Different Distribution of Fluorinated Anesthetics and Nonanesthetics in Model Membrane: A ^{19}F NMR Study

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ABSTRACT Despite their structural resemblance, a pair of cyclic halogenated compounds, 1-chloro-1,2,2-trifluorocyclobutane (F3) and 1,2-dichlorohexafluorocyclobutane (F6), exhibit completely different anesthetic properties. Whereas the former is a potent general anesthetic, the latter produces no anesthesia. Two linear compounds, isoflurane and 2,3-dichlorooctofluorobutane (F8), although not a structural pair, also show the same anesthetic discrepancy. Using ^{19}F nuclear magnetic spectroscopy, we investigated the time-averaged submolecular distribution of these compounds in a vesicle suspension of phosphatidylcholine lipids. A two-site exchange model was used to interpret the observed changes in resonance frequencies as a function of the solubilization of these compounds in membrane and in water. At clinically relevant concentrations, the anesthetics F3 and isoflurane distributed preferentially to regions of the membrane that permit easy contact with water. The frequency changes of these two anesthetics can be well characterized by the two-site exchange model. In contrast, the nonanesthetics F6 and F8 solubilized deeply into the lipid core, and their frequency change significantly deviated from the prediction of the model. It is concluded that although anesthetics and nonanesthetics may show similar hydrophobicity in bulk solvents such as olive oil, their distributions in various regions in biomembranes, and hence their effective concentrations at different submolecular sites, may differ significantly.

INTRODUCTION

The Meyer-Overton rule, that the potency of general anesthetics correlates strongly with their solubility in olive oil, has long been taken to indicate that the sites of action of general anesthetics, whether in the membrane lipid or in excitable proteins, are hydrophobic in nature (Alifimoff and Miller, 1992; Franks and Lieb, 1994; Taheri et al., 1991; Ueda, 1995). Recent studies (Koblin et al., 1994; Liu et al., 1994a,b; Taheri et al., 1993), however, have shown that many compounds, strikingly similar to potent general anesthetics and predicted by the Meyer-Overton rule to be anesthetics, are completely devoid of anesthetic effects (i.e., they produce no anesthesia when administered alone and do not decrease the requirement for conventional anesthetics; Koblin et al., 1994). Although deviation from the prediction by the Meyer-Overton rule has been observed in the potency "cutoff" phenomenon found in many homologous series of anesthetics (Alifimoff et al., 1989; Liu et al., 1994a; Raines et al., 1993), the availability of anesthetic and nonanesthetic pairs that resemble each other structurally permits a closer examination of the molecular characteristics shared by the anesthetics but not by the nonanesthetics. Such examination may shed new light on the molecular and cellular mechanisms of general anesthesia (Raines and Miller, 1994).

Among many novel anesthetic and nonanesthetic pairs are two halogenated cyclobutanes, 1-chloro-1,2,2-trifluorocyclobutane (F3) and 1,2-dichlorohexafluorocyclobutane (F6). These two compounds have a similar molecular shape and size, and according to the Meyer-Overton rule, both should be potent general anesthetics, with predicted minimum alveolar concentrations (MAC) in rats of 0.0073 and 0.042 atm (Koblin et al., 1994), respectively. The measured MAC for F3, however, is 0.014 atm, and no anesthetic effects of F6 have been found, despite the fact that it dissolves in tissues and penetrates into the central nervous system at concentrations exceeding the predicted MAC (Koblin et al., 1994). Similarly, in linear compounds, some halogenated butanes produce anesthesia (Bagnall et al., 1979; Eger et al., 1994; Robbins, 1946), whereas others do not. Of particular interest to us is 2,3-dichlorooctofluorobutane (F8), which has a predicted MAC in rats of 0.073 atm (Koblin et al., 1994; Liu et al., 1994a) but produces no anesthesia.

The pharmacological difference between anesthetics and nonanesthetics may result from their distinct submolecular distribution in neuronal membranes. It has been shown that some anesthetics (e.g., enflurane, methoxyflurane, halothane, and chloroform) distribute preferentially at the membrane interface (Kamaya et al., 1981; Kaneshina et al., 1981; Yokono et al., 1981, 1989; Yoshida et al., 1989) and that they do not mix isotropically with the lipid core (Kaneshina et al., 1981). It has also been demonstrated for *n*-alkane anesthetics (Liu et al., 1994b) that the product of MAC × oil/gas partition coefficient × saline/gas partition coefficient is more constant than that of MAC × oil/gas partition coefficient predicted by the Meyer-Overton rule, suggesting that the site of anesthetic action may be neither purely hydrophobic nor purely hydrophilic, but a combination of the two (Liu et al., 1994b). Indeed, using intermolecular heteronuclear Overhauser effects, we have found

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that xenon, a monatomic anesthetic that is purely apolar in the gas phase, interacts preferentially with the amphiphilic regions after dissolving into a model membrane (Tang and Xu, 1994; Xu and Tang, 1997). Nonanesthetics, on the other hand, have relatively high oil/saline partition coefficients and thus tend to partition into the core of the membrane lipid.

