, until the patch broke or the channel activity disappeared. Typically, around 15 repetitions of the addition step were performed. The series was repeated up to 3 times.

The recorded current curves were analyzed by applying Boltzmann distribution analysis, and determining the negative pressure required to open half of the channels. To obtain comparability between the different patches, the pressure values were normalized using the first measurement from the patch without LA. As parameter to describe the change in opening threshold induced by the LA, we determined the slope of the function given by the opening pressure as a function of added LA.

3. Results and discussion

3.1. Membrane partitioning and critical micelle concentration

The amphiphilic structure and a positive charge are characteristic properties of LAs allowing them to interact with lipid membranes. Octanol/water partitioning and protonation propensity, expressed typically as log P (or the pH dependent log D value, referring to the charged and uncharged fraction) and pK_a value, are regarded as basic pharmacologically relevant parameters describing the ability to reside in and to traverse lipid membranes (see Fig. 1 for characteristic values). More informative for the interaction with membranes, however, is the partitioning into lipid bilayers, as octanol only presents an idealized model for the central region of a bilayer, namely the hydrophobic core. We therefore determined the membrane partitioning coefficients of the selected LAs in zwitterionic DMPC and anionic DMPC/DMPG membranes. LAs were incubated with unilamellar vesicles, or, in a second set of experiments, mixed with lipids before vesicle formation, and the unbound fraction of LAs was quantified using solution ^{1}H NMR (Fig. 2). In the former case of co-incubation with unilamellar vesicles (see Materials and Methods - A), the concentration of the total LA and the unbound fraction were determined from ^{1}H spectra in the absence (Fig. 2b) and presence (Fig. 2c) of lipid vesicles, by integrating selected sharp signals in the aromatic region (Fig. 2c). Any bound LA gives rise to extensively broadened and hardly visible signals in solution ^{1}H NMR due to the slow tumbling of the lipid vesicles and the resulting lack of motional averaging, which is apparent in the broad and largely reduced residual lipid signal (Fig. 2d). In the second preparation method (see Materials and Methods - B), the free LA was determined from ^{1}H spectra of the flow-through after ultrafiltration. Both assays yielded comparable results (Fig. 2e).

At first, when comparing the membrane/water partitioning with the octanol/water partitioning at physiological pH (Fig. 1), we already notice a significant difference. LAs exhibit higher log D values for membrane/water compared to log D for octanol/water, indicating a stronger binding affinity to membranes than to octanol. Even benzocaine, the only LA of the studied LAs which is uncharged, possesses a higher binding to membranes than to octanol, showing that the membrane core, which resembles octanol in hydrophobicity, is not the only lipid region involved in LA binding. Furthermore, whilst the octanol/water log D values vary among the different LA, they cover a narrower range in the case of membrane/water partitioning. Particularly, procaine and QX-314, which differ remarkably from the other LAs in their octanol/water log D values of near 0 and even negative value, exhibit membrane/water partitioning well within the range of the other LAs. The polarity and higher pK_a values of procaine and QX-314 are hence also not determining the membrane binding, the lipid headgroup hence does not play a defining role in LA binding, either. It is, therefore, indeed the amphipathic character of the LAs involving both interactions with the bilayer core and headgroups, which matters for the interaction with membranes.

