Multiple environments of fluorinated anesthetics in intact tissues observed with ^{19}F NMR spectroscopy

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The incorporation of two fluorine-containing general anesthetic agents, halothane and methoxyflurane, into erythrocytes (from three different species), rabbit muscle and rabbit nerve, was followed with ^{19}F NMR spectroscopy. Two major findings emerged from these studies: (1) multiple environments indicative of domain structure in the membrane can be observed depending on the anesthetic and the tissue type; and (2) the ^{19}F chemical shifts of a given anesthetic were characteristic for the tissue examined. Halothane showed a single resonance in erythrocytes and multiple resonances in muscle and nerve, while methoxyflurane showed multiple resonances in both muscle and erythrocytes. The range of the ^{19}F chemical shifts for the multiple peaks was as great as 6 ppm.

Halothane *Methoxyflurane* *Nerve* *Muscle* *Erythrocyte* *NMR*

1. INTRODUCTION

Even though general anesthetics have been used for over a hundred years, the mechanism of action of these agents is still actively debated. The site at which general anesthetics act is widely believed to be the plasma membrane of the brain cells. Disagreements exist about the details of interaction at the molecular level, with both membrane-bound proteins and the lipid bilayer being suggested as the principal locus of action. These and other viewpoints have been presented in two recent reviews [1,2].

Some of the conflicting opinions appear to arise from the fact that only indirect methods have been available for the study of membrane–anesthetic interactions. Model systems such as phospholipid vesicles and bilayers [3–7] have often been studied. As they are made more realistic and thus more complex (e.g., by adding different proteins), the ability to get unambiguous experimental results diminishes. On the other hand, studies on intact or in vivo systems are necessary for the description of these membranes in the living state. Unfortunately, they are limited in intact animals to such physiological measurements as righting behavior and other reflex reactions which provide only crude and indirect assessment of the biochemistry of anesthetic action [8,9].

A new methodological approach to this problem can be gained by applying ^{19}F nuclear magnetic resonance spectroscopy. Fluorine-19 offers distinct advantages over other nuclei: ^{19}F-containing anesthetics can serve as their own probes, the sensitivity for ^{19}F detection is high, and no interfering fluorine compounds exist naturally in tissue. With these considerations in mind, we have initiated a study of fluorinated anesthetics in intact tissues in order to characterize the microenvironment(s) within which anesthetics act. If various microenvironments can be distinguished with the aid of these anesthetics, this technique should have important applications in the general area of membrane analysis as well as in the more specific area of anesthesia research.

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2. MATERIALS AND METHODS

Whole blood was examined either in vitro or in vivo. In vitro examination was performed on samples collected in heparinized syringes from three mammalian species: man, dog, and rabbit. The in vivo sampling was achieved by using an arterio–venous shunt external to the anesthetized dog. An NMR tube was installed in this shunt so that it could be placed in the NMR spectrometer while the dog pumped the blood through the external circuit [10]. This allowed direct observation of the fluorinated anesthetics while the blood was in contact with the body. In order to assign the ^{19}F signals observed in whole blood, the major components of blood were examined individually. Erythrocytes were separated from plasma by low speed centrifugation (3000 × *g*) and washed with 20 mM phosphate-buffered (pH 7.4) saline.

Two examples of excitable tissues which may interact with anesthetics in a manner analogous to the nerve cells in the brain were also examined. The sciatic nerve and soleus muscle were removed from New Zealand White rabbits and stored in cold, well oxygenated Krebs-Ringer bicarbonate buffer (pH 7.4) until they were used.

The anesthetic was delivered to the animal, or to the isolated systems, with standard anesthesia equipment. A North American Dräger-Narcovet machine was used along with a semi-closed rebreathing system for the delivery of halothane. Methoxyflurane levels were regulated with a Hidenbrink number 8 vaporizer.

^{19}F NMR measurements were performed on a Bruker WP-80 NMR spectrometer at ambient temperature (30°C). The following acquisition parameters were used: 2500 Hz sweep width, 90° pulse angle and 1.6 s repetition time. Spectra were collected without proton decoupling. Chemical shifts were measured with respect to a neat trifluoroacetic acid (TFA) external standard.

3. RESULTS

^{19}F NMR spectra of methoxyflurane ($CH_3O–CF_2CH_2Cl$) equilibrated in vitro with blood display multiple peaks as shown in fig.1. The chemical shifts of the resonances observed for each of the blood samples examined are summarized in table 1. Their ranges are 3.5 ppm for human,

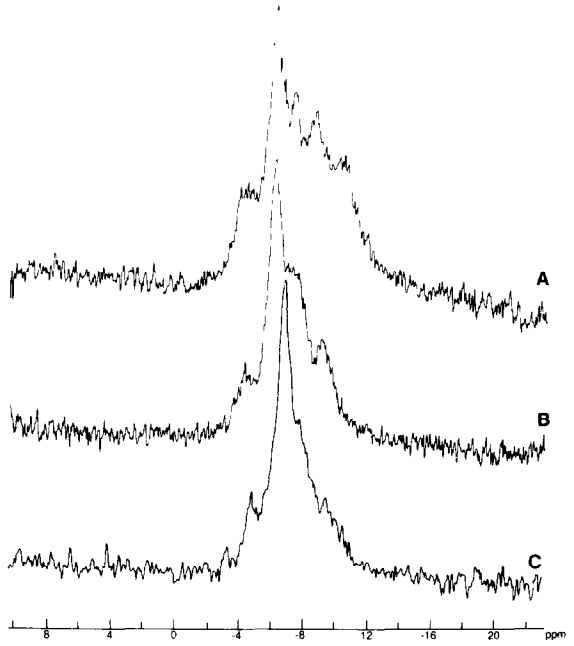

Fig.1. ^{19}F NMR spectra of methoxyflurane in (A) rabbit, (B) dog and (C) human blood.