In the present study, the anesthetic compounds isoflurane and F3, and the nonanesthetic compounds F6 and F8 were equilibrated at clinically relevant concentrations with a phosphatidylcholine (PC) model membrane. Their time-averaged distributions in the model membrane were examined at both 20°C and 37°C, using ^{19}F nuclear magnetic resonance (NMR) spectroscopy. The anesthetics were found to dwell, on the NMR time scale, in regions more accessible to (or easily exchangeable with) water, whereas the nonanesthetics partitioned into the deeper core of the membrane.

MATERIALS AND METHODS

Materials

Lyophilized L-α-PC from egg yolk (purity > 99%) was obtained from Avanti Polar Lipids (Alabaster, AL). Compounds F3, F6 (in a 1:1.2 mixture of *trans*- and *cis*-isomers), and F8 (in a mixture of five different conformational isomers with unknown ratios) were purchased from PCR (Gainesville, FL); isoflurane (AErrane) was from Ohmeda PPD (Liberty Corner, NJ); and deuterium oxide (D_2O) was from Cambridge Isotope Laboratories (Woburn, MA). Other chemicals, of analytical reagent grade, were purchased from Sigma (St. Louis, MO). All agents were used without further purification.

Sample preparation

Phosphatidylcholine lipid vesicles were prepared as previously described (Smith et al., 1981). Briefly, measured amounts of lyophilized egg PC were dissolved in chloroform and subsequently dried into a thin film under a stream of N_2 gas. The chloroform residues were further removed by applying a high vacuum at room temperature for at least 2 h. Thereafter, distilled and deionized water was added to make a homogeneous PC suspension with a final lipid concentration of 80 mg/ml (~105 mM). The suspension was subsequently sonicated into vesicles ~170 nm in diameter, as determined by light scattering (NICOMP 270 submicron particle analyzer; Particle Sizing System, Santa Barbara, CA).

All NMR samples were prepared by mixing measured amounts of PC vesicles (with final lipid concentrations ranging from 0 to 32 mM), fluorinated compounds, H_2O, and D_2O (20% by volume for the purpose of deuterium lock) directly inside the 5-mm, high-precision NMR tubes (Wilmad Glass Co., Buena, NJ), which have a volume of 2.5 ml. High-precision, gas-tight microsyringes (Hamilton Co., Reno, NV) were used to measure either 0.6 μl of F3, 0.8 μl of F6, 0.7 μl of isoflurane, or 0.4 μl of F8 for each NMR sample, resulting in final nominal concentrations of 1.0 mM for F8 and 2.2 mM for F3, F6, and isoflurane. To minimize possible losses of these volatile fluorinated compounds, the tubes were almost completely filled, and rapidly capped and sealed with a paraffin film. A tiny air bubble, <1% of the tube volume, was left in the tubes for the purpose of inversion mixing. After vigorous mixing, the samples were allowed to equilibrate at 4°C for at least 24 h before NMR measurements. Complete dissolution was confirmed by NMR, as the undissolved beads of these compounds precipitate and give rise to distinct NMR spectral peaks.

NMR spectroscopy

^{19}F NMR experiments were conducted at both 20°C and 37°C, using an Otsuka-Chemagnetics (Fort Collins, CO) CMXW-400SLI spectrometer operating at 377.4 MHz. A four-nucleus probe (J S Research, Boston, MA), with an observation channel for ^{19}F, was used to enhance the detection sensitivity. A one-pulse sequence was employed with a nominal 90° flip angle of 29 μs, a repetition delay of 3.5 s, and a spectral width of 10–25 kHz. Typically, 16K complex data points were collected and zero filled once before Fourier transformation. For each series of samples with a given fluorinated compound, changes in resonant frequencies were measured in units of hertz as a function of PC concentrations. Such changes were all relative to the corresponding peaks of the same compound in the PC-free samples and analyzed as follows.

