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INTERACTION OF FLUORINATED ETHER ANESTHETICS WITH ARTIFICIAL MEMBRANES

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Summary

Fluorine-19 nuclear magnetic resonance spectroscopy is applied to the study of the environment of dipalmitoyl phosphatidylcholine-bound fluorinated ether anesthetics (enflurane, fluoroxene and methoxyflurane) both below and above the lipid gel to liquid crystal phase transition temperature. Line widths and spin-lattice relaxation time (T_1) measurements are consistent with substantial immobilization of the lipid-bound anesthetic molecules. Heating anesthetic/lipid mixtures above the lipid transition temperature leads to narrowing of the lipid-bound anesthetic fluorine resonances accompanied by little or no change in anesthetic fluorine-19 chemical shifts, suggesting that although the mobility of the bound anesthetic increases at the higher temperature, the nature of the anesthetic-lipid interaction changes little as a result of this phase change. Differential scanning calorimetric studies of the effects of these anesthetics on the phase transition behavior of the phospholipid indicate that the regions of the bilayer in which volatile anesthetics partition at lower concentrations are different from the regions in which they partition at higher concentrations.

Introduction

In an effort to characterize the forces at play between general anesthetics and the membrane structures into which they partition, we have carried out

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nuclear magnetic resonance and differential scanning calorimetric studies on fluorinated ether anesthetics and halothane. The anesthetics employed in this study were: enflurane, 2-chloro-1-(difluoromethoxy)-1,1,2-trifluoroethane, CHF_2OCF_2CHClF; fluoroxene, (2,2,2-trifluoroethoxy)ethene, $CF_3CH_2OCH=CH_2$; methoxyflurane, 2,2-dichloro-1,1-difluoro-1-methoxy-ethane, $CH_3OCF_2CHCl_2$; and halothane, 2-bromo-2-chloro-1,1,1-trifluoro-ethane, $CF_3CHBrCl$. The methods employed are complimentary in that the ^{19}F nuclear magnetic resonance (^{19}F NMR) spectra observed represent reports arising from the anesthetic molecule itself regarding its environment and differential scanning calorimetric studies report on order-disorder transitions in the phospholipid bilayer and the effects of added anesthetic molecules on these transitions.

Materials and Methods

Enflurane and fluroxene were obtained from Airco, Inc., methoxyflurane and halothane were obtained from Abbott Laboratories and Ayerst Laboratories, respectively. All anesthetics were fractionally distilled and stored at 4°C in the dark. Crystalline synthetic grade I β-γ-dipalmitoyl-DL-α-phosphatidylcholine (99%) was purchased from Sigma Chemical Company. Liposomes were prepared as described previously [1].

NMR tubes were cleaned as previously described [1]. All ^{19}F chemical shifts are reported relative to an external standard of 1% hexafluoroacetone in deuterium oxide sealed in a glass capillary which was run simultaneously with the sample. Chemical shifts were measured either on a Varian XL-100 Fourier transform NMR spectrometer equipped with multinuclear capacity operating at a field of 94.1 MHz and a sweep width of 1000 Hz, or on a Jeol FX60 NMR spectrometer. T_1 values were obtained on the XL-100 instrument employing a 180°-τ-90° pulse sequence, where τ is the waiting period between the 180° and the 90° pulse. T_1 values are estimated to be accurate to 10%.

Anesthetics, 1.27 mol% relative to the calculated molarity of dipalmitoyl phosphatidylcholine, were added to 3 ml containing 250 mg/ml dipalmitoyl phosphatidylcholine in 0.1 M sodium chloride. The external capillary reference was added to each tube, the tube was immediately capped and the ^{19}F NMR spectrum of each anesthetic/phospholipid mixture obtained at ambient temperature and at a temperature above the lipid phase transition temperature (see Table I).