Table 1

^{19}F chemical shifts of signals from methoxyflurane in blood of three mammalian species

Species	Blood (δ, ppm)[a]	Erythrocytes (δ, ppm)[a]	Plasma (δ, ppm)[a]
Human	−5.0	−5.0	
	−6.8[b]	−6.8[b]	
	−7.3		−7.3
	−8.5	−8.5	
Dog	−4.2	−4.2	
	−6.1[b]	−6.1[b]	
	−7.0	−7.0	
	−7.3		−7.3
	−9.0	−9.0	
Rabbit	−4.4	−4.4	
	−6.0[b]	−6.0	
	−7.2		−7.2
	−8.5	−8.5	
	−10.2	−10.2	

[a] Chemical shifts are reported as parts per million from neat trifluoroacetic acid; positive numbers indicate downfield direction

[b] Major peak

4.8 ppm for dog, and 5.8 ppm for rabbit blood. The signals at −7.3 ppm (human and dog blood) and at −7.2 ppm (rabbit blood) with 12–15 Hz line widths can be assigned to methoxyflurane in plasma by comparison with the corresponding plasma fraction. Examination of packed red cells verifies that the remaining signals arise from methoxyflurane distributed in erythrocytes. The appearance of multiple peaks suggests slow exchange (in the ms-range) of methoxyflurane between plasma and the different compartments of erythrocytes. The line widths of these peaks range between 60–200 Hz as a result of differences in correlation times which arise from variations in the constraints on motion of the 'bound' anesthetic. As a result of this broadening, the $^{1}H-^{19}F$ coupling (which is in the order of 5–6 Hz) cannot be observed in any of the spectra reported.

In contrast to methoxyflurane, halothane ($CF_3CHBrCl$) shows only two signals in blood from each of the three species examined (table 2). There is slow exchange of halothane between plasma and the erythrocytes, but there is no evidence for multiple environments for halothane in the erythrocytes. Line widths for halothane in erythrocytes are 80 Hz, whereas those for plasma are only 12–15 Hz. The signals observed for methoxyflurane or halothane with whole blood in vivo are identical to those already described for drawn blood.

Fig.2 shows spectra of rabbit soleus muscle equilibrated with methoxyflurane (2A) and with halothane (2B). Halothane shows peaks at 2.8 and 2.5 ppm (major peak) and methoxyflurane at −7.0 and −8.0 ppm (major peak). Line widths are only 15–20 Hz.

Table 2

^{19}F chemical shifts of halothane in blood of three mammalian species

Species	Blood (δ, ppm)[a]	Erythrocytes (δ, ppm)[a]	Plasma (δ, ppm)[a]
Human	3.4	3.4	
	3.0		3.0
Dog	3.6	3.6	
	2.9		2.9
Rabbit	4.1	4.1	
	3.0		3.0

[a] Chemical shifts are reported as parts per million from neat trifluoroacetic acid; positive numbers indicate downfield direction

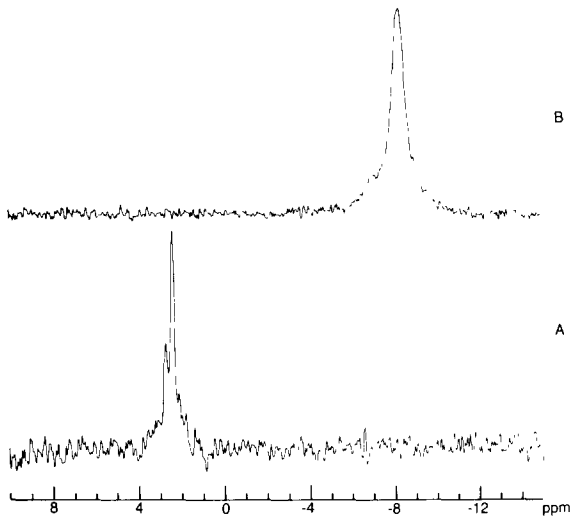

Fig.2. ^{19}F NMR spectra of (A) methoxyflurane and (B) halothane in rabbit soleus muscle.