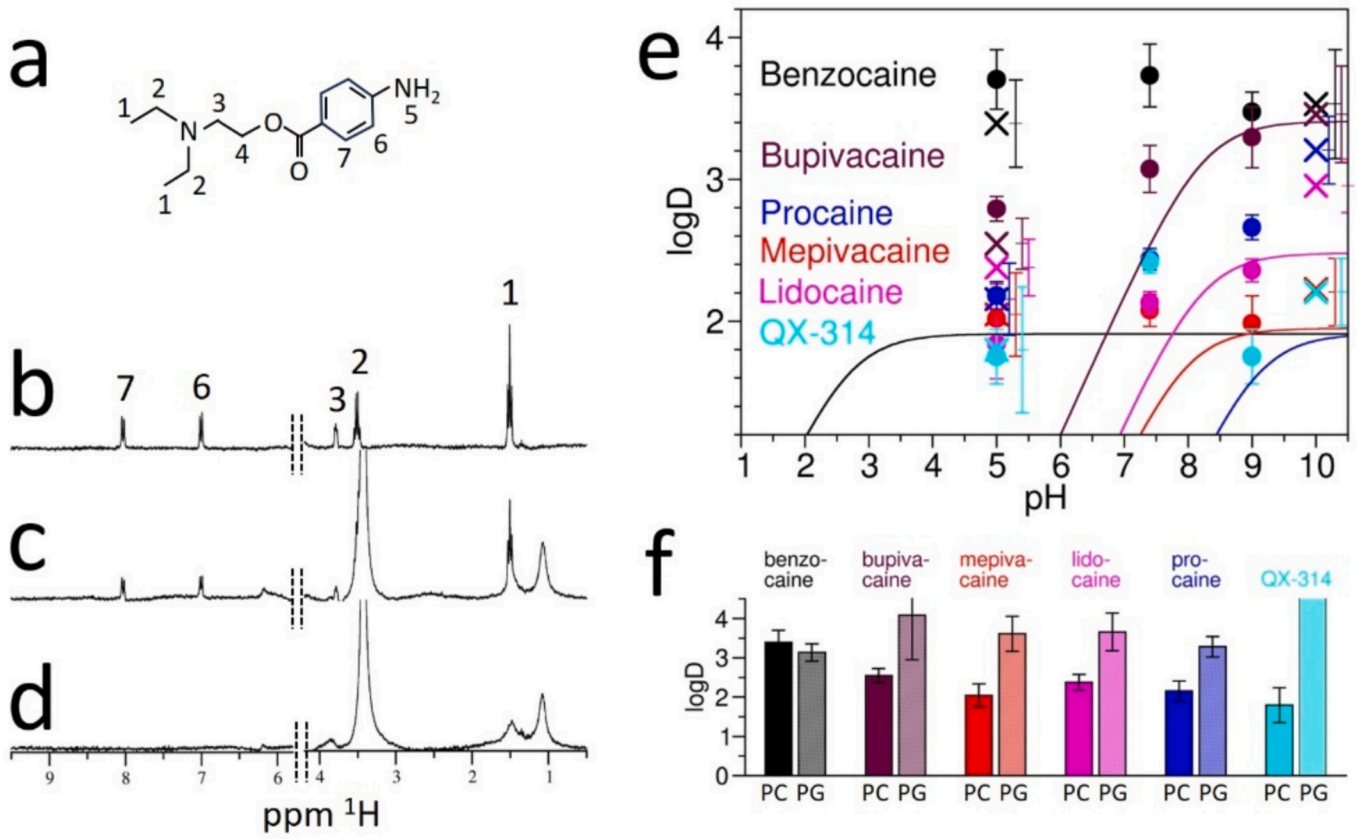


Fig. 2. Membrane partitioning of the local anesthetics examined in this study. To obtain the ratio of LAs inserted into the membrane and in the aqueous phase, solution ^{1}H NMR was employed (a–d). Several signals, which could be assigned to moieties in the LA as shown for procaine as an example, were used for quantification (a). The LA signals were measured in the absence (b) and presence (c) of large unilamellar vesicles of DMPC. In the presence of lipids, the bound LA did no longer contribute to the solution ^{1}H NMR signal due to extensive broadening, resulting into a reduction of the LA signals (b–c). Strongly reduced residual lipid signals were identified using a spectrum of lipid only (d). The membrane partitioning was measured as a function of pH and converted to log D values (e). Besides obtaining the partitioning from ^{1}H NMR in the presence of vesicles (circles in e), we performed additional experiments employing ultrafiltration to separate bound and unbound LA (crosses in e, with error bars displaced to the right for better visibility). For comparison, calculated log D values for octanol/water are shown (e, solid lines). The membrane partitioning in DMPC membranes (f, bars labeled PC) was compared with that in DMPC:DMPG (1:1) membranes (f, bars labeled PG), both measured at pH 5 using the ultrafiltration method.

A deviation of the interaction of LAs with membranes from a mere partitioning into a hydrophobic medium is also seen from the pH dependent log D values (Fig. 2e). Whilst the log D values calculated from octanol/water log P values and pK_a values (Fig. 1) drop below the pH corresponding to the pK_a value, the membrane/water log D values vary only moderately with pH. The charged species hence also partitions into the membrane including a polar headgroup region, whereas it would not dissolve in octanol lacking a similar polar part.

At high pH > 9, the log D values of membrane and octanol partitioning agree well, except for two cases: For benzocaine and procaine, the membrane partitioning is higher than the octanol partitioning. At these pH values, the log D value represents the partitioning of the uncharged species, (the log P value), hence benzocaine and procaine seem to possess a more polar molecular structure when disregarding the protonation, which is indeed reflected in their comparatively low octanol/water log P values (Fig. 1).

To further shed light on the role of the protonation of the LAs, we also compared partitioning of the studied LA selection into zwitterionic DMPC membrane with that into anionic DMPC/DMPG (1:1) membranes (Fig. 2f). These experiments were done at pH 5, where all LAs, except for benzocaine, are present in their protonated form. Except for benzocaine, all LAs showed a higher affinity to membranes with anionic lipids. The difference in log D values between DMPC and DMPC/DMPG lipids was similar for bupivacaine, mepivacaine, lidocaine and procaine, whilst QX-314 exhibited the most pronounced preference for DMPC/DMPG, with 100% partitioning into DMPC/DMPG membranes. These results demonstrate again the important role of the positive charge for the interaction with membranes, as membrane partitioning of the charge-neutral benzocaine is not influenced by the presence of anionic lipids, and that of the permanently charged QX-314 is most affected.

The critical micelle concentration constitutes a further parameter characterizing the solubility in hydrophobic or hydrophilic environments. We determined the critical micelle concentrations of the studied LAs at pH 7.4 using a fluorescent dye soluble in micelles (Fig. 1). The lowest value of ~8 mM was found for benzocaine, whereas all other LAs exhibited critical micelle concentrations in the range of ~50 mM to ~110 mM. The lack of a protonatable amine seems hence to be responsible for the higher propensity of benzocaine to form micelles. Otherwise, the critical micelle concentrations seem not to correlate with pK_a or log P values, indicating that further factors such as molecular packing might play a role as well in micelle formation.

3.2. *Influence on lipid acyl chain order and membrane thickness*

Amphiphilic guest molecules in membranes have been found to influence chain order and membrane thickness. Particularly, when residing near the headgroup region, for example, amphipathic antimicrobial peptides can cause membrane thinning [26,27], whereas deeply inserted molecules, such as cholesterol, have been found to increase the membrane thickness [28]. Membrane thinning is caused when a guest

molecule requires space in the headgroup region, resulting in increased conformational freedom in the chain region, which then expands laterally leading to an overall thinner membrane [26]. Membrane thickening can be the result of increased acyl chain *trans-gauche* order, enforced by a guest molecule residing in the lipid chain region [28]. Both effects are associated with a change in lipid chain order, which we address here by solid-state ^{2}H NMR using chain-deuterated DMPC (DMPC-d_{54}) as a probe (Fig. 3).

