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Interaction of Local Anesthetics with Small Phospholipid Vesicles Investigated by Proton NMR Spectroscopy

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Abstract. The effects of the local anesthetics tetracaine, procaine (both charged at pH 6), and benzocaine (uncharged) on phospholipid liposomes have been investigated by 500 MHz ^{1}H NMR spectroscopy. All the drugs reverse the Pr^{3+} induced shifts of phospholipid resonances in the same sequence as they are shifted by addition of Pr^{3+}: choline $POCH_2-$ > choline$-CH_2N$ > choline$-N(CH_3)_3$ > glycerol β > glycerol γ > acyl C_2 > acyl C_3. The drug effects result from incorporation of positive charges (tetracaine and procaine) and from the induction of a conformational change of the phospholipid head group via an action on the lipid glycerol backbone (benzocaine). From titration experiments with tetracaine on liposomes containing Pr^{3+} inside and outside is derived that the drug passes the bilayer by transverse diffusion. Tetracaine partitions outside/inside at a ratio of 2 : 1. Changes in linewidths of the drug resonances when incorporated into the liposomes allow the conclusion that the tetracaine molecule is located in an elongated way between the lipid acyl chains with its nitrogen group near the glycerol backbone. Benzocaine, showing strong effects on the line shapes of the protons on C_2 and C_3 of the lipid acyl chains is also located near the glycerol backbone, the region with the strongest hydrophobic forces.

Key words: 500 MHz ^{1}H NMR spectroscopy – Liposomes – Local anesthetics

Introduction

Since studies by Skou (1954), who found a correlation between the potency of local anesthetics and their ability to penetrate lipid monolayers, the interaction of local anesthetics with membrane systems has been the object of numerous investigations. When interacting with artificial lipid membranes local anesthetics exert three major affects: they increase the fluidity of phospholipid membranes (Lee 1978; Papahadjopoulos et al. 1975), expand the membrane (Skou 1954;

Haydon et al. 1977), and change the surface potential (McLaughlin 1975; Schlieper et al. 1981). Experiments with quaternary derivatives of local anesthetics on biological tissues led to the receptor hypothesis (Khodorov et al. 1976; Hille 1977). Since none of the theories can explain the complexity of local anesthetic action, the molecular mechanism of anesthesia is assumed to be manifold. Interest has been focused on the way of incorporation of local anesthetic molecules into the lipid phase. ESR and NMR-spectroscopy revealed that local anesthetics interact hydrophobically with the lipid phase, their charged nitrogen being located near the polar head groups of the phospholipids (Eriksson and Westman 1981; Fernandez and Cerbon 1973; Yeagle et al. 1977; Boulanger et al. 1981). The location in and transverse mobility through the lipid bilayer of local anesthetics with different biological activity are still not entirely clear.

Materials and Methods

Phospholipids

Phosphatidylcholine (PC) was extracted from egg yolk as described by Michaelis (1980). Purification was achieved by column chromatography on aluminium oxide (Brockmann 1) with chloroform-methanol 1 : 1 followed by silicic acid (Mallinckrodt CC 4 Special) column chromatography with chloroform-methanol 2 : 1. Oxidation of the phosphatidylcholine was estimated spectrophotometrically (Klein 1970). All fractions showing a ratio greater 0.2 for oxidation were rejected. Lipid phosphorus was determined qualitatively by the method of Dittmer and Lester (1964) and assayed quantitatively by digestion of dried samples with perchloric acid (72%) followed by the determination of inorganic phosphate by the slightly modified method of Gee and Deitz (1953). All preparations were stored under chloroform (containing 1% methanol) at 40–60 mM under nitrogen atmosphere at −23° C.

Preparation of Liposomes

Appropriate volumes of phosphatidylcholine stock solution were taken to dryness in vacuo or under nitrogen at temperatures less than 35° C. The samples were washed three times with carbon tetrachloride to remove methanol traces azeotropically. A volume of D_2O was added and the flask shaken mechanically until all the lipid adhering to the glass had been dispersed. The dispersion was sonicated (Branson B-15; Ti-tip diameter 3 mm), for any length of time or until the contents became opalescent. Temperature was not allowed to exceed 20° C.