Chemical shift analysis

For any given fluorinated molecules, the resonant frequency (i.e., the chemical shift) of resonance peaks for each given ^{19}F nucleus may vary, depending on the microscopic molecular environment that the nucleus experiences. In a water suspension of PC vesicles, the microscopic environment differs from site to site, permitting determination of distribution of the fluorinated molecules at different sites based on changes in the resonant frequencies. In the limit of rapid exchange (Trudell and Hubbell, 1976), in which the characteristic time needed for the fluorinated molecules to relocate from one environment to another is significantly shorter than the reciprocal of the maximum frequency differences for different environments, the measured peak positions are weighted averages of the limiting chemical shifts that are characteristic of each nuclear environment. In a simplified two-site exchange model (Kaneshina et al., 1981; Xu et al., 1996; Yoshida et al., 1989), in which the lipid membrane is collectively taken as one site and the aqueous phase as the other, the measured frequency of a peak, ν, is given by (Fraenkel et al., 1990; Xu et al., 1996)

$$\nu = X_M \nu_M + (1 - X_M)\nu_W, \tag{1}$$

where ν_M and ν_W are the limiting frequencies of the same resonance in pure membrane and in water, respectively, and X_M is the molar fraction of the fluorinated molecule in the membrane. Assuming that D_W and D_M are the solubilities expressed in molar ratio of the dissolved fluorinated molecule to water and to membrane, respectively, and m and m_W are the molarities of PC and water (a H_2O and D_2O mixture) in the NMR tube, respectively, then

$$X_M = \frac{mD_M}{m_W D_W + mD_M}. \tag{2}$$

Substituting Eq. 2 into Eq. 1 and expressing the frequency difference, $\Delta\nu$, as $\nu - \nu_W$ (where ν_W is measured in the PC-free solution), one has

$$\Delta\nu = \frac{mD_M}{m_W D_W + mD_M}(\nu_M - \nu_W). \tag{3}$$

Note that the sign of $\Delta\nu$ is determined by the sign of $\nu_M - \nu_W$. A negative $\Delta\nu$ for a given peak indicates that the frequency change in membrane is upfield relative to the corresponding peak in water.

In the present study, because a 1:4 (v/v) mixture of D_2O:H_2O was used for the deuterium lock, m_W was ~54.4×10^3 mM. Thus Eq. 3 can be rewritten as

$$\frac{1}{\Delta\nu} = \frac{1}{\nu_M - \nu_W}\left[1 + \frac{54.4 \times 10^3}{D_M/D_W} \times \frac{1}{m}\right]. \tag{4}$$

If the assumption of a two-phase fast exchange between membrane and water is valid, then a plot of $1/\Delta\nu$ as a function of $1/m$ (m expressed in mM) will yield a straight line, with the reciprocal of a slope proportional to

D_M/D_W and an intercept of $1/(\nu_M - \nu_W)$. Note that for $\Delta\nu < 0$ (upfield shift), the slope should be negative if the two-site exchange model applies.

A slightly more complicated situation may be considered where the lipid membrane is further divided into two sites. Thus, for a three-site model, Eq. 1 becomes

$$\nu = X_{M_1}\nu_{M_1} + X_{M_2}\nu_{M_2} + (1 - X_{M_1} - X_{M_2})\nu_W, \quad (5)$$

where ν_{M1} and ν_{M2} are the hypothetical limiting frequencies of the resonant peaks at fractional membrane site 1 and site 2, respectively, and X_{M1} and X_{M2} are the molar fractions of the fluorinated molecule at these sites. Clearly, $X_M = X_{M1} + X_{M2}$.

A special case may be considered. Assuming that the molarity of the two fractional membrane sites equals the molarity of the lipids, and the solubilities of a fluorinated compound in regions comprising the two sites, D_{M1} and D_{M2}, respectively, are independent of the lipid concentration, then the $\Delta\nu \sim m$ relationship of the three-site exchange model can be reduced to an apparent water-membrane two-site exchange model. Indeed, it is straightforward to show that Eq. 5 can be rewritten in this special case as

$$\frac{1}{\Delta\nu} = \left[\frac{m_W D_W}{D_{M_1}(\nu_{M_1} - \nu_W) + D_{M_2}(\nu_{M_2} - \nu_W)}\right]\frac{1}{m} + \frac{D_{M_1} + D_{M_2}}{D_{M_1}(\nu_{M_1} - \nu_W) + D_{M_2}(\nu_{M_2} - \nu_W)}. \quad (6)$$

Again, $\Delta\nu = \nu - \nu_W$ (i.e., relative to the corresponding peaks in water). If we define a total solubility in membrane: $D_M = D_{M1} + D_{M2}$, and an apparent limiting frequency in membrane: $\nu_M = (D_{M1}\nu_{M1} + D_{M2}\nu_{M2})/(D_{M1} + D_{M2})$, then Eq. 6 becomes Eq. 4. In other words, if a molecule is in fast exchange among all three sites and its solubility (or affinity) at the two fractional sites in membrane is independent of the lipid concentration, then a two-site exchange model, with the membrane taken collectively as one site, is as good as a three-site model. Clearly, this result can be generalized to one aqueous site and multiple membranous sites.

