

## Unifying characteristics of sites of anesthetic action revealed by combined use of anesthetics and non-anesthetics

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### Abstract

1. The usefulness of nonanesthetics in the study of mechanisms of general anesthesia lies in the possibility to identify the unifying characteristics of molecular sites that are shared by the anesthetics but not by the structurally similar nonanesthetics. 2. In model membranes, pairs of structurally similar anesthetics and nonanesthetics showed distinctly different submolecular distributions. 3. This difference may be the underlying cause for the different anesthetic and nonanesthetic interaction with gramicidin A, a model transmembrane cation channel. 4. Generalization of our findings suggests that the nature of the sites, whether in lipids or proteins, must be neither extremely hydrophilic nor extremely lipophilic, but amphiphilic. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

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### 1. Introduction

The value to use the so-called ‘nonanesthetics’ in the investigation of molecular mechanisms of general anesthesia has engendered considerable debate (Koblin et al., 1994; Eckenhoff, 1995). These nonanesthetics, or nonimmobilizers to be more precise (Eger et al., 1997), belong to a group of molecules that are predicted by the Meyer–

Overton rule to be anesthetics, but neither produce anesthesia by themselves nor reduce the requirement of a known anesthetic to produce anesthesia (Koblin et al., 1994). The debate has centered on whether potential molecular sites of action of general anesthetics can be identified by differentiating anesthetics from nonanesthetics at such sites. Whereas some believe that a molecular event that is shared by both the anesthetics and nonanesthetics is unlikely to be relevant to anesthesia (Koblin et al., 1994; Raines and Miller, 1994), others contend that different mechanisms

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(and hence different sites or multiple sites) are likely involved for different anesthetic agents; to test the anesthesia relevance of any sites solely on the basis of interaction of nonanesthetics at these sites would be premature (Eckenhoff, 1995).

## 2. Meyer–Overton rule and beyond

An important element in the current definition of ‘nonanesthetics’ is that they are predicted by the Meyer–Overton rule to be anesthetics. A countless number of molecules, which are predicted *not to be* anesthetics by the same rule (e.g. extremely hydrophilic molecules), are therefore excluded from the class of agents now referred to as ‘nonanesthetics’. This exclusion is, in essence, to presume that the sites of action are hydrophobic in nature. Because general anesthesia at molecular level is undefined, a more general question to ask is whether making any assumption about the nature of the sites before molecular characterization is fundamentally necessary. There is a distinct possibility that the extremely hydrophobic (or lipophilic) molecules, which disobey the Meyer–Overton rule, fail to produce anesthesia for the same fundamental reason that the extremely hydrophilic molecules fail to do so.

That hydrophobicity alone cannot fully characterize anesthetics has been realized for many years (Franks and Lieb, 1978; Chiou et al., 1990). Recent studies (Taheri et al., 1991; Eger et al., 1994; Liu et al., 1994) have directly indicated the importance of a polar component in anesthetic action. In particular, it has been found that for many volatile anesthetics, the product of potency  $\times$  oil/gas partition coefficient deviates more from a constant predicted by the Meyer–Overton rule (Taheri et al., 1991) than does the product of potency  $\times$  oil/gas partition coefficient  $\times$  saline/gas partition coefficient (Liu et al., 1994). The second product clearly implicates the involvement of water. Using  $^{129}\text{Xe}$  nuclear magnetic resonance (NMR) spectroscopy to quantify site-selective interaction between xenon (a clinical anesthetic) and different regions in a phosphatidylcholine (PC) model membrane, we have shown (Xu and Tang, 1997) that xenon, an apolar molecule with spheri-

cal symmetry in the gas phase and expected to be more compatible with the core of lipid, interacts more favorably with the interfacial choline group than with the aliphatic methylene group. Moreover, xenon interacts specifically with water at the membrane interface. These findings suggest that the selection of target sites by a given molecule, when many sites of different types are available at the same time, depends not only on the properties of the molecule itself, but also more importantly on the possible changes in these properties after interaction with a membrane takes place. In the case of xenon, the *induced* dipole due to xenon-membrane interaction clearly makes xenon more adaptable to the amphiphilic interface than to the lipid core. A similar but opposite argument would be that some molecules, having the ‘right’ solubility in olive oil to be anesthetics based on the Meyer–Overton rule, find themselves more attracted to regions irrelevant to anesthesia and henceforth fall into the category of nonanesthetics.