Differential calorimetric studies were performed as reported earlier [2]. Unsonicated liposomes containing 67 mM dipalmitoyl phosphatidylcholine in 20 mM KCl, 10 mM Tris (hydroxymethyl)aminomethane, pH 7.4 were mixed with an appropriate amount of the anesthetic and incubated for more than 8 h. The calorimetric scans were run in sealed pans on DSC-1B (Perkin Elmer) at 1.25 K/min. Because of the high vapor pressure of these anesthetics it is very difficult to control their concentrations in the sample. The concentration values given in Figs. 2 and 3 are based upon the amount added. These may be higher than the actual concentration.

Results

In the presence of $3.4 \cdot 10^{-1}$ M phospholipid, enflurane, fluroxene, and methoxyflurane (present at 1.3 mol% of the phospholipid) will be largely partitioned into the phospholipid on the basis of octanol-water partition coefficients [3], fat-blood partition coefficients [4], and reported dipalmitoyl and dimyristoyl phosphatidylcholine-anesthetic partition coefficients [5,6]. The broad lines, loss of spin-spin splittings, and relaxation times observed here are consistent with this expectation. The ^{19}F NMR spectra of all of these anesthetic molecules are significantly broadened compared to the line widths observed in organic solvents. ^{19}F chemical shifts and line widths for membrane-bound anesthetic both below and above the dipalmitoyl phosphatidylcholine phase transition temperature (41°C) are collected in Table I. Since proton decoupling was not employed, the measured line widths should be considered in a qualitative manner. Line narrowing occurred in all cases on heating the system above the phospholipid phase transition temperature. In two cases, fluroxene and the enflurane 2-fluoro moiety, spin-spin splitting began to resolve at the higher temperature. Small anesthetic ^{19}F chemical shift changes accompanied the phospholipid phase transition.

As observed for halothane [1], the ^{19}F linewidth of fluroxene increases as the phospholipid fluroxene ratio increases (2.2 Hz, no phospholipid to 20 Hz, $3.4 \cdot 10^{-1}$ M phospholipid). At high phospholipid : fluroxene ratios, when fluroxene is probably largely lipid-bound, the anesthetic ^{19}F T_1 relaxation time is 0.8 s, while the T_1 for fluroxene in buffer in the absence of phospholipid is 3.8 ± 0.2 s.

Chiefly because of its relative insolubility in water, methoxyflurane's distribution and ^{19}F relaxation times in the phosphatidylcholine-water system are complex at anesthetic concentrations somewhat higher than those

TABLE I

F-19 CHEMICAL SHIFTS (ppm) FOR ANESTHETIC/PHOSPHOLIPID MIXTURES

Ambient temperature is 24—27°C, anesthetics were present as 1.3 mol% of the phospholipid. The phospholipid, 0.34 M dipalmitoyl phosphatidylcholine was suspended in 0.1 sodium chloride. Chemical shifts are relative to an external capillary standard of hexfluoroacetone in deuterium oxide, run simultaneously. Proton decoupling was not employed. Linewidths are calculated for the bound anesthetic signal.

Anesthetic	Ambient temperature		56—58°C	
	Shift	Linewidth (Hz)	Shift	Linewidth (Hz)
Methoxyflurane	—3.8	127	—3.9	89
Fluroxene	8.3	28	8.0	45 (12) *
Enflurane a (2-fluroro)	—79.0	>300	—76	63.5 (25) *,**
b (1-fluoro)	—2.3 **	52 ***	—2.3	21
c (difluoro-methoxy)	—2.5 **	47 ***	—2.5	24

* Spin-spin coupling visible in signal; quantity in parentheses indicates apparent linewidth of each component of the signal.