We employed mechanically oriented bilayer samples of DMPC containing 5% DMPC-d_{54}, where LAs were reconstituted at different LA:lipid ratios in the range of 1:40 to 1:0.5. In the ^{2}H NMR spectra, each methylene segment of the deuterated lipid gives rise to a doublet, with a splitting which is narrowed by motional averaging, and thus representing the molecular order of the respective chain segment (Fig. 3a). (Note, that the second, minor component of <20% in some of the spectra, possessing splittings of half width, is due to imperfectly aligned membranes.) The quadrupole splittings were converted into an order parameter profile (see Fig. 3b for a selected LA:lipid ratio), from which the area per lipid molecule (Fig. 3c) and the thickness of the bilayer chain region, 2 D_C, (Fig. 3d) can be derived as described in [26]. The LAs were found to influence the order parameter, lipid area and hydrophobic thickness in two different ways. On one hand, benzocaine was found to increase the chain order slightly near the headgroup region (Fig. 3b), which reflects a decrease in the area per lipid (Fig. 3c). Only a marginal change in the thickness of the hydrophobic bilayer region was observed (Fig. 3d), which is connected to the small overall change in the order parameter (Fig. 3b). All other LAs, on the other hand, were found to lower the order parameter (Fig. 3b), increase the area per lipid (Fig. 3c), and decrease the hydrophobic thickness (Fig. 3d). The largest influence on these membrane parameters was observed for mepivacaine and QX-314. Hence, the influence of all LAs, except for benzocaine, can be explained by membrane thinning, due to the guest molecule residing in the bilayer in the polar region. The LAs exhibiting this reduction in chain order are those among the studied selection which were all protonated under the experimental conditions (pH 7.2). Hence, the positive charge seems to be involved in the positioning of the protonated LAs in the headgroup region, possibly by an interaction of the positive LA charge with the anionic lipid phosphate group. The overall higher polarity of QX-314 and mepivacaine, as apparent in their comparatively lower log P values - or some particularly oblique orientation consuming more space - may lead to the observed strong thinning effect of these two LAs. Interestingly, when comparing the membrane thinning observed for the LAs with that of amphiphilic peptides, LAs exert a similar or even larger effect on membrane thickness when considering the effect per weight unit. For example, for magainin, a prototype example for membrane thinning, a change in bilayer thickness of ~1.2 Å or 2 Å has been observed for a peptide:lipid molar ratio of 1:10 [26,29]. As the molecular weight of LAs is ~10 fold lower, the corresponding LA:lipid ratio would be 1:1, where a thinning by about 2–3.5 Å was observed. The LAs hence may be located at a depth in the membrane, where the separation of the headgroups is most efficient. A localization as described earlier [21] with the aromatic segment near the glycerol backbone and the polar part slightly above might give rise to the observed strong liberation of the chain conformational space.

Benzocaine, on the other hand, does not show a change in membrane thickness, but rather causes an increase in chain order in the upper part of the chain region and an opposite effect of a decrease in area per lipid. Benzocaine therefore likely penetrates at least partially into the hydrophobic core, so that the decrease in order parameter due to allowing more conformational freedom for the chains is balanced by the ordering

