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MOLECULAR DYNAMICS OF THE LOCAL ANESTHETIC TETRACAINE IN PHOSPHOLIPID VESICLES

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Summary

Upon introduction into phosphatidylcholine vesicles, the ^{13}C magnetic resonance peaks of the aromatic resonances of tetracaine are broadened while the T_1 relaxation times show little change. Addition of tetracaine to vesicles containing 30% cholesterol produces a similar broadening in the ^{13}C NMR spectrum of tetracaine. Nuclear magnetic resonance parameters of phosphatidylcholine in vesicles which are unchanged by the addition of equimolar tetracaine include ^{13}C T_1 relaxation time and ^{31}P linewidth, T_1 relaxation time, and nuclear Overhauser effect enhancement. These results are interpreted as indicating a hydrophobic interaction between hydrocarbon portions of the anesthetic and phospholipid bilayer. The rotational correlation time of tetracaine about its long axis in the vesicles has been calculated from the ^{13}C NMR spin lattice relaxation times to be about $10^{-10.3}$ s and is unchanged by incorporation into the phospholipid bilayer. The positively charged ammonium group of tetracaine interacts with the negatively charged phosphate group of the vesicle lipids. Using shift reagents and ^{31}P NMR, tetracaine has been shown to displace cations from the bilayer surface, and does not undergo fast flip-flop across the vesicle bilayer.

Tetracaine is one of a class of local anesthetics which block nerve conduction. It appears to have a variety of effects on the behavior of the membrane surface towards ionic species. Tetracaine has been shown to displace calcium from membranes [1—4] to inhibit calcium transport [5], to decrease excitability of nerve tissue [6] and to inhibit cell fusion presumably by displacement of calcium [7]. The extent of hydrophobic interactions between the local anesthetic and the lipid bilayer, or the ease of incorporation into the bilayer, correlates well with the anesthetic activity [8]. Since the molecular dynamics of tetracaine in membranes have not been thoroughly studied, the object of this work was to investigate both the anesthetic and the phospholipid bilayers to

understand effects on each due to the incorporation of the anesthetic.

Previous studies with ^{1}H nuclear magnetic resonance (NMR) reported some effects on the aromatic protons of tetracaine when incorporated into phospholipid bilayers [8,9], and monitored the displacement of shift reagents from the membrane surface by tetracaine [10]. In order to obtain a more detailed picture of the behavior of this system, we have used ^{13}C and ^{31}P NMR to study properties of all the carbon atoms both in the anesthetic and in the phospholipid, and the phosphate in the phospholipid. With such a complete picture of the molecules of this system, it has been possible to locate the anesthetic in the bilayer, to describe the motional restrictions produced on the anesthetic by the phospholipids, and to show that the anesthetic does not flip-flop across the bilayer.

Experimental

Egg phosphatidylcholine was purified by silicic acid chromatography. Vesicles were prepared by sonication in 100 mM NaCl at 2°C until clear [11]. Tetracaine hydrochloride was purchased from Sigma, ^{13}C and ^{31}P NMR spectra were measured in 10 mm tubes at 24°C using a JEOL-PS100/EC 100 Fourier transform spectrometer operating at 25.15 MHz for ^{13}C and 40.48 MHz for ^{31}P with a JEOL 5 kHz RF crystal filter. Protons were noise decoupled continuously except when gated for nuclear Overhauser experiments. T_1 measurements were made using the $180°—\tau—90°$ pulse sequence, with a delay time of five-times the longest T_1 reported. Carbon-13 chemical shits were measured using internal methanol or acetone and converted to the tetramethylsilane scale.

Results

The carbon-13 spectrum of tetracaine in $^{2}H_2O$ appears in Fig. 1. The spectral assignments given by the small letters in the figure and chemical shifts listed in Table I were obtained by off-resonance decoupling and comparison with known compounds. The terminal methyl and *N*-methyl groups were easily identified by the quartet structure in the off resonance decoupled spectrum and by their chemical shifts. The methylenes in the butyl chain were assigned by comparison with other alkyl chains. The remaining resonances were assigned by comparison with benzocaine [12].