Another special case is when the solubility of a fluorinated molecule in regions comprising the two membrane sites varies with the lipid concentration. In this case, dependence of X_M (or X_{M1} and X_{M2}) on m is more complicated than as indicated explicitly by Eq. 2, because D_{M1} and D_{M2} are also functions of m. Rearranging Eq. 5 to express frequency change relative to ν_W, one has

$$\Delta\nu = \nu - \nu_W = X_M(\nu_{M_2} - \nu_W) + X_{M_1}(\nu_{M_1} - \nu_{M_2}). \quad (7)$$

This equation indicates that the measured frequency change relative to ν_W is a weighted superposition of the frequency difference between water and one of the two membrane sites (the first term in Eq. 7), and the difference between the two membrane sites (the second term in Eq. 7).

If in the presence of lipids, the membrane sites are strongly favored by a given fluorinated compound, then $X_M \to 1$, and Eq. 7 can be approximated by

$$\nu - \nu_{M_2} \approx X_{M_1}(\nu_{M_1} - \nu_{M_2}). \quad (8)$$

Note the resemblance of Eq. 8 to Eq. 3. This equation describes a two-site exchange within the membrane, without involving the aqueous phase.

RESULTS

Spectral assignment

Representative NMR spectra of isoflurane in a PC-free solution, and of neat F3, F6, and F8 are shown in Fig. 1. The spectral assignment for isoflurane has been well documented (Dubois and Evers, 1992; Mills et al., 1987; Xu et al., 1996). For F3, F6, and F8, the peak assignments shown in Fig. 1 were based on theoretical consideration of through-bond shielding and through-space deshielding to the ^{19}F nuclei by the Cl electron clouds (Cavalli, 1976; Feeney et al., 1966; Williamson and Braman, 1967) and were confirmed by the ratio of peak integration for the *trans* and *cis* forms of F6 and by ^{19}F homonuclear decoupling spectroscopy. The same number of peaks, with a similar spectral pattern, was found for fully dissolved F3, F6, and F8 in water. No additional peaks were found when the PC concentration was varied, suggesting that all of the compounds, once completely dissolved, were either in a single environment or in rapid exchange among multiple environments.

Changes in resonance frequencies as a function of PC concentration

Changes in resonant frequencies of all peaks were referenced to the corresponding peaks of the same compound in a PC-free solution. In the case of multiplets, the midpoints were used to measure the resonant frequency, so that errors due to variation in J-coupling constants at different PC concentrations were minimized. Fig. 2 *a* compares the frequency changes as a function of PC concentration at 20°C of various spectral peaks in the anesthetic and nonanesthetic pair F3 and F6. Similar changes for isoflurane and F8 are depicted in Fig. 2 *b*. Note that for both anesthetics (F3 and isoflurane), changes in frequency as a function of lipid concentration were gradual. In particular, the frequency change of the $-CF_3$ resonance in isoflurane was minute over the range of PC concentrations studied. In contrast, for both of the nonanesthetics, especially the ^{19}F atoms vicinal to Cl in F6 (i.e., F_{aa}, F_{bb}, F_{cc}, and F_{dd}, Fig. 1) and those of the various $-CF_3$ groups and the F_a group in F8, there were abrupt changes in resonant frequencies when the PC concentration was varied from zero to a nonzero value. This finding suggested that once the PC was present, the nonanesthetics F6 and F8 were predominantly in the lipid phase.

Submolecular distribution

The data presented in Fig. 2, *a* and *b*, are replotted in Fig. 3 to show the dependence of $1/\Delta\nu$ on $1/m$. Because $\Delta\nu$ is unchanged ($\Delta\nu = 0$) for the $-CF_3$ resonance of isoflurane, the double-reciprocal plot cannot be made for this group. Solid lines are linear least-squares fits to the data using Eq. 4, yielding D_M/D_W and $1/(\nu_M - \nu_W)$ values (at 20°C) for all of the resonance peaks of F3 and the $-CF_2H$ peak of isoflurane. Similar linear $1/\Delta\nu \sim 1/m$ plots are also constructed (not shown) for F3 and isoflurane at 37°C. The fitting results are summarized in Table 1. The excellent fit to the anesthetic data by Eq. 4 suggests that the two-site exchange model is reasonably realistic for both the linear and cyclic anesthetics used in this study. Although the F_{cis} and F_{trans} peaks of F6, and the F_b/F_c peak of F8 showed a linear $1/\Delta\nu \sim 1/m$ dependence, with the same sign of slope as