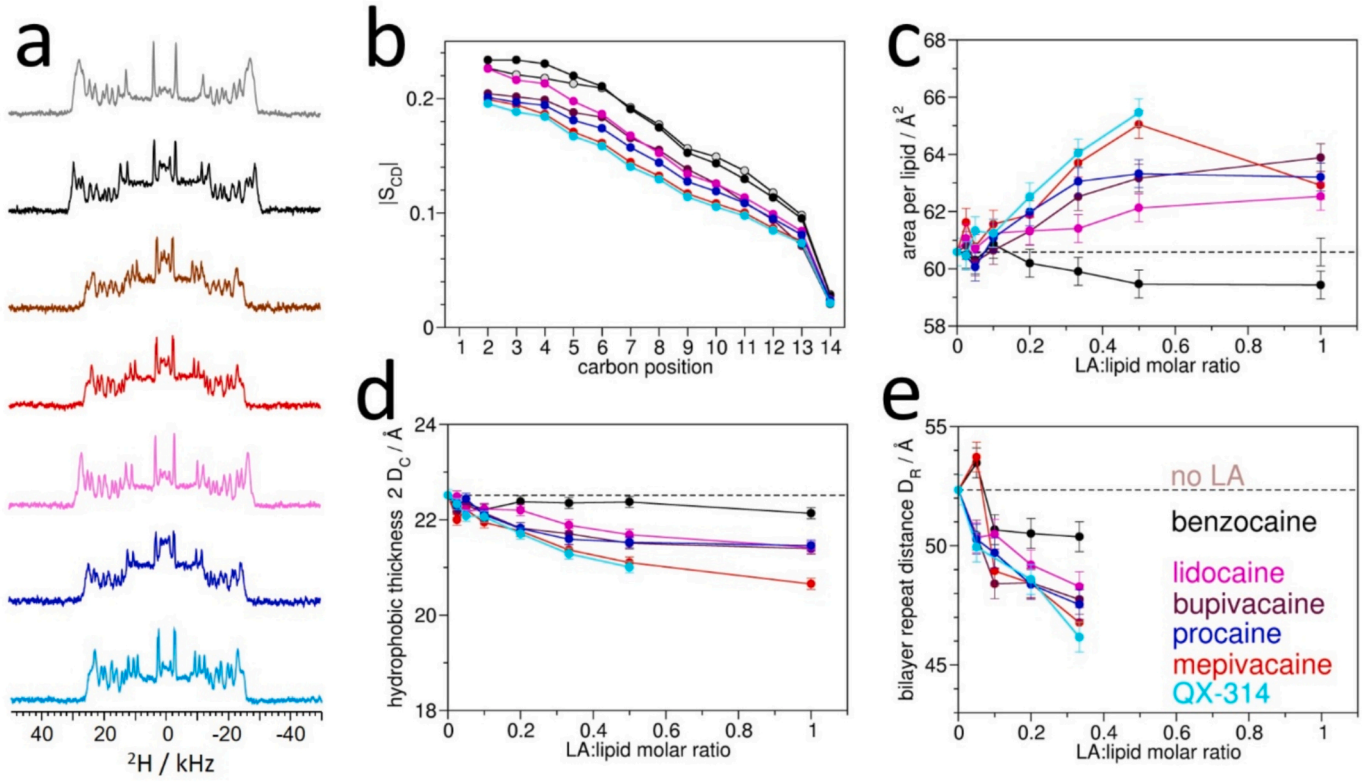


Fig. 3. Chain order and bilayer thickness. Solid-state ^{2}H NMR on oriented samples of hydrated DMPC bilayer stacks with reconstituted LAs were used to determine the influence of LAs on chain order. Using chain-deuterated DMPC as NMR probe, solid-state ^{2}H NMR spectra were obtained which show a doublet for each labeled methylene segment with a splitting corresponding to the local mobility (a). Samples with different LA:lipid ratios were measured, example spectra are shown for a molar LA:lipid ratio of 1:3 (a, colors refer to different LA as indicated in e). The splittings were converted into order parameter profiles (b, example shown for LA:lipid molar ratio of 1:3). From the order parameter profiles, the area per lipid (c) and the thickness 2 D_C of the chain region (d) were determined. The bilayer-to-bilayer repeat distance D_R comprising the bilayer and interstitial water layer was determined from GISAXS X-ray scattering experiments on oriented DMPC samples (e).

effect of interaction with the chains. A penetration deeper into the membrane as compared to ionizable LAs has also been described for benzocaine earlier [23].

As the thickness 2 D_C, derived from the ^{2}H NMR order parameters, reflects only the hydrophobic part of the membrane, we conducted additional X-ray scattering GISAXS (grazing incidence small angle x ray scattering) experiments to obtain the bilayer repeat distance D_R, which includes as well the polar headgroups and interstitial water layer (Fig. 3e). The experiments were conducted using mechanically oriented samples, and employing a grazing incidence geometry which allowed us to assess the layer profile in the direction perpendicular to the bilayer stack. The repeat distance D_R, comprising the thickness of both polar headgroups and hydrophobic chains of the bilayer and the interstitial water layer, was confirming in general the influence of the LAs on membrane thickness observed in ^{2}H NMR. Benzocaine showed the least change in repeat distance, and QX-314 as well as mepivacaine exhibit the largest decrease in repeat thickness at the highest measured LA:lipid ratio of 0.33:1. Regarding the extent of the thickness change, however, a large difference can be noted between the hydrophobic thickness and the repeat distance. Whilst the former changed by ~0.5 to 2 Å, the change in the bilayer repeat distance was 2 to 8 Å. A similar difference has been noticed previously for amphiphilic α-helical peptides [26]. The difference might be explained as follows. Using NMR, the thickness of the chain region of the bilayer, 2 D_C, represents an average of larger and smaller values of 2 D_C. The repeat distance D_R, on the other hand, is rather the maximum of the range of D_R values, as, for example, it is the peak values in membrane undulations which define the distance between adjacent bilayers.

3.3. Influence of local anesthetics on the lipid headgroups

The headgroup of zwitterionic lipids such as phosphatidylcholines constitutes an electric dipole formed by the negatively charged phosphate and the positively charged amine. By labeling the choline bridge between the two charges with ^{2}H, Joachim Seelig has developed a solid-state ^{2}H NMR tool, termed "electrometer", which is sensitive to the orientation and mobility of this dipole. We used this approach, which has already been applied to local anesthetics [16,17,19], to characterize the influence of our set of LAs on the lipid headgroup.

As for the determination of the chain order parameters, we employed oriented samples of DMPC bilayers, containing 5% of DMPC labeled in the choline (DMPC-d_4), with varying amount of LA. Except for benzocaine, the LA were added as hydrochloride unless otherwise stated, to avoid having mixtures of protonated and unprotonated molecules. The ^{2}H NMR spectra show two doublets caused by quadrupolar interaction, which correspond to the two labeled methylene segments of the choline (Fig. 4a). In the absence of any guest molecule (Fig. 4a topmost spectrum), the doublet with the larger splitting and broader linewidth was assigned to the ^{2}H at the α-carbon, the inner doublet with narrower linewidth was assigned to the β-carbon [17]. Addition of LA led to a change of one or both splittings (see Fig. 4a for ^{2}H NMR spectra measured for a LA:lipid ratio of 1:3). We monitored the changes of both splittings as a function of LA:lipid ratio, where the assignment of the two signal pairs could be followed in the gradual changes and using the different linewidths of the signals of the α and β methylenes. Following Akutsu et al., we compared the splittings Δ_α and Δ_β obtained for the different LAs with varying LA:lipid ratios on the basis of so-called α-β plots (Fig. 4b) [17]. The splittings Δ_α and Δ_β of the α-segment and the β-segment, respectively, were found to behave rather differently. With increasing LA amount (indicated by colors from purple to cyan in Fig. 4b), Δ_β was increasing. On the other hand, Δ_α showed an opposite trend as it decreased with increasing LA:lipid ratio in almost all cases. Exceptions were benzocaine and lidocaine when added as pure compound (not as hydrochloride), where Δ_α stayed nearly constant. Hence, two different behaviors were found in the α-β plots (Fig. 4b) for LAs in their protonated or unprotonated form. The charge seems to dominate