Pretreated liposomes were obtained by adding small amounts of lanthanide or local anesthetic stock solution to the samples prior sonication. Shift reagents, tetracaine and procaine were dissolved in D_2O. Benzocaine was in dimethylsulfoxide (DMSO). A PC concentration of 34.2 mM was used, if not otherwise

stated. Homogeneity of the liposome suspension was checked by electron microscopy and laser light scattering (Michaelis and Schlieper 1982). The suspensions were found to consist of at least 90% of small unilamellar vesicles.

NMR Spectroscopy

Experiments were carried out on a 80 MHz proton NMR continuous wave spectrometer (Bruker, WP 80) at the Department of Organic Chemistry, the University of Düsseldorf, and on a 500.13 MHz proton NMR Fourier transform spectrometer (Bruker, Spectrospin) at Messrs. Bruker GmbH, Forchheim, West Germany. All shifts are given in parts per million (ppm) relative to tetramethylsilane (TMS) or sodium-2,2-dimethyl-2-silapentane-5-sulphonate (DSS).

Partition Coefficients

Suspensions of mechanically shaken liposomes in H_2O, pH 6.4, containing the different drugs at molar concentrations of 3 : 1 (lipid : drug) were centrifuged at 5.5×10^4 rpm for 2 h (average g = 2.8×10^5). Drug concentrations in supernatants and pellets were determined spectrophotometrically (312 nm tetracaine, 299 nm procaine, 285 nm benzocaine). The pellets were dissolved in ethanol. Partition coefficients (P) were calculated:

$$P = \frac{[C_l]}{[C_s]}$$

$[C_l]$ = anesthetic concentration in the lipid phase,
$[C_s]$ = anesthetic concentration in the supernatant.

Results

Partition coefficients for the three drugs are: 80.3 ± 4.8 (tetracaine), 34.5 ± 3.7 (procaine), and 172.5 ± 9.2 (benzocaine).

Titrations of phospholipid vesicles with trivalent lanthanide ions like Pr^{3+} or Eu^{3+} cause a shift of various lipid proton resonance frequencies (Bystrov et al. 1971; Hauser et al. 1975; Hunt and Tipping 1980). In addition to the results already described in the literature we observed a shift of the membrane outside glycerol β and one glycerol γ resonance (assigned γ_2) as well as the resonances adjacent to acyl C_2 and C_3 (Michaelis and Schlieper 1982). Shifts of $POCH_2- > -CH_2N > -N(CH_3)_3 >$ glycerol $\beta >$ glycerol $\gamma_2 > C_2$ and C_3 are observed. The shifts induced by Pr^{3+} or Eu^{3+} can be reversed by tetracaine, procaine, and benzocaine to a different degree. In Fig. 1a tetracaine reverses the Pr^{3+} induced

