General Anesthetic Binding to Gramicidin A: The Structural Requirements

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ABSTRACT There is a distinct possibility that general anesthetics exert their action on the postsynaptic receptor channels. The structural requirements for anesthetic binding in transmembrane channels, however, are largely unknown. High-resolution ^{1}H nuclear magnetic resonance and direct photoaffinity labeling were used in this study to characterize the volatile anesthetic binding sites in gramicidin A (gA) incorporated into sodium dodecyl sulfate (SDS) micelles and into dimyristoylphosphatidylcholine (DMPC) bilayers, respectively. To confirm that the structural arrangement of the peptide side chains can affect anesthetic binding, gA in nonchannel forms in methanol was also analyzed. The addition of volatile anesthetic halothane to gA in SDS with a channel conformation caused a concentration-dependent change in resonant frequencies of the indole amide protons of W9, W11, W13, and W15, with the most profound changes in W9. These frequency changes were observed only for gA carefully prepared to ensure a channel conformation and were absent for gA in methanol. For gA in DMPC bilayers, direct [^{14}C]halothane photolabeling and microsequencing demonstrated dominant labeling of W9, less labeling of W11 and W13, and no significant labeling of W15. In methanol, gA showed much less labeling of any residues. Inspection of the 3-D structure of gA suggests that the spatial arrangements of the tryptophan residues in the channel form of gA, combined with the amphiphilic regions of lipid, create a favorable anesthetic binding motif.

INTRODUCTION

Sensitivity to general anesthetics has placed a superfamily of ligand-gated ion channels at the top of the list of candidate sites for general anesthetic action. These include glycine, γ-aminobutyric acid type A ($GABA_A$), neuronal nicotinic acetylcholine (nACh), and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors. Although the functional characteristics of these channels have been extensively studied (Eckenhoff and Johansson, 1997; Dilger et al., 1997; Forman et al., 1995; Franks and Lieb, 1994; Harrison et al., 1993; Jenkins et al., 1996; Mihic et al., 1997), the anesthetic binding sites at the molecular level have still not been revealed. The difficulty is associated directly with the lack of structural information about these complicated ion channels. Before a 3-D structure of these channels becomes available, it is advantageous to use simplified and structurally well-characterized membrane channels as models to analyze the structural requirements for anesthetic binding in transmembrane channels. The characteristics of the binding site or sites learned from the simplified models may possibly be generalized to the authentic receptors.

Gramicidin A (gA) is a simple model of an ion channel (Tang et al., 1999a,c; Cross, 1997; Laio and Torre, 1999; Lundbaek and Andersen, 1999; Tian and Cross, 1999; Cotten et al., 1997). It is one of the best-characterized transmembrane peptides and can adopt various conformations in different solvents (for a review, see Killian, 1992). Gramicidin A is a 15-amino-acid peptide of the sequence HCO-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-$NHCH_2CH_2OH$. When incorporated into lipid membranes, gA forms monovalent cation channels in the form of a right-handed, single-stranded $\beta^{6.3}$ helical dimer. In sodium dodecyl sulfate (SDS) micelles, the secondary structure of gA is the same as that of the channel. The 3-D structures of gA in both SDS micelles and lipid bilayers have been determined by NMR to atomic resolution (Arseniev et al., 1985; Cross, 1997). In organic solvents, however, gA is predominantly in one of the nonchannel forms. For example, in methanol, gA occurs as an equilibrium mixture of interwound double-helical conformers (Chen and Wallace, 1996; Veatch et al., 1974). This conformational difference allows for analysis of the structural requirements for general anesthetic binding in the channel.