The carbon-13 spectrum of phosphatidylcholine vesicles appears in Fig. 1b similar to previously reported results [13]. Fig. 1c shows a spectrum of vesicles containing tetracaine (1 : 1). Similar spectra were obtained with a tetracaine to lipid ratio of 1 : 2. The anesthetic was added as a dry powder to a solution containing sonicated vesicles and a spectrum was taken after the tetracaine was completely dissolved. The dramatic change in the tetracaine resonances is evident and the lack of any narrow components in those broadened resonances demonstrates that most, if not all, the tetracaine has entered the vesicle. Fig. 1d shows a difference spectrum between that due to vesicles with incorporated tetracaine and vesicles alone. T_1 relaxation times were obtained for the carbon atoms in the anesthetic in $CH_3OH/^{2}H_2O$ (1 : 1) and in phosphatidylcholine vesicles. The T_1 values are reported in Table I for all the carbon atoms except

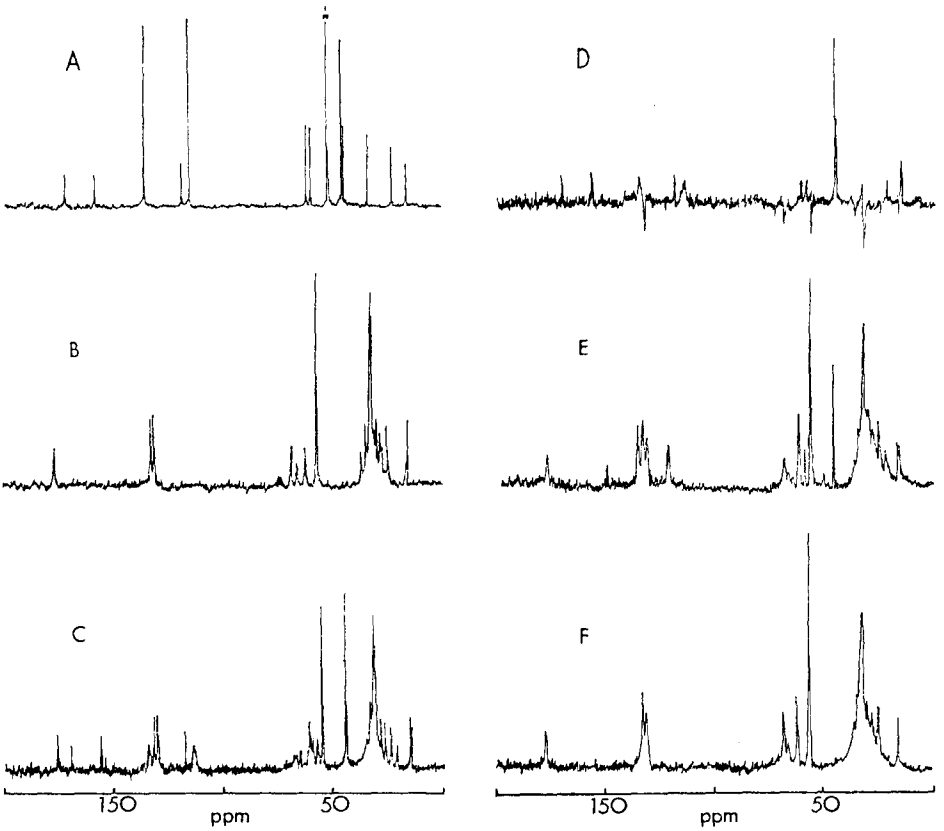


Fig. 1. 25 MHz ^{13}C spectra of tetracaine and phosphatidylcholine vesicles at 24°C. (A) Tetracaine in $^{2}H_2O$; (B) phosphatidylcholine vesicles in 0.1 M NaCl, 0.01 M EDTA; (C) vesicles as in (B) with tetracaine, 1 : 1; (D) difference spectra C-B; (E) vesicles as above with tetracaine and cholesterol (phosphatidylcholine/tetracaine, 1 : 1, phosphatidylcholine/cholesterol, 3 : 1); (F) vesicles with cholesterol, 3 : 1.

the three unprotonated carbons (e, h, and i) with long T_1 values. Three other tetracaine carbon atoms (c, d, and j) were not well defined in the phosphatidylcholine-tetracaine sample, and their T_1 values do not appear in Table I. In both environments the butyl group shows the segmental motion characteristic of long chain molecules [14]; the T_1 values decrease progressively up the chain from the terminal methyl group.