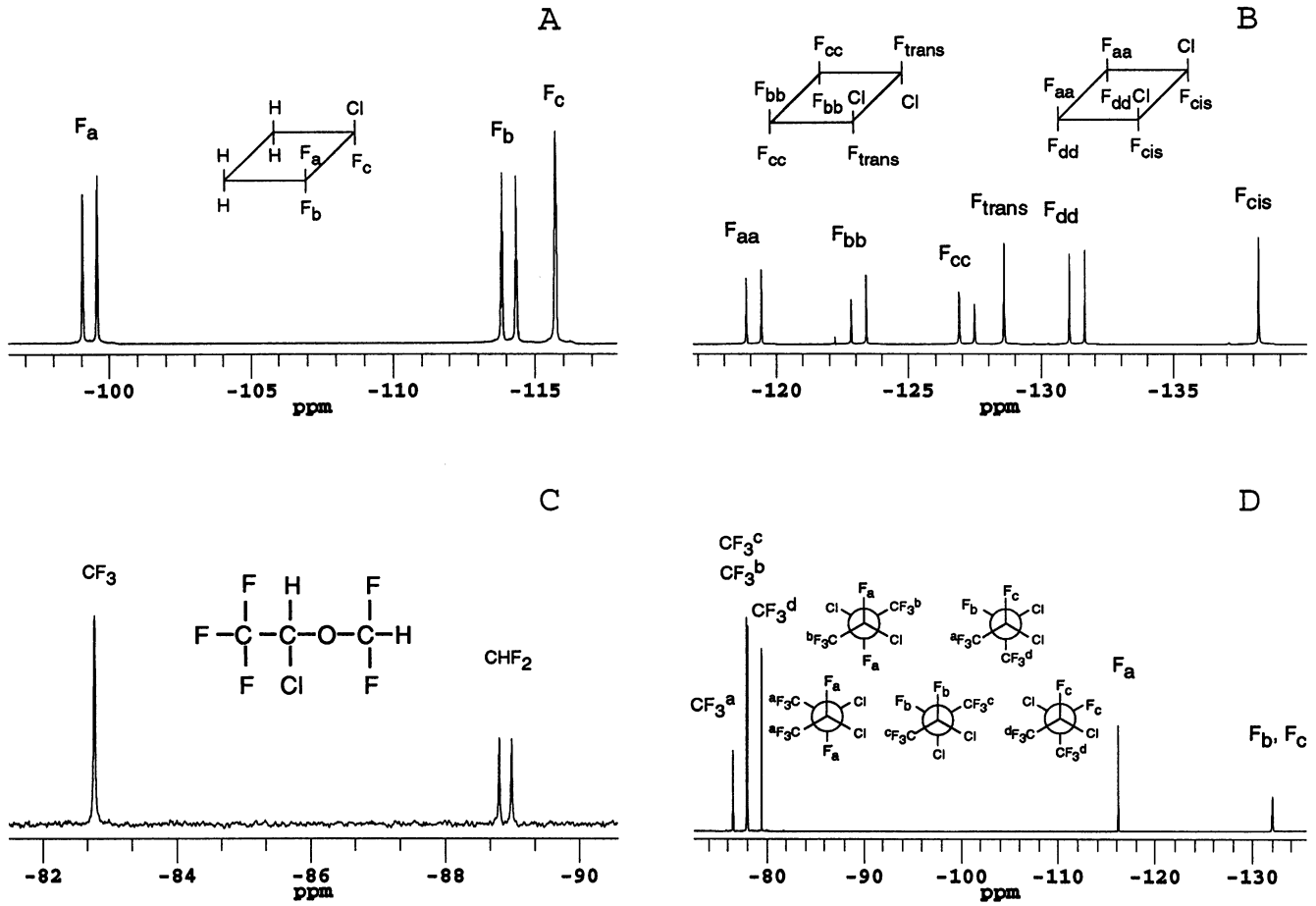

FIGURE 1 Representative ^{19}F NMR spectra at 9.4 T (377.4 MHz) and molecular structures of (*A*) F3, (*B*) F6, (*C*) isoflurane, and (*D*) F8. Labels for F atoms are indicated in the structures and are used for the peak assignments shown here and in the text.

predicted by Eq. 4, the majority of resonance peaks of these two nonanesthetics deviated greatly from the water-membrane exchange model (i.e., they either have a wrong slope, or do not fit a straight line). This is also the case at 37°C. Because F6 and F8 are small molecules, it is unlikely that portions of the nonanesthetic molecules are in fast exchange between membrane and water, whereas the rest of the molecules are not. Thus a model of two-site exchange between water and membrane is not applicable to the nonanesthetic distribution.

As suggested by Eq. 8, the double reciprocal dependency of resonant frequencies of F6 and F8 on PC concentration reflects the fast exchange of the nonanesthetics between the two fractional membrane sites. However, the complexity of the X_{M1}-m relationship, as implicated by the nonlinear double reciprocal function for most of the resonance peaks, precludes a quantitative analysis of D_{M1} and D_{M2}. For those nonanesthetic resonances that can be fitted with a linear function, the reciprocal of the intercept, which equals $\nu_{M1} - \nu_{M2}$ according to Eq. 8, and the reciprocal of the slope, which relates in a rather complicated way to the partitioning of the nonanesthetics at the two membrane sites, are listed in Table 2.