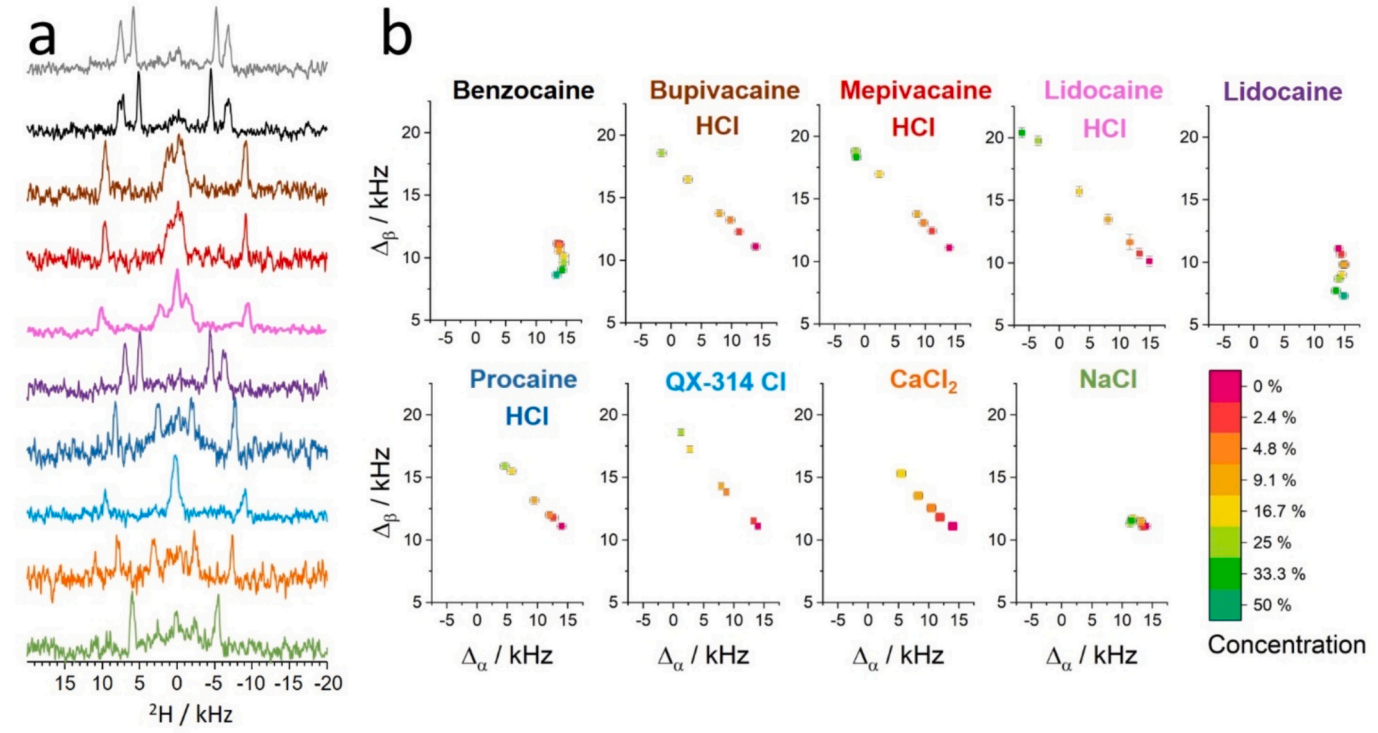


Fig. 4. Influence of local anesthetics on lipid headgroups. The conformational change exerted by the LAs on the P-N dipole of the headgroup was measured using ^{2}H NMR employing DMPC labeled in the choline of the headgroup (a, shown are ^{2}H spectra of samples of LA:lipid molar ratio of 1:3). The splittings Δ_α and Δ_β of the two doublets, referring to the α and β segment of the choline, were plotted as α-β plots (b). For each LA, the quadrupole splittings were determined for different LA:lipid molar ratios, where the different LA:lipid molar ratios are shown in different colors as indicated in the color scale on the bottom right.

the influence on the headgroup manifested in the electrometer experiments, as within each of the two groups, the α-β plots do not vary much. For comparison, we conducted the same experiment with $CaCl_2$ and NaCl. Whilst $CaCl_2$ exhibited the same behavior like the protonated LA, NaCl was not causing much change in the ^2H NMR splittings and resembled rather the behavior of the unprotonated LA (Fig. 4b). As Ca^{2+} ions are known to strongly interact with the phosphate of lipid headgroups, these results confirm that the presence of the positive charge of the LA near the lipid headgroup is decisive for the observed changes in the headgroup. Na^+ ions do not bind to the bilayer, hence the effect on the headgroup conformation is much lower, in agreement with the minimal changes in the ^2H NMR splittings observed in this case.

The results are in line with earlier ^2H NMR electrometer experiments by Seelig and others [16,17,30]. Charged compounds such as metal ions or ionic amphiphiles have been found to give rise to a straight line with a negative slope in the α-β plot, whereas neutral compounds such as cholesterol or chloroform have come to lie on a line of constant Δ_α, similar to the LA in charged or uncharged state in this study [17,30]. Also, earlier studies of selected LAs have shown a similar influence on lipid headgroups [19,30].

In these examples, the intercalation of a guest molecule near the polar/apolar interface leads to a charge being placed near the phosphocholine of the lipid, below the positive end of the P-N dipole. A positive charge then pushes the amine towards the aqueous medium, leading to a more upright conformation of the P-N dipole vector [30]. This change is then reflected in characteristic changes in the ^2H NMR splittings. We can thus conclude that the protonated LAs of this study are inserted into the bilayer such that their positive charge is localized near the phosphate moiety of the lipid headgroup.