We showed recently (Tang et al., 1999a) that the function of the gA channel can be modulated differently by a volatile anesthetic, 1-chloro-1,2,2-trifluorocyclobutane (F3), and a structurally similar nonimmobilizer (nonanesthetic), 1,2-dichlorohexafluorocyclobutane (F6). We also showed with 2D NMR and truncated driven nuclear Overhauser effects (Tang et al., 1999c) that while both F3 and F6 can perturb gA residues deep inside the hydrophobic region in SDS micelles, only F3 can interact specifically with tryptophan residues and significantly alter the NMR resonance frequencies of the tryptophan indole N-H protons near two ends of the channel. In the present study, we determined whether the preferred anesthetic interaction with the interfacial tryptophan residues in gA was structure-dependent. The halothane binding was analyzed by both ^{1}H NMR spectroscopy and [^{14}C]halothane direct photoaffinity labeling techniques for gA in the channel form in SDS and dimyristoylphos-

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phatidylcholine (DMPC) bilayers, and in the double-stranded helical dimer form in methanol. We also attempted to determine whether halothane binding prefers the lipid-protein interface, by varying the gA-DMPC ratio. If halothane prefers to localize at the protein-lipid interface, normalized labeling of lipid will depend on protein concentration.

The experimental methods used in this study are complementary to each other. The NMR experiments focus on the changes in the gA channels due to anesthetic binding, whereas the photolabeling measurements focus on the anesthetic halothane. The former offers the advantage of measuring anesthetic-protein interaction at equilibrium without perturbing the measured system, whereas the latter, although destructive, can pinpoint the actual location at which the interaction takes place. The combination of the two approaches provides unambiguous assignment of the anesthetic binding sites at high resolution.

MATERIALS AND METHODS

Materials

Purified gA was purchased from Calbiochem (La Jolla, CA). Deuterated sodium dodecyl sulfate (SDS-d_{25}) and deuterated methanol were obtained from Cambridge Isotope Laboratories (Andover, MA). Halothane was purchased from Ayerst Laboratories (Philadelphia, PA). [^{14}C]Halothane ([1-^{14}C]2-bromo-2-chloro-1,1,1-trifluoroethane; 6.6 mCi/mmol) was purchased from Dupont NEN as the neat compound. DMPC was purchased from Avanti Polar Lipids (Alabaster, AL). 3-Trimethylsiyl-1-propanesulfuric acid (DSS) and other chemicals, of analytical grade, were from Sigma Co. (St. Louis, MO). SDS was recrystallized in ethanol before use. All other compounds were used without further purification.

NMR

Samples for NMR experiments were prepared in two different groups: 1) 4 mM gA dissolved in deuterated methanol (90% MeOH, 10% H_2O) and 2) 2.5 mM gA in 500 mM SDS micelles dissolved in 90% H_2O and 10% D_2O. To ensure a channel conformation for gA in SDS micelles, the same preparation procedure as described previously (Tang et al., 1999c) was followed. For both groups, 1 mM DSS was added to each sample as a reference for ^{1}H resonant frequencies. The pH was adjusted to 4.8, and the solution volume was 0.5 ml in a 5-mm high-precision NMR tube, which was later sealed, leaving a 2-ml vapor space above the solution.

Halothane was titrated directly into the samples in the NMR tube, using a Hamilton microsyringe. After equilibrating with the vapor phase, the total halothane concentrations in the sample solution were estimated using ^{19}F NMR, with reference to an external standard of 0.19 mM trifluoroacetic acid (TFA) in a 10-mm NMR tube, which was coaxial to the 5-mm sample tube.

The NMR experiments were conducted at 30°C, using an Otsuka-Chemagnetics (Fort Collins, CO) CMXW-400SLI spectrometer equipped with a ^{1}H detection probe (Nalorac Co., Martinez, CA). The resonance frequency for ^{1}H was 401.102 MHz. Typical experimental parameters were 12-μs 90° pulses, 1.5-s repetition delays, a 6.67-kHz spectral width, and WATERGATE (Piotto et al., 1992) for water suppression. For each spectrum, 128 scans were accumulated in 4096 complex points. The data were zero-filled once before Fourier transformation. Resonance frequencies (i.e., chemical shifts) were determined by multipeak Lorentzian curve fitting, using the Origin 6.0 analysis program (Microcal Software, Northampton, MA), and averaged among four repeated measurements.