DISCUSSION

The results presented here show that a novel pair of cyclobutanes, one anesthetic and one not, exhibit different distributions in a model membrane. Two linear compounds, again, one anesthetic and one not, follow the same patterns of anesthetic and nonanesthetic distribution. For the anesthetics (F3 and isoflurane), resonance from all ^{19}F spins showed a gradual change in resonant frequency from a PC-free aqueous solution to vesicle suspensions of various PC concentrations, suggesting that all ^{19}F spins, and hence the whole molecules, remained in contact with the aqueous phase and could exchange easily between the water and lipid environments. Indeed, results from all resonance peaks of F3 and the -CHF_2 resonance of isoflurane showed an excellent agreement with the two-site exchange model. The D_M/D_W ratios for these two compounds are in line with those found by others for other inhaled anesthetics (e.g., halothane, enflurane, methoxyflurane, and chloroform), confirming that the anesthetics dwell in regions close to the lipid-water interface (Kamaya et al., 1981; Kaneshina et al., 1981; Xu and Tang, 1997; Yokono et al., 1981, 1989; Yoshida et al., 1989). Interestingly, the resonant frequency

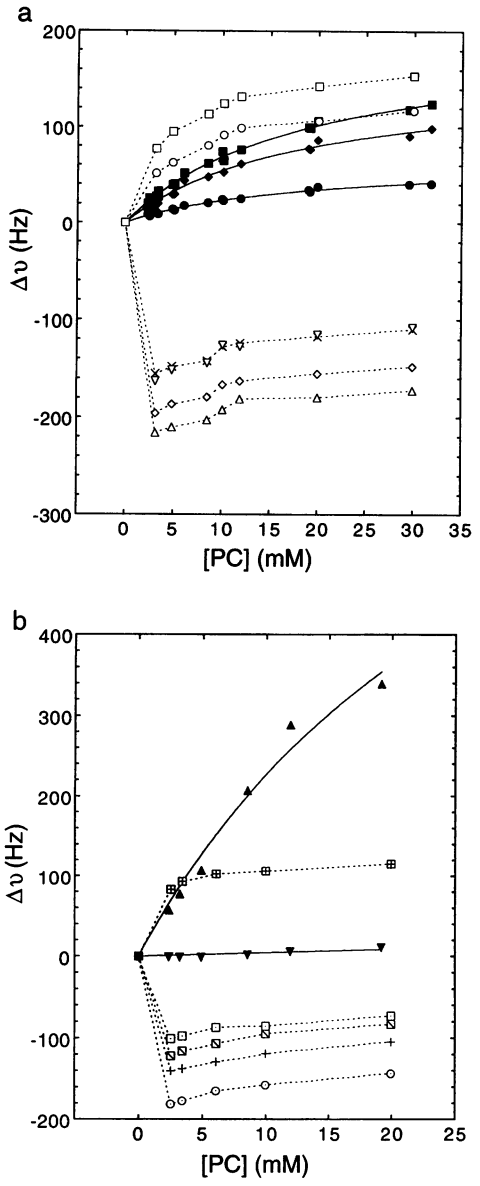

FIGURE 2 Changes in resonant frequencies (Hz at 9.4 T) as a function of PC concentration at 20°C for (*a*) compound F3 (F_a, ●; F_b, ■; F_c, ♦) and compound F6 (F_{cis}, □; F_{trans}, ○; F_{aa}, ×; F_{bb}, ▽; F_{cc}, Δ; F_{dd}, ◇); and (*b*) isoflurane (-CF_2H, ▲; -CF_3, ▼) and compound F8 (CF_3^a, ◫; CF_3^b/CF_3^c, +; CF_3^d, ⊙; F_a, ⊡; F_b/F_c, ⊞). All changes are referenced to the corresponding resonance of the same peak in a PC-free solution (the points at the origin). The solid lines are a nonlinear regression to the anesthetic data using Eq. 3. The dotted lines are a visual guide for the nonanesthetic data.