Thus, deviation from the Meyer–Overton rule may represent more refined characteristics of the sites of anesthetic action. The value to use nonanesthetics as a distinct group of molecules, in contrast to anesthetics, lies in the possibility to identify the unifying nature of molecular sites that are shared by all anesthetics but not by the structurally similar nonanesthetics.

## 3. Different distribution of anesthetics and nonanesthetics in model membrane

To determine if such unifying characteristics exist, we have recently studied the time-averaged, submolecular distributions of two volatile anesthetics, 1-chloro-1,2,2-trifluorocyclobutane (F3) and isoflurane, and two volatile nonanesthetics, 1,2-dichlorohexafluoro-cyclobutane (F6) and 2,3-dichlorooctofluorobutane (F8), in a suspension of PC lipid vesicles (Tang et al., 1997). Fluorine-19 ( $^{19}\text{F}$ ) NMR was used to measure the changes in resonant frequencies of these agents as a function of PC concentration. The high sensitivity of  $^{19}\text{F}$  NMR allows for measurements of these agents at a clinically relevant concentration (i.e.  $\approx 1\text{--}2\text{ mM}$

total concentration in a 3–30 mM lipid vesicle suspension). NMR experiments were conducted at 20 and 37°C, using a Chemagnetics CMXW-400SLI spectrometer operating at 377.4 MHz. For each series of experiments, in which the PC concentration was varied from 0 to 30 mM, the changes in resonant frequencies,  $\Delta\nu$ , were measured in unit of hertz relative to the corresponding peaks of the same compound in the PC-free samples. For data analysis, the following two equations were derived to describe rapid exchanges between two sites (i.e. aqueous and lipid phases) and three sites (i.e. one aqueous phase and two membrane sites), respectively:

$$\Delta\nu = \nu - \nu_W = \frac{mD_M}{m_W D_W + mD_M} (\nu_M - \nu_W) \quad (1)$$

$$\Delta\nu = X_M(\nu_{M_2} - \nu_W) + X_{M_1}(\nu_{M_1} - \nu_{M_2}) \quad (2)$$

where  $\nu$  and  $D$  represent resonant frequency and solubility expressed in molar ratio of the dissolved fluorinated molecule to the corresponding solvent (i.e. the site), respectively,  $m_W (= 54.4 \times 10^3 \text{ mM})$  and  $m (= [\text{PC}])$  are the molarity of water and PC, respectively, and the subscripts W, M,  $M_1$ , and  $M_2$  indicate aqueous phase, membrane phase collectively taken as one site, and the first and second sub-sites in the membrane, respectively.

When changing from a lipid-free environment to one with various PC concentrations, the resonant frequencies of all fluorine groups in the two anesthetics changed gradually, but those in the two nonanesthetics changed abruptly. This is true at both 20 and 37°C. Fig. 1 depicts representative changes in  $\Delta\nu$  as a function of PC concentration for a typical peak from F3, isoflurane, F6, and F8 at 20°C. For both volatile anesthetics, irrespective of cyclic or linear in shape, the changes can be fitted remarkably well by Eq. (1) (the solid lines in Fig. 1), suggesting that the anesthetics are in rapid exchange between aqueous and membranous phases. For both F3 and isoflurane, the ratios of solubility in membrane and in water,  $D_M/D_W$  are in good agreement with those found by others for other inhaled anesthetics (Kaneshina et al., 1981; Kamaya et al., 1981), confirming that anesthetics dwell in regions close to the lipid–water interface

(Xu and Tang, 1997). We have shown (Tang et al., 1997) that the  $\Delta\nu \sim [\text{PC}]$  dependency given by Eq. (1) is also valid for cases with multiple sites in membranes, as long as the anesthetics are in rapid exchange among all these sites and the aqueous phase. In contrast, the nonanesthetics, F6 and F8, showed a  $\Delta\nu \sim [\text{PC}]$  relationship that greatly deviates from the prediction of Eq. (1), but is qualitatively in agreement with Eq. (2) under the special condition of  $X_M \rightarrow 1$ . The abrupt changes in resonant frequency indicate that the molecular environments for the nonanesthetics are completely different when changing from the aqueous to lipid phase—once the PC is present, the nonanesthetics are predominantly in the lipid phase with negligible access to water.