** Spin-spin splitting observed, separation of the two broad peaks observed was approximatly 30 Hz.

*** Peaks due to enflurane fluorines designated b and c were broad and poorly separated.

employed to obtain the results in Table I. The integrated ^{19}F NMR spectrum of methoxyflurane in the presence of unsonicated dipalmitoyl phosphatidylcholine vesicles (Fig. 1) indicates the presence of at least three readily distinguishable species at −3.4 ppm, −4.0 ppm, and at −4.9 ppm. We have tentatively assigned these to methoxyflurane molecules dissolved in water, lipid-bound methoxyflurane, and undissolved methoxyflurane, respectively. Peak assignments were made as a result of a series of experiments involving: (1) varying the amount of added methoxyflurane in the presence of fixed amounts of phospholipid (0.07 M dipalmitoyl phosphatidylcholine, varying anesthetic from $8.6 \cdot 10^{-3}$ to 0.14 M; 0.34 M dipalmitoyl phosphatidylcholine, using $8.6 \cdot 10^{-3}$ M to 0.53 M anesthetic) and (2) varying the amount of phospholipid in the presence of a fixed amount of methoxyflurane ($2.9 \cdot 10^{-2}$ M anesthetic at phospholipid concentrations ranging from 0 to 0.28 M dipalmitoyl phosphatidylcholine). Integrated ^{19}F NMR spectra were obtained for each sample. The results were fully consistent with a system containing the three species noted in equilibrium with one another and in which the water solubility of the methoxyflurane is limited. For example, the free and bound methoxyflurane signals level off at the point of appearance of the signal due to undissolved anesthetic. At fixed methoxyflurane, increasing the amount of phospholipid leads to increasing amounts of bound anesthetic up to about 0.11 M dipalmitoyl phosphatidylcholine above which the amount bound levels off. Prior to this leveling off, the peak due to undissolved anesthetic disappears and the peak due to water-dissolved methoxyflurane begins to decrease in intensity above 0.14 M dipalmitoyl phosphatidylcholine and disappears between 0.20 M and 0.24 M dipalmitoyl phosphatidylcholine. Further substantiating these assignments are the T_1 values of the dissolved, lipid-bound, and neat methoxyflurane in 0.28 M phosphatidylcholine in 0.1 M sodium

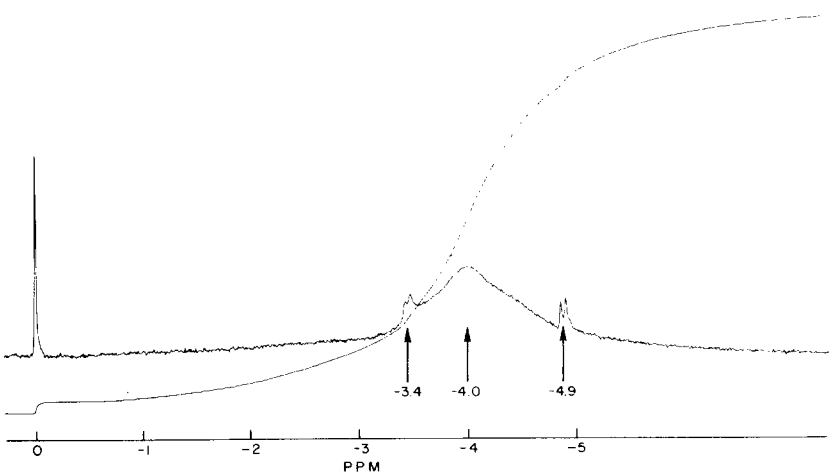

Fig. 1. Integrated ^{19}F NMR spectrum of $8.6 \cdot 10^{-2}$ M methoxyflurane (2,2-dichloro-1,1-difluoro-1-methoxyethane) in the presence of 0.28 M unsonicated synthetic dipalmitoyl phosphatidylcholine multilayer liposomes in 0.1 M sodium chloride and five drops of deuterium oxide/3 ml (to provide the internal lock). Chemical shifts are reported relative to an external capillary standard of hexafluoracetone in deuterium oxide run simultaneously.

chloride: 3.7 s, 0.7 s and 1.5 s, respectively. Thus, the motion of all the anesthetics investigated thus far by ^{19}F linewidth and relaxation time measurements indicate that upon transfer from aqueous solution to the phospholipid structure, a substantial decrease in mobility of the anesthetic agent ensues. Perhaps because of the effect of hydrogen bonding in aqueous media, the chemical shifts of lipid bound and unbound anesthetic are similar in cases of halothane, enflurane, and fluroxene. In the case of methoxyflurane, however, the difference in chemical shift between water and lipid is significant. From Fig. 1 it is possible to estimate a phosphatidylcholine-water partition coefficient, P, for methoxyflurane of 42. This value compares favorably with that estimated for methoxyflurane in an erythrocyte membrane-water system [3].