3.4. Membrane curvature induced by local anesthetics

Spontaneous membrane curvature and the lateral pressure profile have been frequently discussed as parameters which are important for the conformational and functional states of membrane proteins. Molecules inserting into the lipid bilayer have the ability to change the lateral pressure and spontaneous curvature in a specific way depending on their depth of insertion, thereby influencing membrane protein function. Here, we evaluated the capacity of the studied LAs to modulate membrane curvature, employing an assay based on the lamellar-to-hexagonal phase transition of DOPE lipids [31–33]. DOPE forms a lamellar (L_α) phase at low temperature, which converts into an inverse hexagonal phase (H_{II}) above a temperature of ~290 K. If an inserted guest molecule favors negative curvature thermodynamically, the H_{II} phase with negative curvature will be stabilized and the transition is shifted to lower temperatures. Vice versa, insertion of a molecule preferring positive or zero curvature would increase the transition temperature.

The transition temperature was determined using solid-state ^{31}P NMR on oriented samples of DOPE containing LAs at different LA:DOPE ratios (Fig. 5). The two phases can be identified from the signal position, where the ^{31}P signal of the L_α phase occurs at 27 ppm and that of the H_{II} phase at 5 ppm (Fig. 5a/b). Pure DOPE in our setup using oriented samples was found to possess a phase transition temperature of ~292 K, where the midpoint of the transition was used to define the transition temperature (Fig. 5a). In the presence of LA, the transition was shifted (see Fig. 5b for procaine as an example). We found an increase in transition temperature for bupivacaine, mepivacaine, procaine and QX-314, with QX-314 exserting the largest effect on the phase transition (Fig. 5c). Lidocaine was showing no significant effect on the lamellar-to-hexagonal transition, and benzocaine was behaving oppositely to the majority of LAs and decreasing the transition temperature (Fig. 5c). Hence, most of the studied LAs are seen to stabilize the lamellar phase with no curvature, whereas benzocaine energetically favors the hexagonal phase with a negative curvature, and lidocaine exhibits no preference. Interestingly, the LAs favoring zero or positive curvature are, with the exception of lidocaine, exactly those which are protonated at neutral

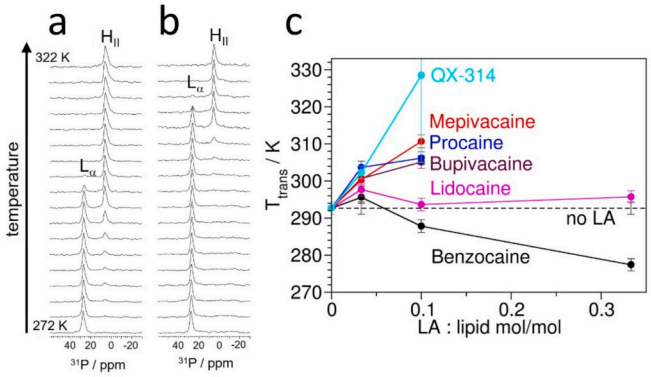


Fig. 5. Curvature preference of local anesthetics. The influence of LAs on the transition temperature T_{trans} of the lamellar-to-hexagonal phase transition of DOPE was determined using solid-state ^{31}P NMR performed on oriented samples. As examples, the ^{31}P NMR spectra of DOPE (a) and of procaine reconstituted into DOPE at a molar ratio of 1:10 (b) are shown for temperatures in the range of 272 K to 322 K in steps of 3 K. The signals of the lamellar and inverse hexagonal phases are labeled with L_α and H_{II}. The transition temperatures were determined for different LA:DOPE ratios (c). The transition temperature in absence of LA is indicated by a dashed line in (c).

pH, and which showed an effect on the lipid headgroup P-N dipole due to their positive charge. Moreover, this subset of LAs also showed membrane thinning and an increase in the area per lipid, due to a localization of the LA near the glycerol backbone or slightly above, as discussed above. The compatibility with zero or positive curvature thus seems to be connected to a localization of the LAs near the apolar/polar interface, i.e., above the hydrophobic acyl chain region. Given that the induction of a positive curvature leaves more space in the headgroup region of a membrane than a negative curvature, it is plausible that a localization of the LA molecules more in the headgroup region leads to the observed preference for positive/zero curvature.

Benzocaine, on the other hand, was found to promote negative curvature, which can be analogously explained by its preferred deeper localization within the membrane. Benzocaine was found to reside deeper in the membrane, penetrating partially into the chain region, where it causes no membrane thinning, and where the influence on the P-N dipole was rather different from the other LA molecules. Likely, the partitioning of benzocaine into the chain region is rather pronounced to cause the observed preference for negative curvature.

The indifference in terms of curvature preference found for lidocaine might indicate a deeper insertion of lidocaine compared with bupivacaine, mepivacaine, procaine and QX-314, but not as deep as benzocaine. At the other end of the scale, the largest preference for zero/positive curvature found for QX-314 is taken to indicate that this LA stays near the hydrophilic membrane surface.

The curvature modulating ability of the LAs of this study constitutes a sizable effect when compared with similar studies of other membrane-active compounds. For amphiphilic peptides, similar shifts in the transition temperature have been found as for the LAs in this study. For example, the peptide BP100 increased the transition temperature by around 10 K when added at a molar peptide:lipid ratio of 1:100. Taking the different molecular weights into account, this peptide:lipid ratio corresponds to roughly a molar LA:lipid ratio of 1:10, where we found an equivalent change in transition temperature for the majority of the studied LAs. In another example, the small molecule drug squalamine has been characterized in its curvature modulating properties, where a change about 20 K has been observed for a drug:lipid ratio of 1:10 [33]. The rather large increase in the transition temperature found for this compound compares well with that observed for QX-314. The polycationic squalamine has been found to stay at the membrane surface without penetrating into the bilayer, hence showing some similarities

with the cationic QX-314 in terms of charge and localization in the membrane, which could explain the strong curvature effect found in both cases.