#### 4. Gramicidin A as a model for transmembrane channel proteins

The distinctly different distribution of anesthetics and nonanesthetics in the lipid membrane may

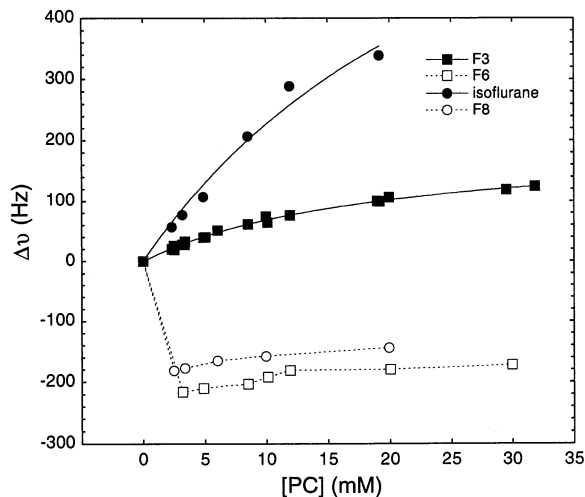


Fig. 1. Different anesthetic and nonanesthetic distribution in model membrane. Plotted are changes in resonant frequencies (Hz at 9.4 T) as a function of phosphatidylcholine lipid concentration at 20°C for anesthetics, F3 (■) and isoflurane (●), and nonanesthetics, F6 (□) and F8 (○). All changes are referenced to the corresponding resonance in a lipid-free solution (the points at the origin). The solid lines are a nonlinear regression to the anesthetic data using Eq. (1). The dotted lines are a visual guide for the nonanesthetic data.

ultimately affect their affinity for other membrane constituents, such as membrane proteins. Gramicidin A, a 15-amino-acid polypeptide that forms cation-conducting channels with highly resolved three-dimensional transmembrane structure (Fig. 2), offers a unique model for analyzing interactions at a submolecular level between anesthetics (or nonanesthetics) and integral proteins. We have found that F3 and F6 modulate  $\text{Na}^+$  transport differently across the gramicidin A channel. With  $75 \mu\text{M}$  gramicidin A in 66 mM PC/phosphatidylglycerol vesicle suspension, 6.9 mM F3 (equivalent to 1.5 mM aqueous concentration) increased  $\text{Na}^+$  unidirectional influx and efflux rates by 22 and 28%, respectively. In contrast, a similar concentration of F6 had no effects on either influx or efflux rate.

To account for this difference, we have: (1) investigated the surface polarity map of the gramicidin A channel in the lipid bilayer, using the known 3D coordinates of the channel conformer (1 gra, from the Protein Data Bank at the Brookhaven National Laboratories); and (2) measured the specific interaction strength between F3 or F6 and individual amino acid residues of the channel, using intermolecular, truncated driven  $^{19}\text{F}\{^1\text{H}\}$  nuclear Overhauser effects (NOE) (Xu and Tang, 1997). Fig. 2 shows the top and side views of the surface map calculated using the sidechain hydrophobicity and hydrophilicity values on a residue-by-residue base. Notice the four amphiphilic domains at each end of the channel. These domains are attributable mainly to the four tryptophan residues in each gramicidin molecule. (Each channel consists of two monomeric  $\beta$  helices, which meet head-to-head in the interior of the membrane bilayer.) If the different anesthetic and nonanesthetic preferences for the amphiphilic interfacial region in lipid bilayer hold also true for protein sites, then one would expect a different affinity for F3 and F6 at the amphiphilic domains of the channel. Indeed, we found that the anesthetic F3, but not the nonanesthetic F6, can interact specifically with the four tryptophan residues at the membrane interface. Parallel studies using two-dimensional NOE spectroscopy (NOESY) in the presence and absence of isoflurane, F3 and F6 also showed that while all three compounds could affect the residues in the deeper lipophilic regions,