Photolabeling

Gramicidin A was dissolved in trifluoroethanol along with DMPC in a 1:100 mole ratio, dried with argon, and exposed to high vacuum overnight. The dried protein-lipid mixture was resuspended (1 mg/ml) in argon-equilibrated, 50 mM potassium phosphate buffer (pH 7.0) by vigorously mixing and sonication until the mixture was opalescent. [^{14}C]Halothane was added to a concentration of 0.3 mM (~1% gas v/v at 37°C). The mixture was then exposed to a 254-nm light from a pencil calibration Hg(Ar) lamp driven at 18 mA (Oriel, Stratford, CT) for 60 s at a distance of 5 mm in capped quartz cuvettes ($1 \times 0.5 \times 4$ cm^3). The solution was lyophilized and resuspended in methanol. The components were separated on a C4 column with a methanol mobile phase. The gA fraction was desformylated with methanolic 0.4 N HCl for 1 h and then sequenced on an Applied Biosystems sequencer (model 473A; Applied Biosystems, Norwalk, CT). Small aliquots were run to ensure proper identity of the residues. Thereafter, larger fractions (10 nmol) were run and pre-HPLC fractions were collected to determine the release of radioactivity by liquid scintillation.

Lipid-protein photolabeling

To determine the possibility of preferential localization of halothane binding at the lipid-protein interface, photolabeling was measured with a varying mole ratio of gA to DMPC, ranging from 0 to 0.05. Labeling was carried out as above, but with 50 μM ^{14}C-halothane (0.2% gas v/v at 37°C). The DMPC was separated from the gA by thin-layer chromatography. The radioactivity (in units of disintegrations per minute or dpm) incorporated into each fraction was normalized to either mole phosphorus or to microgram of protein. An aliquot of labeled lipid was hydrolyzed at the carbonyl oxygen, using mild alkaline conditions and heat. The radioactivity in each phase of the extraction was determined using liquid scintillation.

RESULTS

Changes in NMR frequency of gA due to anesthetics are indicative of anesthetic-gA interaction. We found that the effects of halothane on the resonance frequencies of the indole amide protons of gA were strongly dependent on gA conformation. Fig. 1 depicts representative ^{1}H-NMR spectra in the indole amide proton region of gA in SDS micelles and methanol (90% methanol and 10% H_2O) before and after the addition of 16 mM halothane, with frequencies referenced to the internal standard of DSS at 0 ppm.. Similar spectra were also acquired at other concentrations of halothane. The spectral assignment was made based on 2D ^{1}H NOESY experiments (Tang et al., 1999c) and H-D exchange experiments (Tang et al., 1999b). Comparable to our previous findings with F3 (Tang et al., 1999c), the only gA resonance frequencies that were significantly affected were those of the indole amide protons. Fig. 2 compares the chemical shift changes of the indole amide protons as a function of halothane concentration in SDS and methanol. Solid lines are linear least-squares fit to the data. In SDS micelles, in which gA forms β6.3 channel dimers, all of the