The experiment described in Table I for methoxyflurane revealed two signals: one relatively narrow, still showing spin-spin coupling, and a very broad signal due to bound anesthetic. The integral suggests that a peak may exist in the position characteristic of water-dissolved anesthetic. The peak due to undissolved methoxyflurane disappears above 41°C, suggesting that the membrane-water partition coefficient for methoxyflurane changes appreciably on going from the phospholipid gel to liquid crystalline states. Although only a broad ^{19}F signal is apparent in a sample containing $2.9 \cdot 10^{-2}$ M phosphatidylcholine at 26°C, it is possible that the T_1 relaxation times vary across the peak from 0.64 ± 0.5 to 0.74 ± 0.02 s (moving away from the external reference) indicating that even in this case the anesthetic may not be completely lipid-bound.

Typical differential scanning calorimetric profiles of liposomes containing enflurane are shown in Fig. 2. Similar profiles are obtained with halothane, fluroxene and methoxyflurane. The main transition is lowered in temperature while the heat capacity (the calorimetric enthalpy) remains constant with

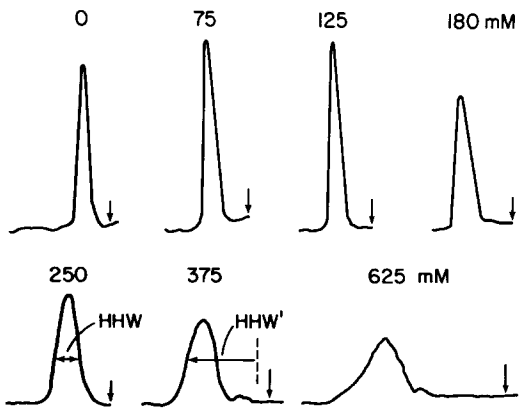

Fig. 2. The order-disorder transition profiles of dipalmitoyl phosphatidylcholine liposomes containing 0, 75, 125, 180, 250, 375 and 625 mM enflurane. The arrow on the side of each profile marks 315 K, that is the temperature at the end of the transition of the unmodified liposomes. It may be noted that not only the transition profiles become broader (as refected in larger HHW values) but they also shift towards lower temperature (as reflected by HHW' < HHW) at higher anesthetic concentrations. The size of the cooperative unit is larger if the midpoint slope of the integrated transition profiles is larger, than if the half-height width (HHW) is smaller.

increasing anesthetic concentrations. At lower anesthetic concentrations the transition profile remains sharp, that is, the lowering of the transition peak is accompanied by a shift of the whole transition peak. At higher anesthetic concentrations the shift of the transition peak towards lower temperature is accompanied by distinct broading of the peak. This trend is represented in the values of half-height width (HHW) and in the values of the shifts at half-height width (HHW′) as a function of anesthetic concentrations. As shown in Fig. 3, the difference between HHW and HHW′ values at several concentrations for the four volatile anesthetics is quite different. The lowering of the transition temperature (related to HHW′) represents a weaker packing of the cooperative unit undergoing the transition, whereas an increased width of the transition (related to HHW) reflects a decrease in the size of the cooperative unit (or cluster) of the lipid undergoing the transition (1a). The results in Fig. 3 thus suggest that the inhalation anesthetics are not only incorporated into the lipid bilayer as evidenced by altered transition parameters, but they also bring about subtle organizational changes without disrupting the gross bilayer organization as reflected in values of the half-height width parameters without any noticeable effect upon the enthalpy of the transition. A sharp shift in the values of HHW′ at more than 150 mM anesthetic suggest that the packing of the cooperative unit is altered drastically above this concentration. A change in the HHW values is also observed at above 150 mM indicating that the size of the cooperative unit undergoing the order-disorder (gel-to-liquid crystalline) transition is also smaller at higher anesthetic concentrations. As discussed elsewhere [2] these two types of changes demonstrate that the regions of the bilayer in which volatile anesthetics partition at lower concentrations is different from the regions in which they partition at higher concentrations.