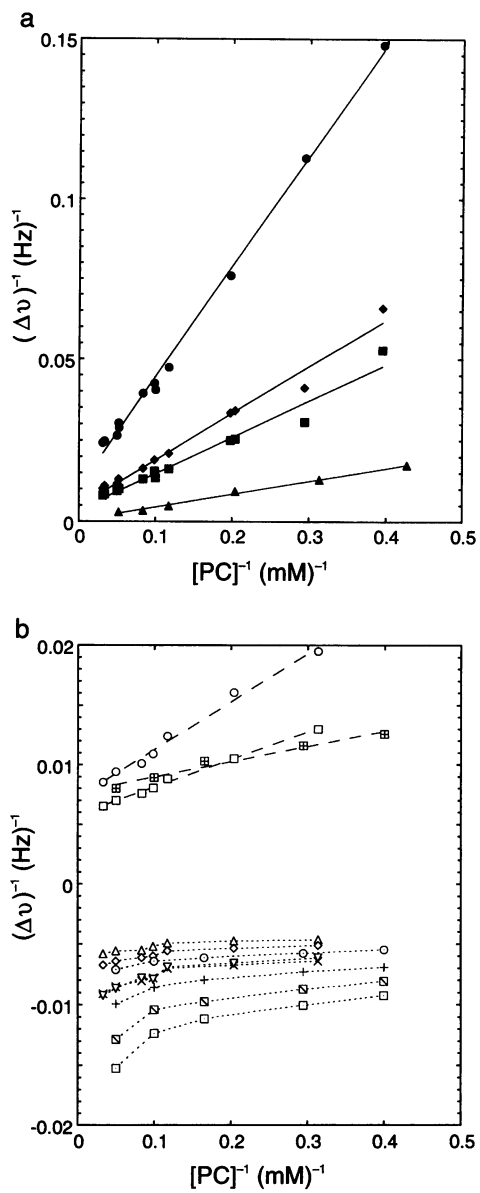

FIGURE 3 Plot of reciprocal of frequency changes, $\Delta\nu^{-1}$, as a function of reciprocal of PC concentration, $[PC]^{-1} \equiv m^{-1}$, for (*a*) anesthetics F3 and isoflurane; and (*b*) nonanesthetics F6 and F8. Symbol assignments are the same as in Fig. 2. Solid lines in *a* are linear least-squares fit to Eq. 4, showing an excellent agreement of the anesthetic data with the model of rapid exchange of the anesthetics between aqueous and membranous environments. The same model fails to fit the nonanesthetic data. In *b*, dashed lines are a linear fit with Eq. 8, and dotted lines, showing nonlinearity, are a visual guide.

of the -CF_3 peak of isoflurane did not change with PC concentration. It has been pointed out (Kaneshina et al., 1981; Yoshida et al., 1989) that many linearly shaped halogenated anesthetics are amphiphilic, with one end being relatively more hydrophilic and the other end more hydrophobic. It is thus of interest to compare the changes in resonance frequency of the -CF_3 resonance in halothane ($CF_3CClBrH$) and in isoflurane. The -CF_3 group in halothane represents the hydrophobic end of the molecule and has been shown (Yoshida et al., 1989) to be immersed in the membrane with the opposite end extending outward, making the halothane molecule perpendicular to the membrane surface. In isoflurane, however, the -CF_3 group belongs to the more hydrophilic end of the molecule. The nearly constant resonant frequency, independent of the lipid concentration, suggests either that the limiting shifts in water and at the membrane surface are incidentally identical for this group, or that the -CF_3 is in close contact with the aqueous phase in the vesicle suspension.

TABLE 1 Summary of D_M/D_W and $\nu_M - \nu_W$ of F3 and isoflurane at 20°C and 37°C

		20°C		37°C	
Compound	F atom	$D_M/D_W \times 10^{-3}$	$\nu_M - \nu_W$ (Hz)	$D_M/D_W \times 10^{-3}$	$\nu_M - \nu_W$ (Hz)
Isoflurane	CHF_2	3.3 ± 0.3	520 ± 22	3.2 ± 0.3	433 ± 18
F3	F_a	3.0 ± 0.3	66 ± 3	1.9 ± 0.3	44 ± 5
	F_b	2.9 ± 0.2	199 ± 8	2.5 ± 0.2	155 ± 5
	F_c	2.9 ± 0.4	156 ± 11	2.4 ± 0.2	132 ± 8

TABLE 2 Summary of 1/slopes and ($\nu_{M1} - \nu_{M2}$) of the double-reciprocal plots for F6 and F8

		20°C		37°C	
Compound	F atom	1/Slope	$\nu_{M1} - \nu_{M2}$ (Hz)	1/Slope	$\nu_{M1} - \nu_{M2}$ (Hz)
F6	F_{tran}	24.9 ± 1.2	138 ± 6	15.4 ± 1.0	124 ± 7
	F_{cis}	43.3 ± 1.2	171 ± 6	31.3 ± 1.4	155 ± 5
F8	F_b, F_c	77.8 ± 8.0	122 ± 1	47.6 ± 7.4	96 ± 3