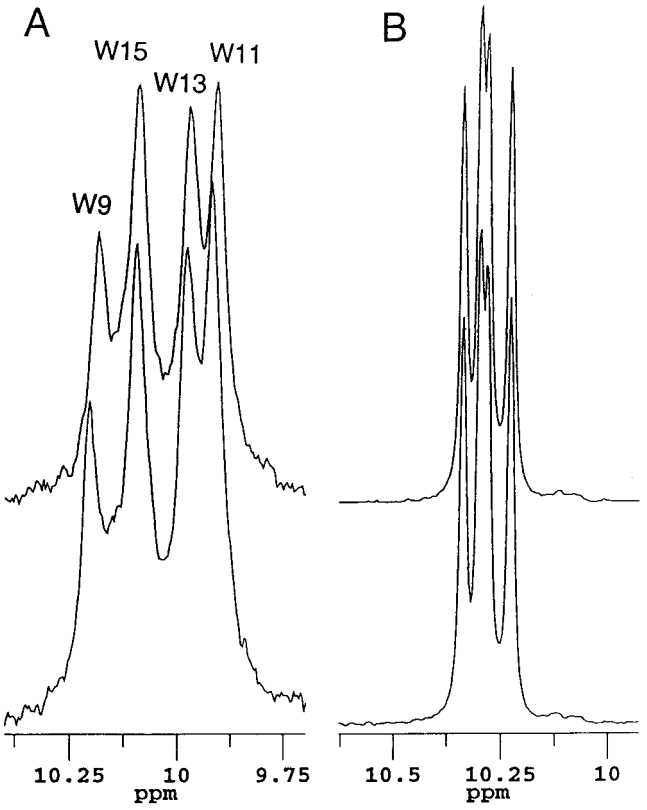

FIGURE 1 Stacked plots of ^{1}H-NMR spectra of the indole N-H proton region acquired at 30°C before (*bottom traces*) and after addition of 16 mM halothane. (*A*) 2.5 mM gA in 500 mM SDS micelles. (*B*) 4 mM gA in deuterated methanol (90% MeOH, 10% H_2O). All resonant frequencies are referenced to the DSS peak at 0 ppm.

indole N-H protons were shifted by halothane in a concentration-dependent manner. The extent of the shifts correlated with the location of the indole N-H protons along the gramicidin channel. W9, which is located furthest from the surface, showed the largest shift, as depicted in Fig. 2 *A*. In contrast, the anesthetic effect on resonance frequency is undetectable for gA in the form of double-stranded dimers in methanol. The slopes in Fig. 2 *B* are essentially not significantly different from zero.

Fig. 3 demonstrates the results of [^{14}C]halothane photolabeling of gA in DMPC bilayers and methanol. Consistent with NMR frequency change in SDS, the majority of halothane label in gA in DMPC bilayers was found on the tryptophan residues near the two ends of the channel. The amount of labeling on W9, W11, W13, and W15 followed the same trend as the anesthetic effect on indole N-H chemical shifts: W9 showed the most labeling, whereas W15 showed the least labeling among the four tryptophan residues. Only the background dpm levels were observed for residues from the N-terminus to V8. Photolabeling under identical conditions in methanol showed a large reduction of incorporated dpm, but a small preference for labeling W9 was still noted. This residual preference might be due to

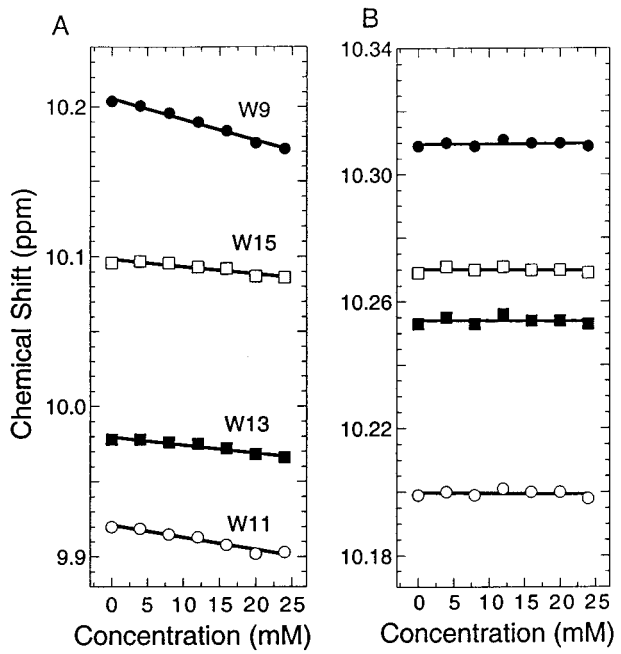

FIGURE 2 Comparison of resonant frequencies of gramicidin indole amide protons as a function of halothane concentrations in (*A*) SDS micelles and (*B*) methanol. Resonant frequencies are determined by repeated measurements, using multiple-peak Lorentzian fitting. Error bars are smaller than the size of the symbols.

persistent structure in this region of the peptide, as photochemical selectivity for the tryptophan residues should have been apparent in the other tryptophan residues as well.