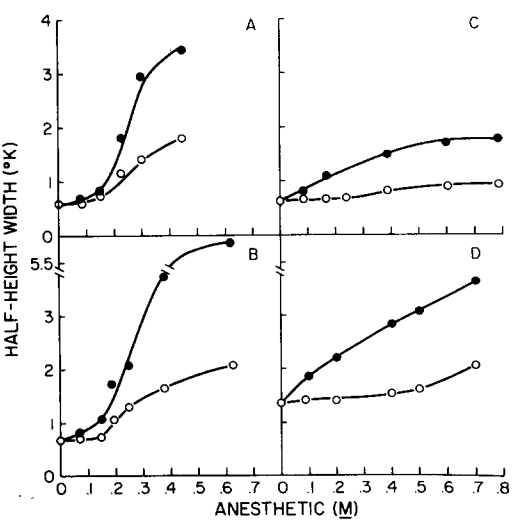

Fig. 3. Dependence of the half-height width (HHW, o———o) and of shifts at half-height widths (HHW′, ●———●) of the transition profiles for halothane (A), enflurane (B), fluroxene (C), and methoxyflurane (D) at several concentrations. The phospholipid concentration in each sample is 67 mM.

Discussion

The partitioning of nonelectrolytes in the presence of cells and artificial membranes has been discussed by Diamond and Wright [7]. They suggest that Van der Waals forces between solute and membrane will be the predominant attractive forces between solute and the hydrocarbon tails of membrane phospholipids. On the other hand, partitioning out of the aqueous phase involves, to a great extent, hydrogen-bonding interactions. Thus, the final partitioning is a balance between solute-water interactions and solute-solvent (in this case membrane) interactions. These considerations are paralleled and supported by Hansh's conclusion [8,9] that anesthetic action is brought about by both hydrophobic and polar properties of anesthetic molecules. Thus, although hydrogen-bonding or other solute-solvent interactions are important determinants of the partitioning behavior of an anesthetic, it is not unreasonable to suggest as a working hypothesis that membrane-solute interactions do not primarily involve such specific interactions.

It must further be assumed that no change in the type of anesthetic-membrane interaction occurs as a function of temperature, lipid concentration, or anesthetic concentration. Clearly these matters are not now determined, in fact, our results suggest that at least the distribution of anesthetic molecules in the bilayers alters at higher anesthetic concentrations. However, experimental support for the assumption that hydrogen bonding interactions may not be of importance for nonpolar solute-membrane interactions derives from the observations of Katz and Diamond [10—12] on the partitioning of somewhat less hydrophobic solutes (than the anesthetics employed in this study) into dimyristoyl phosphatidylcholine liposomes. The membrane site at which these solutes partition appears to be isolated from water molecules [13]. In the comparisons we make below, bulk isotropic solvent properties are ascribed to particular locations within the bilayer assembly, which is a highly anisotropic structure. Nonetheless, as is the case with microviscosity measurements on membranes by fluorescence spectroscopy or NMR techniques, useful deductions concerning both the behavior of solutes and membrane structure itself are possible from such data. The measurements reported here have not required the introduction of potentially perturbing reporting molecules, such as fluorescent probes or spin labels. This is clearly a distinct advantage in an already very complex system.

Due to its relative insolubility in water, the methoxyflurane spectrum in the presence of phospholipid (Fig. 1) illustrates several important points about the equilibria involved. While independent variation of anesthetic and phospholipid concentrations indicate that the equilibria involved are readily reversible, the observation of distinct aqueous and bound methoxyflurane resonance indicate that the exchange of methoxyflurane between the lipid and bulk aqueous phases is not rapid on the NMR time scale. Dix et al. have observed slow exchange of di-*t*-butyl nitroxide between dipalmitoyl phosphatidylcholine and water on the ESR time scale [14]. Since distinct broadening is observed in the aqueous methoxyflurane resonance in the presence of phospholipid compared to the same signal in the absence of phospholipid, it is clear that the exchange rate is likely to be moderately slow [15]. Thus, the lipid-bound

anesthetic signal probably does not represent weighted average contributions from bound and free anesthetic molecules. Similar conclusions have been reached with regard to the exchange of halothane between lipid-bound and free states [16].