3.5. Membrane-mediated influence of local anesthetics on mechanosensitive channels

To conclude this study, we evaluated whether the observed changes of physical membrane properties induced by the LAs are indeed able to alter the function of membrane proteins through a membrane-mediated mechanism. We thus employed the mechanosensitive channel of small conductance MscS as a model protein, using patch clamp to readout its channel activity in the absence and presence of LA. MscS is part of the bacterial defense system against osmotic pressure, and is able to open a nanometer size pore in response to changes in the lateral pressure profile, which renders this channel an ideal candidate to probe a membrane-mediated activity of LAs [9,34,35]. Furthermore, being of bacterial origin and unrelated to other ion channels, there is no reason to expect any specific homologous binding site(s) for LAs, so any functional changes in the presence of LAs ought to be attributable to their unspecific, membrane-mediated interactions.

We determined the negative pressure needed to open MscS channels, which were reconstituted into azolectin liposomes, in the absence and presence of LAs. The LAs were added stepwise to obtain the concentration dependent response of the channels, which we quantified as the relative change of suction needed to open the channels in presence versus in absence of LA, per added LA (Fig. 6).

We found indeed indications for an influence of some of the studied LAs on the opening threshold of MscS (Fig. 6). Whilst the change in opening threshold was within experimental error for benzocaine, bupivacaine and lidocaine, the other LAs - mepivacaine, procaine and QX-314 - showed a significant change in the opening propensity of MscS. Mepivacaine was found to lower the pressure needed to open MscS, whilst procaine and QX-314 increased this threshold. The error margins, in part due to experimental challenges, call for further experimental refinement, but nonetheless these early results seem to indicate a modulation of the opening threshold of MscS, which may well depend on the insertion depth of the LA. Mepivacaine, which we found to insert more deeply into the membrane than QX-314, exhibits an opposite effect on the channel function, which could be related to a change in lateral pressure in different depths in the bilayer.

4. Conclusion

In this study, we characterized how six representative LAs are changing the physical properties of lipid membranes. Comparing the LAs regarding their behavior in different experiments, some patterns are recognizable (Fig. 7).

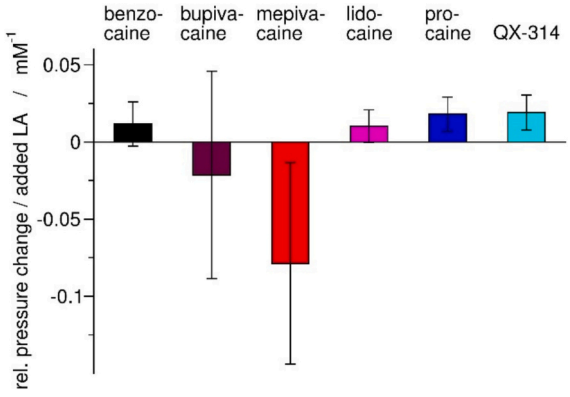


Fig. 6. Effect of local anesthetics on opening threshold of mechanosensitive channels. The change of membrane tension needed to open MscS channels was probed using patch clamp. LAs were added successively to azolectin membranes with reconstituted MscS, and the change in suction needed for channel opening was determined.

When ranking the LAs in terms of physicochemical parameters characterizing their effects on the membrane, we notice a similar ordering of the LAs with regard to all parameters, particularly the same two LAs always make up the respective lowest or highest values in all cases. Namely, benzocaine and QX-314 are always found at either end of the resulting parameter scales in all of the studied membrane effects. Benzocaine possesses the highest membrane partitioning expressed as the highest log D value at pH 7.4 (Fig. 7a), it caused the smallest change in membrane thickness (Fig. 7b), was different from all other LAs in its influence on the headgroup P-N dipole (Fig. 7c), and it showed, as the only LA of the studied compounds, a preference for negative spontaneous curvature (Fig. 7d). On the other hand, QX-314 was always found at the other end of the scale, as it is one of the four LAs exhibiting the lowest membrane partitioning (Fig. 7a), it caused the largest membrane thinning (Fig. 7b), had a distinctly different effect on the headgroup from benzocaine (Fig. 7c), and showed by far the highest lamellar-to-hexagonal phase transition temperature indicating the strongest preference for positive curvature (Fig. 7d). Among the remaining LAs, an arrangement on a scale between the two extremes of benzocaine and QX-314 is less obvious. Considering the overall similarities in their membrane-bound behavior, however, bupivacaine and lidocaine may be placed adjacent to benzocaine. Bupivacaine was found to possess a membrane partitioning log D value between benzocaine and the other LAs (Fig. 7a). Lidocaine had neither a lowering nor an increasing effect on the lamellar-to-hexagonal transition temperature, and thus behaved most similar to benzocaine. Furthermore, regarding the membrane thinning effect, bupivacaine and lidocaine were found in the group in between the extreme values, placing these two LAs overall closer to benzocaine than to QX-314 in terms of membrane influence. Hence, the LAs may be arranged here regarding the similarity of their effects on the bilayer as: benzocaine – bupivacaine/lidocaine –mepivacaine/procaine – QX-314.

Most of the membrane properties addressed in our experiments are related to the localization of the LAs within the membrane. Membrane thinning, for example, is caused by guest molecules residing in the headgroup region. The conformation of the headgroup as detected in the re-alignment of the P-N dipole responds to the positive charge of the LA being near the phosphate moiety of the headgroup. Also, the preference for positive or negative membrane curvature depends on whether the guest molecule resides near the headgroup or deeper in the membrane. As we observed benzocaine to insert most deeply into the bilayer, partially penetrating into the chain region, and QX-314 to stay near the bilayer surface, it is a plausible concept that the characteristics of the interaction of LAs with membranes should be a consequence of their insertion depth. A further important determinant is the protonation state of the LA. Benzocaine lacks a protonatable amine, which allows it to penetrate deep into the membrane, and thereby causes the difference in interaction with the headgroup P-N dipole.