Fig. 4 shows that the stoichiometry of labeling was ~40 nmol labeled halothane per μmol of gramicidin A, or a halothane-gA ratio of 1:25 at a [^{14}C]halothane concentration of ~0.05 mM. The stoichiometry of labeling was

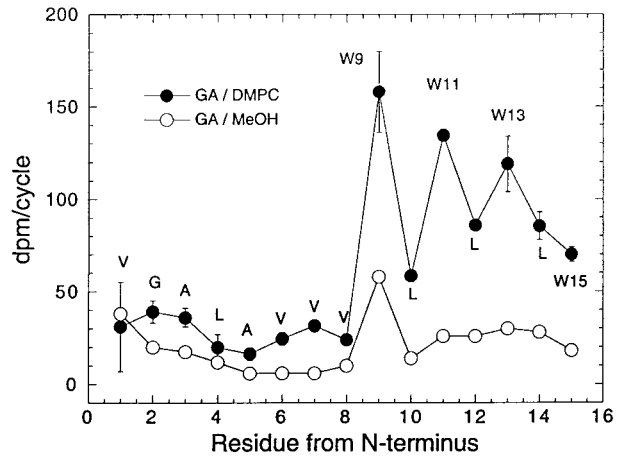

FIGURE 3 Disintegrations per minute (dpm) per cycle for each cycle of the HPLC fractions are plotted from microsequencing of gA in DMPC (●) and in methanol (○). All standard error bars are included for gA in DMPC, but some are smaller than the size of the symbols. Letters denote amino acids.

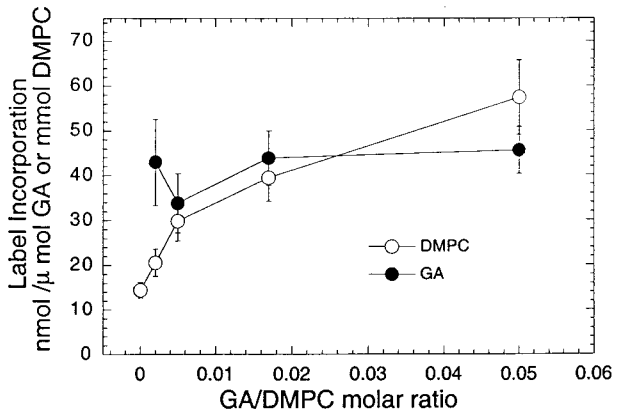

FIGURE 4 The stoichiometry of ^{14}C-halothane labeling to gA (●) and to DMPC (○) as a function of mole ratio of gA to DMPC.

independent of gramicidin concentration in the lipid. Labeling of DMPC, although of much lower stoichiometry, was strongly dependent on the mole ratio of gA to DMPC. Furthermore, hydrolysis of the phospholipid revealed that 91% of the dpm was associated with the acyl chain region.

DISCUSSION

The strong dependence of indole amide proton frequencies on halothane concentration indicates that structurally selective interaction between gA and the anesthetic occurs near the tryptophan residues in the channel form. The predominant photolabeling of [^{14}C]halothane on the tryptophan residues of the channel further rules out the possibility that NMR frequency change is caused by the nonspecific perturbation of the SDS or DMPC lipids surrounding the channel. Both NMR and photolabeling results suggest that the anesthetic-gA channel interaction is strongest at or near W9 and decreases gradually in the order of W11 > W13 > W15. The selective anesthetic interaction with the tryptophan residues diminishes almost completely when gA becomes double-stranded helical dimers in methanol. The halothane photolabeling on gA in methanol falls within the background labeling level, and halothane at sufficiently high concentrations fails to produce a significant change in the NMR frequencies of gA. These results imply that tryptophan residues per se are not the sole determinant of the anesthetic-gA interaction, but that the structural arrangement of the tryptophan side chains in the channel conformer might also play a role in creating the specific anesthetic target sites.

Fig. 5 depicts the structure of gA channel in DMPC (Ketchem et al., 1993) and the structure of gA crystal grown from methanol (Langs et al., 1991; Burkhart et al., 1998). Both were drawn using the RasMol program (Sayle and Milner-White, 1995), based on the coordinates deposited at the Protein Data Bank. Careful examination of Fig. 5 *A* (i.e., the structure for photolabeling) reveals that the tryptophan side chains in DMPC are uniquely oriented relative to the lipid-water interface. In particular, W9 and W15 side chains are arranged such that an amphiphilic pocket is created in the space between the side chains and the lipid headgroups. Although W9 and W15 do not stack in gA in SDS (Arseniev et al., 1985), the side chain association with the lipid headgroups is similar. We showed previously (Xu and Tang,