Although the two-site exchange model fits the anesthetic data well, our results do not contradict the possibility that multiple membrane sites exist for anesthetics. As Eq. 6 indicates, if the anesthetic distribution among these sites is independent of the lipid concentration, then the result of fast exchange among two or more membrane sites is effectively the same as taking the membrane as a single collective site (compare Eq. 6 with Eq. 4). Using ^2H NMR and ^1H-^1H NOE spectroscopy, Baber and colleagues showed in a recent study (Baber et al., 1995) that the anesthetic effects on the order parameter for segments 1–6 and segments 7–16 in a perdeuterated palmitoyl(d_{31})oleoylphosphatidylcholine (POPC-d_{31}) membrane were different, suggesting that segments 1–6 and 7–16 may represent different sites for anesthetic binding. A careful examination, by the same authors, of the average population of anesthetics at these sites revealed that the fractional occupations at these two sites were almost equal, with a slight preference of anesthetics for the interface. Because the linear $1/\Delta\nu \sim 1/m$ relationship fulfills both Eq. 4 and Eq. 6, our result with F3 and isoflurane further confirms that the anesthetics are in rapid exchange not only between aqueous and membranous phases, but also among submolecular sites within the lipid membrane (Trudell and Hubbell, 1976).

The situation, however, was entirely different for the nonanesthetics. For both F6 and F8, most resonance peaks could not be approximated by the two-site exchange model (see Figs. 2 *a*, 2 *b*, and 3 *b*). Almost all spins showed abrupt changes in resonant frequency from the aqueous phase to the lipid membrane. Such abrupt changes indicate that the molecular environments for these spins are completely different when changing from the aqueous phase to the lipid phase—once the lipid is present, the chance that these spins will approach water is greatly reduced. Because F6 and F8 are small in size, this result also implies that the accessibility of whole nonanesthetic molecules to the aqueous phase may be limited.

This conclusion is also qualitatively in agreement with Eqs. 7 and 8. A close examination of the data for F6 and F8 in Fig. 2, *a* and *b*, especially those with negative $\Delta\nu$, reveals that the frequency changes of the nonanesthetics can all be treated as a superposition of a large, abrupt jump relative to ν_W and a small change over the range of the PC concentration studied. Clearly, the large jump can be accounted for by the first term in Eq. 7, whereas the small increases are dictated by the second term of Eq. 7. Thus, if the nonanesthetics do indeed escape from the aqueous phase when PC is present, then it is conceivable, according to Eq. 8, that the small frequency change after the abrupt jump is due to the two-site exchange process of nonanesthetics inside the membrane, probably within the core of the lipid bilayer without involving the water phase.

Temperature had only small effects on the anesthetic and nonanesthetic distribution in the egg PC membrane. As the temperature was increased from 20°C to 37°C, the D_M/D_W and $\nu_M - \nu_W$ of the two anesthetics, and the slopes and intercepts of the double-reciprocal plots for the two nonanesthetics were all decreased slightly (see Table 1 and Table 2). The overall distribution pattern, however, remained the same: the anesthetics dwelled in regions closer to the aqueous phase, whereas the nonanesthetics partitioned deeply into the membrane.

The distinct distribution of anesthetics and nonanesthetics may ultimately affect their affinity for other membrane constituents, such as membrane proteins. Although the lipid portions of membranes have been implicated by the Meyer-Overton rule as the target sites for general anesthetics, recent evidence suggests that anesthetics may inhibit neuronal activity by modulating the function of specific proteins. A superfamily of neurotransmitter-gated receptor channels, including the γ-aminobutyric $acid_A$ ($GABA_A$) receptor, the glycine receptor, the neuronal and muscular nicotinic acetylcholine receptor, and 5-hydroxytryptamine$_3$ receptor, are particularly sensitive to general anesthetics (Wood et al., 1995), with potentiation of postsynaptic inhibitory channel activity best fitting the pharmacological profile of general anesthesia (Franks and Lieb, 1994). Indeed, it has been shown (Mihic et al., 1994) that F3, but not

F6 and F8, can strongly potentiate GABAergic currents in *Xenopus* oocytes expressing $\alpha1\beta2$ or $\alpha1\beta2\gamma2S$ $GABA_A$ receptors. Because a structurally diverse range of anesthetics can enhance the same current, but structurally comparable nonanesthetics cannot, the anesthetic action at these receptors must not be structurally specific. Rather, it may simply be controlled by the submolecular solubility to some amphipathic sites on the protein: the strong hydrophobicity of the nonanesthetics, as shown in this study, renders them less likely to distribute to these sites.

In conclusion, we have shown that the distributions of anesthetics and nonanesthetics in the membrane differ. The anesthetics dwell in the more amphiphilic regions with easy access to the aqueous phase, whereas the nonanesthetics prefer the lipid core of the membrane.

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