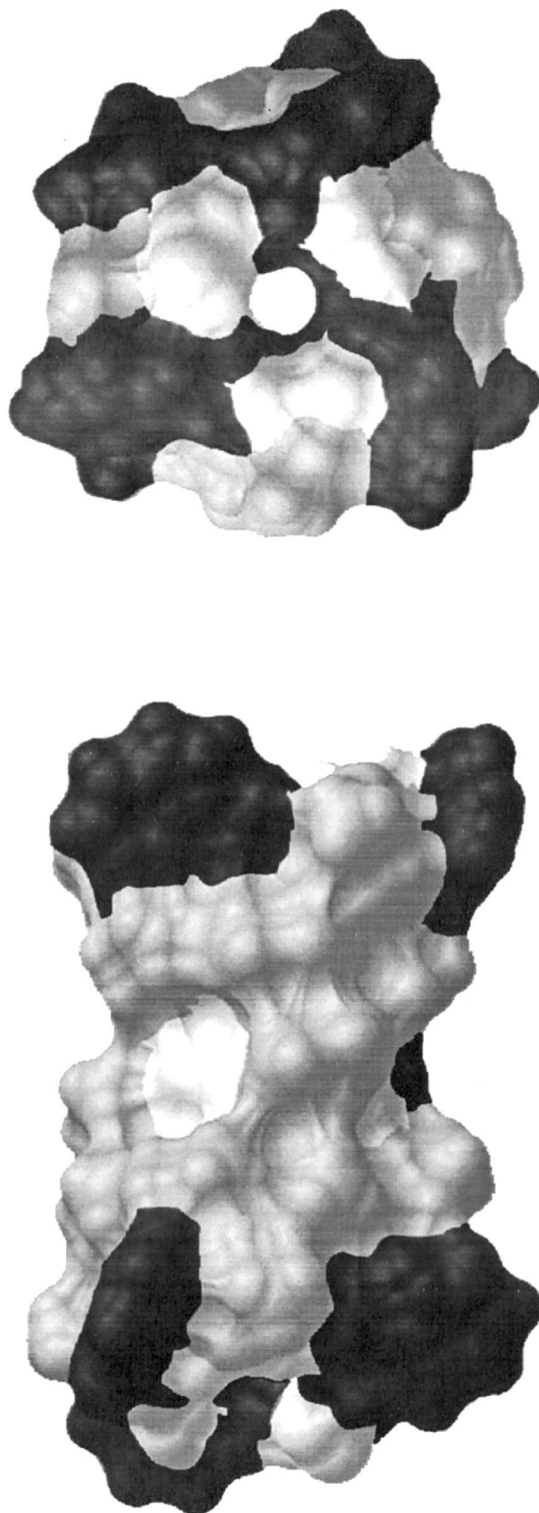


Fig. 2.

only isoflurane and F3 caused concentration-dependent changes in the  $^1\text{H}$  resonant frequencies of the sidechains at the interface. These changes are believed to result from changes in hydrogen bonding of these sidechains with water or lipid head groups, rather than from changes in the secondary structure of the channel. It is conceivable that association of the critical residues of the channel with the interfacial “solvent” (i.e., lipid and water) can affect the channel dynamics, thereby changing the channel functionality.

Extensive NMR structural studies of gramicidin A have shown that the four tryptophan residues play a pivotal role in channel function. First, each of the indoles is uniquely oriented with its N–H bond directed toward the bilayer surface (Hu et al., 1993; Hu and Cross, 1995). Such orientation favors hydrogen bonding to the hydrophilic interface, presumably to water molecules that are either at the membrane surface or penetrate into the amphiphilic domain along the lipid–peptide interface. Second, the sidechains of the tryptophan residues are extended parallel to the bilayer surface, and are likely to be hydrogen-bonded to the lipid molecules (Meulendijks et al., 1989; Scarlata, 1991). These extended tryptophan sidechains anchor the channel in the bilayer and orient the channel with respect to the surface. Thus, if our finding with F3 and F6 can be generalized, that is, only anesthetic, but not the structurally similar nonanesthetic, can preferentially target the amphiphilic sidechains to alter the functional association of these sidechains with their environment, then it is no surprise that only the anesthetic, but not the nonanesthetic, can change the channel conductivity. It should be noted that although gramicidin A plays no clinical role in general anesthesia, the pharmacological profile found in our studies suggests that this channel-forming peptide can offer unique properties and typical

structural motifs for testing general theories of anesthetic–protein interaction.

## 5. Conclusion

Our studies with lipid vesicles and a model transmembrane protein all indicate one mechanism, that only the anesthetics, but not the structurally similar nonanesthetics, can target the amphiphilic regions in the membrane and keep constant access to the aqueous phase. This feature may prove to have important structural and functional consequences on neuronal channel proteins.

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Fig. 2. Surface polarity map of gramicidin A channel: top view and side view (bottom). Notice the amphiphilic domains (black) near each entrance of the channel and the hydrophobic domain (gray) that spans the membrane. Note that because the dark grey scale was added to highlight the amphiphilic domains, the abrupt boundaries between domains are artificial.

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