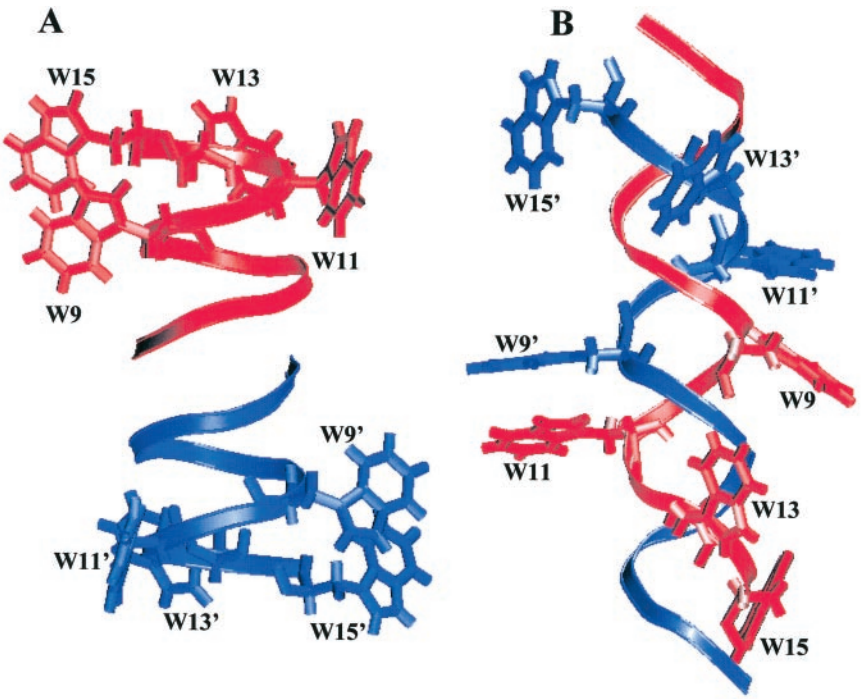

FIGURE 5 Ribbon structures of gA. (*A*) gA in DMPC; the β6.3 head-to-head dimer forms a channel across the membrane. Notice the unique orientation of the tryptophan side chains. The coordinates are from the Protein Data Bank file 1MAG. (*B*) gA in methanol; the coordinates are from 1ALX.

1997; Xu et al., 1998; Tang et al., 1997) that anesthetics are in rapid exchange among different sites within the lipid bilayers, with a certain preference for the amphiphilic regions. The photolabeling shown in Fig. 3 suggests that in the case of the gA channel, the specific targeting effects are such that only the four tryptophans at the interface are significantly labeled. Although photochemical selectivity for aromatics is well known, the previously demonstrated ability of halothane to photolabel other residues (Eckenhoff, 1996; Johansson and Eckenhoff, 1996), combined with the consistent NMR evidence of this study, suggests that adduct position is reliably reporting sites of equilibrium binding. The structural arrangement of W9 and W15 side chains relative to the membrane interface may also explain why the W9 chemical shift is affected the most by the anesthetics. The adjacent locations of W9 and W15 indole rings might hinder the water interaction with W9. This hindrance is conceivably reduced by the amphiphilic anesthetic molecules that might facilitate the coupling between the W9 indole side chain and the water molecules that penetrate the lipid headgroup. Indeed, we found recently (Tang et al., 1999b), with gA in SDS and D_2O, that the H-D exchange rate of indole amide protons with water can be modulated by volatile anesthetics.