T_1 relaxation times for easily measurable phosphatidylcholine carbon atoms in the vesicles containing tetracaine are similar to those values previously reported for pure phosphatidylcholine vesicles [13]. ^{31}P NMR spectra were collected as a probe of the phosphate region of the lipid. In addition to linewidths and T_1, the $^{31}P\{^{1}H\}$ nuclear Overhauser effect is a measure of headgroup conformation and motion [15]. These three parameters are unchanged upon incorporation of tetracaine into the vesicle.

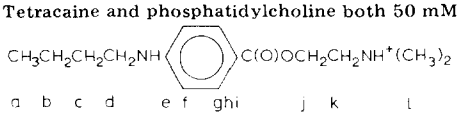
Addition of tetracaine to vesicles containing 25% cholesterol produced the spectrum displayed in Fig. 1e. Line broadening effects in the tetracaine are similar to those observed in vesicles without cholesterol. However, in the presence of cholesterol chemical shifts of aromatic carbons are notable, carbon e upfield and carbons f and h downfield. This result suggests that the aromatic portion of

TABLE I

TETRACAINE CARBON-13 CHEMICAL SHIFTS AND T_1 RELAXATION TIMES

Carbon	Shift [a]	T_1 relaxation times	
		$CH_3OH/2\,H_2O$ [b]	Vesicles [c]
a	14.1	2.0	0.9
b	20.8	1.5	0.7
c	31.6	0.9	
d	44.1	0.4	
e	154.9		
f	112.2	0.36	0.2
g	132.7	0.27	0.3
h	115.6		
i	188.5		
j	57.2	0.38	
k	59.5	0.33	0.3
l	44.1	1.0	0.5

[a] In $CH_3OH/^2H_2O$ (1 : 1) in ppm downfield from tetramethylsilane to ±0.1 ppm.
[b] In $CH_3OH/^2H_2O$ (1 : 1) in s to ±10%.
[c] Tetracaine and phosphatidylcholine both 50 mM in water.



tetracaine experiences a different environment in vesicles with and without cholesterol.

Lanthanide shift reagents can be used to differentiate resonances due to lipids residing on either side of the vesicle [16]. When Pr^{3+} was added to a solution of vesicles the ^{31}P phosphate resonance due to lipids on the exterior of the vesicle shifted downfield. Addition of tetracaine with the lipids (1 : 1) caused the resonance due to exterior lipids to shift back to the original position.

When sonicated in the presence of Pr^{3+} and then dialyzed to remove exterior Pr^{3+}, the lipids form vesicles with Pr^{3+} trapped on the inside. In the ^{31}P NMR spectra, the resonance due to interior lipids is shifted downfield from exterior lipids and an outside/inside ratio of lipids (measured with gated decoupling to eliminate the nuclear Overhauser effect [17]) of 2.2 was measured in good agreement with previous results [18,19]. Addition of tetracaine (1 : 2, tetracaine/lipid) to these vesicles did not change the outside/inside ratio of lipids. In addition over a period of four hours the ratio was constant and the chemical shift of the shifted resonance was unchanged. Since incorporating tetracaine in the bilayer displaces the Pr^{3+} shift reagent from the side of the bilayer in which the anesthetic is inserted, an insignificant amount of the anesthetic could have reached the inside during those four hours. As a control to demonstrate that tetracaine had actually been incorporated into those vesicles, shift reagent was added to the exterior of the vesicles and no shift of the ^{31}P resonance was observed.