The behavior of the three ether anesthetics in the presence of phospholipid is similar to the behavior of halothane in a similar system. Anesthetic line widths increase substantially, bound fluoroxene and methoxyflurane T_1 relaxation times shorten, implying slower isotropic motion, ^{19}F chemical shifts of bound anesthetics are different from shifts in water, and at the same time the anesthetics modify the order-disorder transition profile of the lipid. This implies that the incorporation of the anesthetics modifies the organization of the acyl chains in the bilayer.

Characterization of the anesthetic membrane interaction site may be achieved through a comparison of anesthetic ^{19}F chemical shifts in a series of *n*-alkanes and carbon tetrachloride [17] *. For the comparisons intended here any solvent property such as refractive index or solvent cohesive energy density, δ, [18] may be employed. The latter is related to the energy required to form a solvent cavity of sufficient size to contain the solute. The slopes of linear plots of δ vs. chemical shift were as follows: halothane (0.73), methoxyflurane (0.65), fluroxene (0.66), enflurane 2-fluoro moiety (0.82), enflurane difluoromethoxy moiety (0.79) [17]. Thus, from the observed ^{19}F chemical shifts of the lipid-bound anesthetics, corresponding δ values were estimated for the lipid site into which the anesthetics partition. Both above and below the phospholipid phase transition temperature for all of the anesthetics noted above, $\delta = 8.3 \pm 0.02$ $(cal/cm^3)^{1/2}$. This suggests that the local environment of the anesthetic molecules is affected very little by the phospholipid order-disorder transition.

Increasing the temperature of the phospholipid above its phase transition temperature leads to considerable narrowing of the anesthetic ^{19}F resonance in the absence of significant shifts in the position of the resonance. Thus, although the anesthetic-membrane interactions which lead to anesthetic line broadening, resulting from a lengthened correlation time due to restriction of anesthetic motion, are altered by the lipid phase transition, those forces determining anesthetic ^{19}F chemical shifts of membrane-bound anesthetics do not change appreciably.

The differential scanning calorimetric data presented in this paper relates to the anesthetic induced perturbations in the organization of the lipid bilayer as reflected in the order-disorder transitions. At low concentration (lower than 100 mM) the anesthetic appears to perturb only the packing of the acyl chains as reflected in lowering of the transition temperature without any effect on the half-height widths of the transition profiles. At higher concentrations (higher than 200 mM) the anesthetics also reduce the size of the cooperative unit undergoing the transition as reflected in increased half-height width or lowered mid-point slopes of integrated transition profile. The packing effects could arise from a localization of the solutes in the methyl end region of the acyl chains.

* The point of cyclohexane fell consistently above the line defined by the *n*-alkanes. A δ value of 7.7 was used for decane.

Reduction in the size of the cooperative units would involve a disruption of the hydrophobic interactions in the C_1-C_8 region of the acyl chains [2]. The calorimetric data are thus consistent with an interpretation that invokes different regions of localization of an anesthetic in the bilayer. Since little or no change in ^{19}F NMR chemical shift is detectable in a bilayer below or above the transition temperature, it would appear that the anesthetic is always outside the gel region. Below the phase transition temperature and/or at low anesthetic concentrations it may be in the defect region between the cooperative units. At high anesthetic concentrations it creates more of such defect regions by disrupting the cooperative units, and these smaller cooperative units are stabilized by distribution of the anesthetic molecules in the C_1-C_8 region.

The physiological significance of the order-disorder transitions in biomembranes remains to be understood [19]. If, however, such transitions were to regulate and control the various protein-mediated functions in the membrane, the anesthetic-induced changes in the transitions would exert physiological influence. The potential role of an anesthetic could thus not only be to modify the packing with the cooperative unit but also to reduce the size of the cooperative unit in biological membranes.

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