The lack of an anisotropic environment allows gramicidin to adopt double-helical conformations in methanol. Although only a left-handed antiparallel double helix is shown in Fig. 5 *B*, other types of double helices of gramicidin are also possible in methanol in the absence of ions. Why are the tryptophan residues of gramicidin in these double helices not as favorable sites for halothane binding as those in the channel form (Fig. 5 *A*)? In addition to the structural difference, at least two factors may be important. First, W9, W11, and W13 in the channel form are located in the amphiphilic regions of lipid, whereas all tryptophan residues in double helices are exposed to an isotropic solvent phase. Because of halothane's preferential distribution to amphiphilic environments, the probability of finding halothane molecules adjacent to the tryptophan residues is increased. Consistent with this is the finding that over 90% of the halothane adducts are found below the carbonyl oxygen, where W9–W13 are thought to reside. Moreover, the interface between gA and lipid might also help to immobilize halothane, increasing the photolabeling affinity at the protein-lipid interface. Indeed, as shown in Fig. 4, the incorporation of dpm on gA remained constant but that on lipid increased with an increasing gA-lipid ratio. The additional incorporation into the lipid can be attributed to more interfacial region at higher gA-lipid ratios. In contrast, the halothane distribution in methanol has no preference, reducing the probability that halothane targets specific sites in gA. Second, methanol molecules may compete with halothane for binding sites. Hydrogen bonding between the tryptophan and solvent molecules was observed for seven of eight tryptophan residues in the methanol complex in a recent study (Burkhart et al., 1998). A structure favoring isotropic association with the solvent may reduce the chance of selective interaction at a particular site.

Although leucine residues L10, L12, and L14 are also located near the lipid interface, our NMR and photolabeling data indicate that the anesthetic binding to these so-called spacer residues (Jude et al., 1999) is weaker. This suggests that anesthetic interaction with gA is controlled not only by the preferential anesthetic distribution to the interfacial region. Preferential selection of different residues within a given structure also plays a critical role in anesthetic-protein interaction. The difference in anesthetic binding capacity to leucine and tryptophan residues might be attributed to a weak cation-π type of interaction (Cubero et al., 1998). It has been suggested that the aromatic residues in proteins, particularly tryptophan, constitute attractive cation-binding sites because of the intense region of negative electrostatic potential. Most anesthetic molecules, particularly halogenated ones, undergo either permanent or inducible displacement of partial atomic charges (Eckenhoff and Johansson, 1997; Trudell and Bertaccini, 1998). The positive moiety of the partial atomic charge acts as a partial cation that interacts with the π-current of the indole ring. A recent study of halothane binding in a specifically designed anesthetic binding pocket also suggests the preferential interaction between halothane and aromatic side chains (Johansson et al., 1998).

Although gA plays no role in clinical anesthesia, it serves as a reasonable model of a transmembrane helix to test popular hypotheses about where volatile anesthetics may interact with ion channel proteins. Site-directed mutagenesis experiments have implied that sites of anesthetic interaction with the postsynaptic ligand-gated ion channels may locate within the channel pore (Forman et al., 1995) or at a putative pocket between TM2 and TM3 domains (Mihic et al., 1997). In the case of gA, our results seem to favor the idea that the sites of anesthetic interaction are at the lipid-protein interface. The significant modulation of the W9 frequency by anesthetics and abundant labeling of halothane at W9 further narrow the location to the region between the methyl groups and headgroups. Heterogeneous anesthetic interaction in this region has been suggested to alter the lateral pressure of the membrane, thereby changing the function of transmembrane channels (Cantor, 1997).

Although the halothane concentrations used for photolabeling are comparable to the clinical values, those for the NMR experiments cover 0–4 times the clinical range. The halothane partition coefficient in SDS solution depends on SDS concentrations. For 500 mM SDS used for NMR experiments, the SDS_{500}/gas partition coefficient of halothane is 10.9 (unpublished data). Given the halothane saline/gas partition coefficient of 1.2 (Firestone et al., 1986), it can be estimated that the equivalent halothane concentration in saline ranges from 0 to 2.2 mM.

In summary, both high-resolution NMR and direct photoaffinity labeling measurements revealed that volatile an-

esthetics interact specifically with the tryptophan residues of gA in the channel conformation. This interaction largely disappears when gA becomes double-stranded helical dimers, suggesting that a specific structural motif is required for anesthetic-channel interaction. This motif, when combined with the lipid interface, creates an amphiphilic environment that is preferred by the anesthetics. Tryptophan residues in an amphiphilic environment, with their prominent ring current for a cation-π type of interaction, may provide an electrostatic contact for anesthetic binding. Our finding that a specific side-chain conformation of the channel near the membrane interface is required for anesthetic binding may be relevant to the function of anesthetic-sensitive neuronal receptor channels.

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