Discussion

Considerable detail of the interaction of tetracaine with phospholipid bilayers can now be described. Upon simple addition of tetracaine to pre-

formed phosphatidylcholine vesicles the anesthetic incorporates readily into the outside layer, apparently without seriously disrupting the vesicle structure. For example, at a ratio of 1 : 2, tetracaine/phospholipid, the outside/inside phospholipid ratio remains constant, the vesicles do not become leaky to cations, and no obvious aggregation of vesicles occurs when tetracaine is incorporated. Apparently tetracaine occupies some available free volume on the exterior of the vesicle, possibly arising from the unsaturated hydrocarbon chains.

It has been previously suggested that at least part of the driving force for incorporation comes from hydrophobic interactions [8], and our data are consistent with this suggestion. The motional properties of the hydrophobic portion of tetracaine are altered by interaction with the phospholipids in an interesting way: certain linewidths are selectively broadened, but T_1 changes are small in comparison. The protonated aromatic carbons (f and g) display this contrast most dramatically (see Results). The tetracaine methylene carbons j and k, also exhibit broadened resonances, while the resonances due to carbons in the butyl tail of the anesthetic are not substantially affected. The linewidths reflect motional parameters and therefore the central portion of the molecule must be motionally restricted by the surrounding phospholipids, while the tail remains free. The central portion of a phospholipid bilayer is quite fluid but considerable order is encountered when progressing up the fatty acyl chains toward the headgroup [20]. It is likely that the aromatic portion of tetracaine is located in the ordered middle and upper portion of the phospholipid chains, and the butyl tail is located either in a defect due to the aromatic ring which confers mobility on the tail, or in the more fluid region toward the center of the bilayer. Consistent with this view the positive charge of the tetracaine is at the same level as the negatively charged phospholipid phosphate.

Selective motional effects so clearly reflected by the linewidths are not mirrored in the T_1 values. If those results are discussed in terms of a previously proposed motional model [21] then segmental motions along the chain of tetracaine are little affected by incorporation into the bilayer, but large deviations of the anesthetic from an orientation perpendicular to the bilayer surface are damped. This is consistent with the location of tetracaine proposed above, where the aromatic ring is constrained due to its location in a relatively highly oriented section of the bilayer.

The affect on the rotation of tetracaine about its long axis from incorporation into the phospholipid bilayer appears to be minimal. The T_1 values for the carbons of tetracaine are little changed in the bilayer from the values for tetracaine free in solution, so the motion governing the spin lattice relaxation rate must not change significantly. The one motion most favored by a long molecule of this kind both in solution and in a bilayer, particularly as reflected in the relaxation of the aromatic carbon atoms which cannot undergo kink formation, is rotation about the long axis of the molecule. Analysis of the T_1 values for the protonated aromatic carbon atoms according to Woessner [22], using an isotropic vesicle rotational correlation time of about 10^{-6} s and a much faster anisotropic axial rotation, yields a rotational correlation time around the long molecular axis of about $10^{-10.3}$ s.

The behavior of the phospholipid was simultaneously monitored with the tetracaine in order to assess the affect of the anesthetic on the phospholipid.

To the extent to which the ^{13}C and ^{31}P NMR parameters reflect the motional properties of the lipids, the phospholipids are unaffected by the introduction of the anesthetic.

Recent results indicate that flip-flop of lipids across the two surfaces of a bilayer is slow [23—25]. It is of interest to determine whether a foreign, anesthetic molecule introduced into vesicles might itself undergo flip-flop. As described in the results section, over a period of four hours no transfer of tetracaine to the inside of the vesicle bilayer occurred. If it had, Pr^{3+} would have been displaced from the phosphates of the interior lipids and the resonance due to interior lipids would have shifted towards its original position. Since no changes occurred, flip-flop of the anesthetic must be slow. In these vesicle model systems, tetracaine remained on the side of the bilayer to which it originally had access, and the vesicle membrane is asymmetric.

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