The ^{19}F NMR spectrum of halothane equilibrated with rabbit sciatic nerve (fig.3A) shows several peaks which span the chemical shift

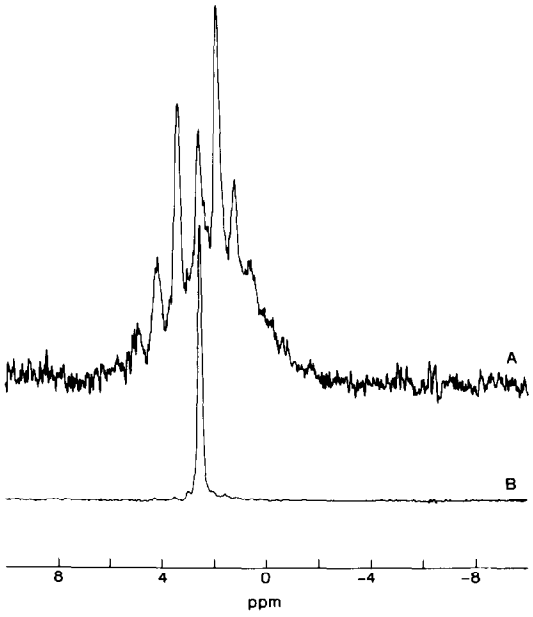

Fig.3. ^{19}F NMR spectrum of halothane in (A) fresh and (B) aged rabbit sciatic nerve.

range from 5.0 to −1.0 ppm, with different line widths. As the excised nerve ages, the pattern of NMR peaks changes until only one signal is seen at 2.5 ppm (3B). These signals reported from whole tissue originate only from anesthetic incorporated in the muscle or nerve, since buffers in which these tissues are suspended during the NMR experiments show no ^{19}F signals after tissue is removed.

4. DISCUSSION

A range of chemical shifts is observed for both halothane and methoxyflurane in the tissues examined which is comparable to that seen in pure solvents (table 3) [11,12]. The chemical shifts observed are dependent upon both the anesthetic agent and the type of tissue used.

Variation of chemical shift with tissue type can be illustrated with the data for halothane in rabbit tissues. In muscle, the major and minor peaks are at chemical shifts 2.5 and 2.8 ppm. The nerve has a multiplet with at least eight components on a broad base over the range 5 to −1 ppm. In blood, the chemical shift is 4.1 ppm for erythrocytes and 3.0 ppm for plasma. Methoxyflurane shows similar variation in chemical shift with tissue type.

A more detailed analysis is possible for the data on blood and its components. Each anesthetic shows a single narrow line with a characteristic chemical shift when equilibrated with plasma. The position of the signal is independent of species. This behavior would be consistent with either fast exchange of anesthetic among several sites in the plasma, or with a single site.

Table 3

^{19}F chemical shifts of halothane and methoxyflurane in pure solvents

Solvent	Halothane (δ, ppm)[a]	Methoxyflurane (δ, ppm)[a]
H_2O	2.85	−7.03
n-Octanol	2.14	−8.59
Hexane	1.16	−9.50
Heptane	1.25	−9.43
Neat	1.41	−8.60
Methanol	0.67	−9.75

[a] Chemical shifts are reported as parts per million from neat trifluoroacetic acid; positive numbers indicate downfield direction

The signals from erythrocytes are species specific and consist of 3 or 4 lines for methoxyflurane, while halothane gives only single line, also species specific (see tables 1 and 2). This difference in signal characteristics may be due to the different solubilities of these agents in blood [13]; methoxyflurane is five times as soluble as halothane. We propose that the greater solubility of methoxyflurane is a reflection of a larger number of sites to which it can be distributed in the erythrocyte membrane. These distinct environments are sufficiently different that they give rise to unique ^{19}F chemical shifts. Chemical exchange between these environments must be slow on the NMR time scale, in order that multiple peaks be observed. In contrast, the single line observed for halothane is consistent with either fast exchange among several sites or with a single site. In both cases the ^{19}F chemical shift reflects the environment of the anesthetic in the membrane, and the characteristic composition [14] of lipids and proteins in the erythrocyte membrane is the source of species specificity. We regard the chemical shift of the anesthetic as an index to characterize that average environment.

The characteristic differences in line widths for anesthetics in plasma (<20 Hz) and in membrane (60–200 Hz) reflect differences in mobility at these sites. In general, lower mobility will result in broader lines.

We now turn to regularities in the ^{19}F chemical shifts. Halothane shows a consistent downfield shift of fluorine resonance relative to the value in water (2.85 ppm). These downfield chemical shifts may indicate protein–anesthetic interactions, since fluorine-containing amino acids incorporated into proteins have been reported to show a wide range of signals downfield from their chemical shifts free in solution [15,16]. Methoxyflurane ^{19}F signals are distributed both down- and upfield from the signal observed (−7.03 ppm) for this compound in water. The additional upfield peaks not observed with halothane may be indicative of interactions with the lipid portions of the erythrocyte membrane.

Further systematic studies are required to assign the environments occupied by the anesthetics on the basis of their ^{19}F chemical shift and other NMR parameters. Erythrocytes provide a more tractable system to work with since their membrane com-

position can easily be manipulated to aid in the spectral assignment. The spectra of halothane in nerve show that anesthetic exists at multiple sites and that those sites depend on maintenance of metabolic activity. Whether one or more of these sites is critical for neural activity will be a point to investigate in future research. The NMR technique potentially can contribute to elucidation of the mechanism of anesthesia.

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