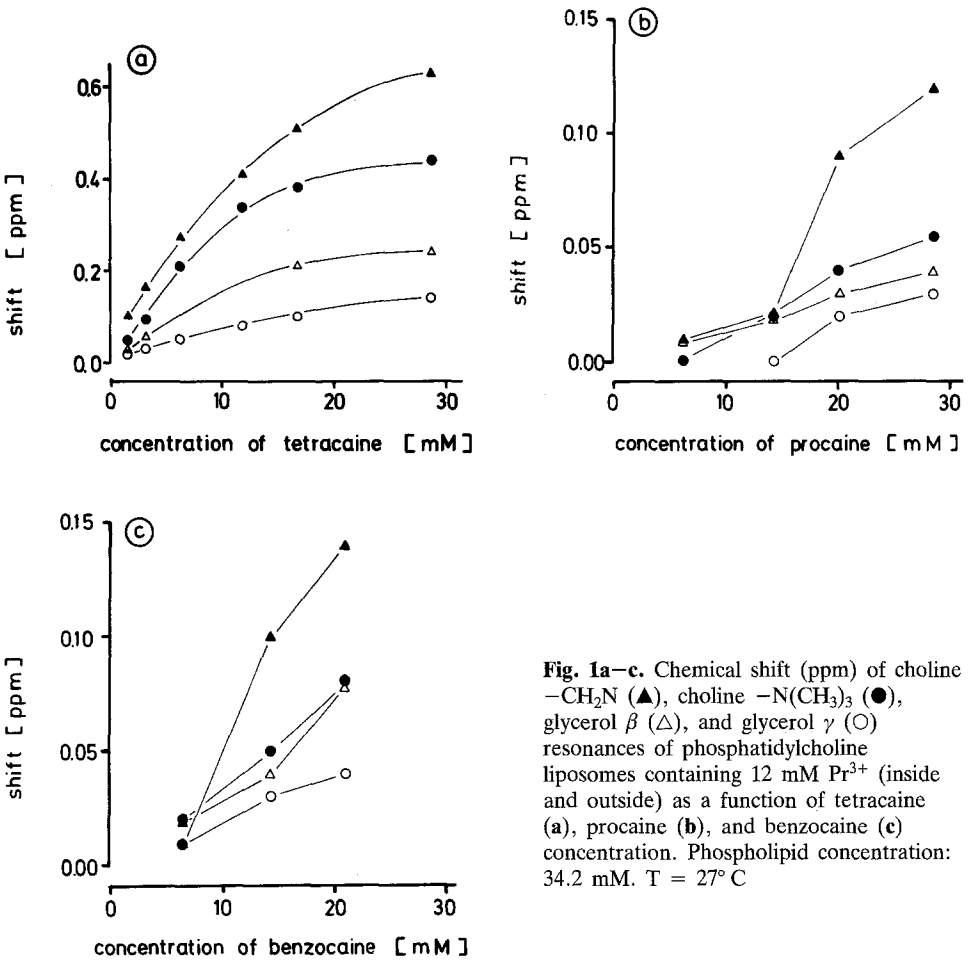

Fig. 1a–c. Chemical shift (ppm) of choline $-CH_2N$ (▲), choline $-N(CH_3)_3$ (●), glycerol β (△), and glycerol γ (○) resonances of phosphatidylcholine liposomes containing 12 mM Pr^{3+} (inside and outside) as a function of tetracaine (**a**), procaine (**b**), and benzocaine (**c**) concentration. Phospholipid concentration: 34.2 mM. T = 27° C

shifts. Procaine and benzocaine only show weak effects (Fig. 1b and c). The resonances are reversed in the same sequence as they are shifted by addition of Pr^{3+}. Control experiments show no interaction between the drugs and the lanthanide ions. In the case of benzocaine, the effect of DMSO alone was checked. No shifts in the resonances were observed when DMSO without the drug was added to the liposomes.

Figure 2 shows the titration of liposomes with tetracaine containing Pr^{3+} on both sides (membrane inside and outside). The $-N(CH_3)_3$ resonance from the inside as well as from the outside phospholipids is shifted to higher magnetic fields (upfield) at low tetracaine concentrations. At 3 mM tetracaine concentration the resonance splits into an inside and outside component. The outside component of the $-N(CH_3)_3$ resonance is reversed at high drug concentrations.

When tetracaine, procaine, or benzocaine are incorporated into the liposomes and then titrated with Pr^{3+} no splits of the drug resonances could be observed.

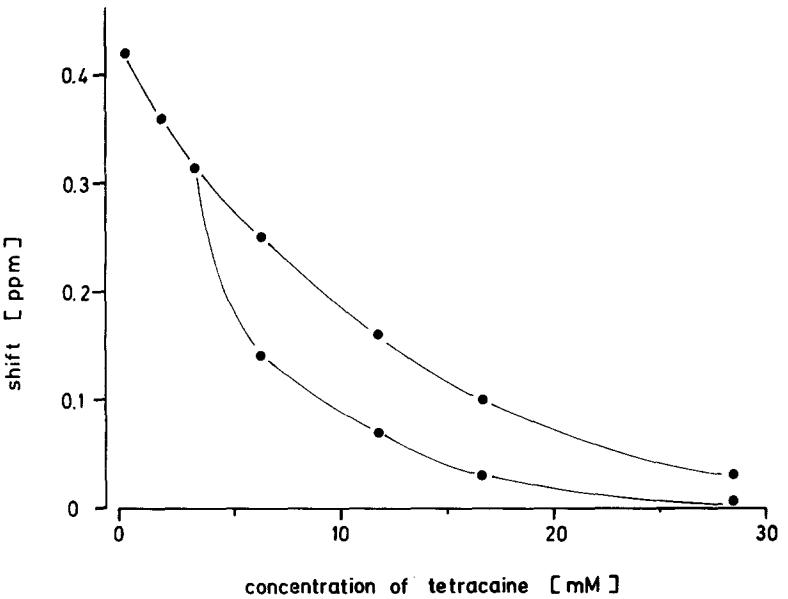

Fig. 2. Shift reversal of the choline $-N(CH_3)_3$ resonance by tetracaine. The phosphatidylcholine (34.2 mM) liposomes contain 12 mM Pr^{3+} inside and outside. T = 27° C