The positioning of bupivacaine and lidocaine, however, still remains an open question, as we found a different behavior in the membrane compared to mepivacaine and procaine, which is likely due to different insertion depths despite their very similar structures (Fig. 1). It might be conceivable that the molecular shape and/or conformational preferences result in a different divide of polar and apolar molecular segments, leading to a different molecular alignment in the bilayer which relays the center of mass closer or farther to the bilayer surface. The position of procaine could also be influenced by the polar amine residue at the aromatic moiety.

Given the observed ability of LAs to modulate physical properties of lipid membranes, it is conceivable that LAs can influence membrane protein function indirectly this way. Depending on their insertion depth, they will reshape the lateral pressure profile and modify the spontaneous curvature of the bilayer, which may lead to changes of membrane

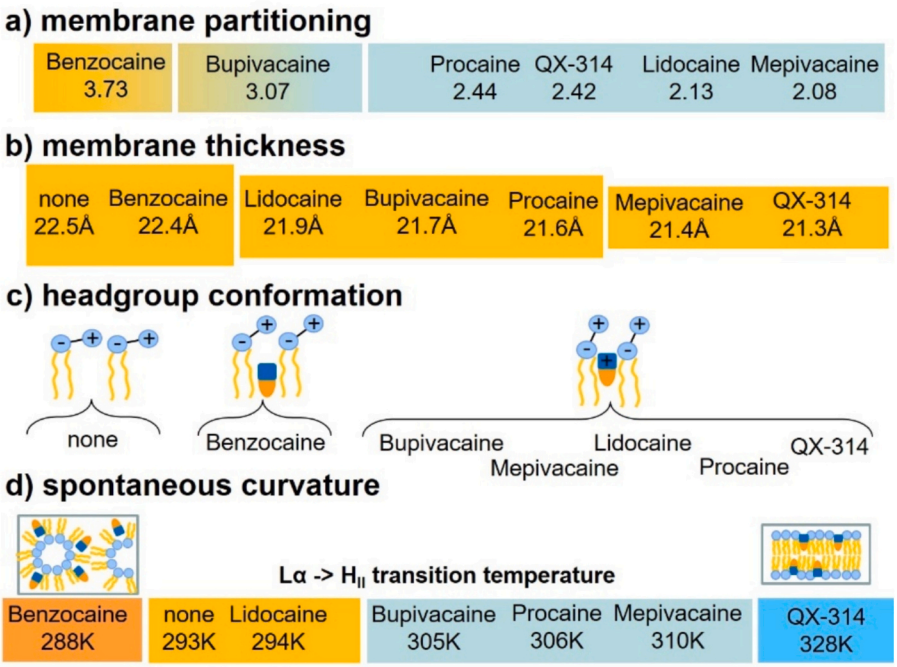


Fig. 7. Comparison of the local anesthetics in our study in terms of their influence on lipid membranes. The LAs are ordered according to their membrane partitioning log D values (a), their influence on membrane thickness parameterized by the thickness of the chain region (b), the induced change in headgroup conformation as determined from ^{2}H NMR of headgroup labeled lipids (c), and the preference for negative or positive membrane curvature, represented by the transition temperature of the lamellar-to-hexagonal transition of DOPE (d). The values of membrane thickness (b) and transition temperature (d) refer to LA:lipid molar ratios of 1:3 and 1:10, respectively.

protein conformation as well as function, in order to adapt to the new pressure profile [7]. Indeed, we observed indications that function of a mechanosensitive channel can be differentially altered by LAs.

Also, as has been pointed out by Seelig et al. [16], the positive charges introduced in the bilayer by most LAs under physiological conditions will alter the physicochemical conditions of the bilayer, with implications for many embedded membrane proteins. It has been argued that the surface potential is augmented by some 20 mV even at moderate LA concentrations, and that the dipole fields are changed by 50–100 mV [16,36]. These changes in potential may in turn shift the cellular Ca^{2+} balance and trigger conformational changes in various other membrane proteins.

The question may be asked whether such a membrane-mediated interaction of LAs with membrane proteins can have aspects of a specific interaction as found for ligand-receptor interactions. A rather basic way of influencing membrane properties by mere molecular presence and/or introduction of a charge might not allow enough differentiation for LA molecules to interact differently with different membrane proteins. Including further molecular parameters in the kind of general analysis presented here may be necessary to judge if a membrane-mediated interaction of LAs with channel proteins can indeed have the characteristics of a specific interaction, such as underlying anesthesia or analgesia in the conventional explanation of LA activity. Nonetheless, the interaction of LAs with lipid bilayers should certainly be regarded as an important part in the activity of LAs either way.

In summary, we characterized the interaction of six representative LAs with lipid membranes. The way how LAs affect membranes was found to depend strongly on their insertion depth and protonation state. It seems likely that the observed changes in membrane properties by LAs are able to alter membrane protein function in an indirect, membrane-mediated way, as indicated by the changes in function of mechanosensitive channels.

CRediT authorship contribution statement

Anke Mautner-Culetto: Writing – original draft, Investigation. **Marcel Huhn:** Investigation. **Simon Schwarz:** Investigation. **Li Tian:** Investigation. **Mert Hamballer:** Investigation. **Sergii Afonin:** Investigation. **Boris Martinac:** Supervision, Investigation. **Gernot Buth:** Investigation. **Anthony Watts:** Writing – review & editing, Methodology. **Stefan Weinschenk:** Writing – review & editing, Conceptualization. **Anne S. Ulrich:** Writing – review & editing, Supervision, Resources, Conceptualization. **Stephan L. Grage:** Writing – original draft, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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