Table 1. Percent change in linewidth at half height of tetracaine, procaine, and benzocaine resonances after incorporation of the drugs into PC liposomes at concentrations of 3.1 mM. Concentration of phosphatidylcholine: 34.2 mM

Chemical group		Tetracaine	Procaine	Benzocaine
Ring protons	a)	40	14	114
	b)	64	14	145
$-COCH_2-$	c)	56	5	*n.d.*
$-N(CH_3)_2-$	f)	76	*n.d.*	–

n.d. = not detectable

Figure 3a and b show the 500 MHz spectra of tetracaine in D_2O and of tetracaine containing liposomes titrated with Pr^{3+}. Several resonances of tetracaine are substantially broadened. The PC $-N(CH_3)_3$, $-CH_2N$, $POCH_2-$ and glycerol γ_2 peaks are split into a highfield and lowfield component, due to Pr^{3+}.

Titration with tetracaine also shows an influence on the multiplet of the PC acyl terminal methyl groups, since at high drug concentrations (45 mM) a triplet is observed (same as PC in $CDCl_3$).

When titrating liposomes with tetracaine, procaine, or benzocaine the drug resonances are differentially broadened. Table 1 shows the percent change in peak linewidths of various resonances. Changes for procaine are very small.

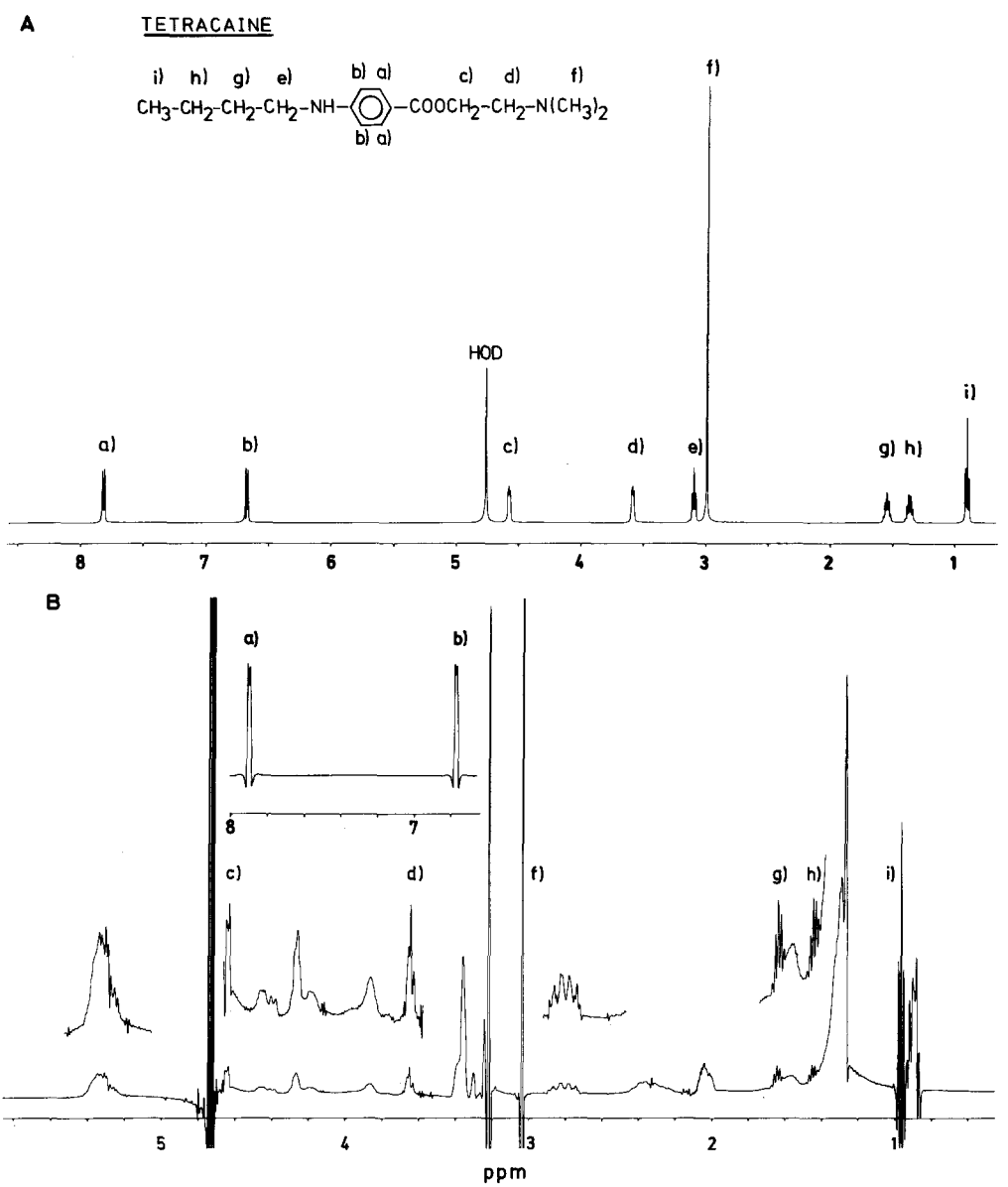

Fig. 3A and B. 500 MHz proton NMR spectra of tetracaine (10 mM) in D_2O (**A**) and of phosphatidylcholine (34.2 mM) liposomes titrated with 5.9 mM Pr^{3+} and 8.1 mM tetracaine (**B**). T = 27° C. *Transients:* 32 for tetracaine, 512 for phosphatidylcholine. Assignments of posphatidylcholine and tetracaine resonances (values for D_2O in brackets): phosphatidylcholine: terminal methyl: 0.89 (0.88); $(CH_2)_n$ of β and γ chains highfield: 1.267 (1.267), lowfield 1.30 (1.30); $-CH_2$ C = C: 2.04 (2.01); acyl C_3: 1.56 (1.59); acyl C_2: 2.36 (2.37); $-CH_2$ (C = C)$_2$: 2.80 (2.80); choline $-N(CH_3)_3$ inside membrane: 3.22 (3.23), outside membrane: 3.36 (3.25); choline $-CH_2N$ inside membrane: 3.65 (3.70), outside membrane: 3.86 (3.71); glycerol α: 3.96 (4.01); glycerol γ_1 highfield: 4.19 (4.23); glycerol γ_2 lowfield: 4.40 (4.44); choline $POCH_2-$ inside membrane: 4.27 (4.28), outside membrane: 4.62 (4.31); glycerol β: *n.d.* (5.29); vinyl (CH = CH): 5.32 (5.30); tetracaine: *a*) 7.90 (7.87); *b*) 6.77 (6.75); *c*) 4.64 (4.62); *d*) 3.65 (3.62); *e*) n.d. (3.18); *f*) 3.03 (3.0); *g*) 1.64 (1.60); *h*) 1.44 (1.40); *i*) 0.96 (0.92). *n.d.* = not detectable

The assignments of the resonances of the drugs incorporated into liposomes do not significantly vary when compared with spectra of the drugs dissolved in D_2O (also legend to Fig. 3).

Discussion

Local anesthetics (tetracaine, procaine) increase the surface potential of phosphatidylcholine liposomes (Schlieper et al. 1981). The drugs (charged form) incorporate into the lipid membrane by hydrophobic forces and by electrostatic interactions between the protonated amine of the drug and the phosphorus oxygen of the phospholipid (Boulanger et al. 1981).

Competing for the same binding site (phosphorus oxygen) di- and trivalent ions are antagonists to local anesthetics (Grasdalen et al. 1977; Browning and Akutsu 1982; Hauser and Dawson 1968; Papahadjopoulos 1970). We support these findings by measuring the reversal of the trivalent lanthanide induced shift of the PC $-N(CH_3)_3$, $-CH_2N$, $POCH_2-$ and the glycerol β and γ_2 proton resonances by local anesthetics.

The sequence for the magnitude of the local anesthetic shift reversal ($POCH_2- > -CH_2N > -N(CH_3)_3 >$ glycerol $\beta >$ glycerol γ_2) is the same as in titration experiments with Pr^{3+}. This means that tetracaine, procaine, and benzocaine completely displace Pr^{3+} from the liposome surface. Tetracaine and to a much smaller extent procaine displace lanthanide cations by changing the electrostatic conditions at the liposome surface. But also benzocaine displaces Pr^{3+} from the bilayer surface (Fig. 1c). The measured nominal pH of the samples was between 6.2 and 6.4. With a pK of 2.5 more than 99.9% of the benzocaine is in its uncharged form. Separate experiments with benzocaine (2 mM) at pH 6.2 did not show any change in the surface potential of PC or PC-phosphatidylserine liposomes. Consequently the displacement of Pr^{3+} cannot be attributed to a charge effect of benzocaine. Entering the lipid palisade structure, benzocaine alters intramolecular energetics, leading to a change of the lipid headgroup configuration which affects Pr^{3+} pseudocontact interaction (Hauser et al. 1978; Hauser 1981; Browning and Akutsu 1982).

The effects of tetracaine, procaine, and benzocaine on the displacement of Pr^{3+} from the liposome surface differ in magnitude. In the case of the $-CH_2N$ resonance ten times higher concentrations of procaine and five times higher concentrations of benzocaine are needed to induce the same shift reversals as for tetracaine. This is due to the different physico-chemical properties of the three drugs (partition coefficients and charge conditions).

After addition of tetracaine to Pr^{3+} containing liposomes, a shift of the outside and inside PC $-N(CH_3)_3$ resonances is observed (Fig. 2). This demonstrates that tetracaine when added to the liposome suspension rapidly crosses the bilayer by transverse diffusion within a time shorter than is required for an NMR measurement (3–5 min). At the membrane inside lipid aqueous interface tetracaine displaces lanthanide cations, as a shift reversal of the inside resonances is observed. The ratio of the membrane inside to outside tetracaine concentration is about 1 : 2 as derived from the results in Fig. 2.

Any increase or decrease in linewidths generally indicates increased or decreased molecular interaction. Changes in linewidths of the various PC-resonances after incorporation of the drugs were not found to be significant for tetracaine and procaine. However, clear changes in linewidths of the drug resonances could be observed (when compared with the spectra of the drug in a convenient solvent). The benzene ring resonances of tetracaine appear as single, strongly broadened peaks. This indicates a strong hydrophobic interaction with the hydrophobic part of the phospholipid molecules. The c) resonance shows about the same broadening than benzene ring protons and the f) resonance shows the strongest broadening. This leads to the conclusion that the tetracaine molecule is incorporated in an elongated way with its f) protons near the glycerol backbone of the phospholipids (the region with the strongest hydrophobic interaction) and the rest of the molecule parallel to the acyl chains. The bulky benzene ring causes a stronger broadening of its resonances compared to the c) resonances because of a stronger interaction with neighbouring acyl chains so that the protons are restricted in rotational mobility. There is more evidence that tetracaine deeply penetrates into the lipid bilayer. At high drug concentrations the PC terminal methyl multiplet changes into a well resolved triplet, indicating a higher mobility of the lipid acyl $-CH_3$ protons. This supports the view by other authors (Lee 1978; Papahadjopoulos et al. 1975) that a decrease in lipid phase transition temperature caused by local anesthetics, results in a fluidization of the lipid acyl chains.

In case of benzocaine also the benzene ring protons are strongly restricted in mobility. In titration experiments a strong broadening of the proton resonances on C_3 and C_2 of the lipid acyl chains is observed, whereas the terminal methyl, acyl $(CH_2)_n$, $-CH_2-C = C$ and choline resonances are unaffected. This indicates that the benzocaine molecule must be located near the glycerol backbone between the lipid acyl chains. Finally, little can be said about the position of procaine in the lipid bilayer. Because of its low partition coefficient a high amount stays in the aqueous phase. Therefore the NMR signals are composed of resonances exhibited by the drug molecules in D_2O and in the lipid phase. The whole spectra become indistinguishable.

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