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Structural Consequences of Anesthetic and Nonimmobilizer Interaction with Gramicidin A Channels

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ABSTRACT Although interactions of general anesthetics with soluble proteins have been studied, the specific interactions with membrane bound-proteins that characterize general anesthesia are largely unknown. The structural modulations of anesthetic interactions with synaptic ion channels have not been elucidated. Using gramicidin A as a simplified model for transmembrane ion channels, we have recently demonstrated that a pair of structurally similar volatile anesthetic and nonimmobilizer, 1-chloro-1,2,2-trifluorocyclobutane (F3) and 1,2-dichlorohexafluorocyclobutane (F6), respectively, have distinctly different effects on the channel function. Using high-resolution NMR structural analysis, we show here that neither F3 nor F6 at pharmacologically relevant concentrations can significantly affect the secondary structure of the gramicidin A channel. Although both the anesthetic F3 and the nonimmobilizer F6 can perturb residues at the middle section of the channel deep inside the hydrophobic region in the sodium dodecyl sulfate micelles, only F3, but not F6, can significantly alter the chemical shifts of the tryptophan indole N-H protons near the channel entrances. The results are consistent with the notion that anesthetics cause functional change of the channel by interacting with the amphipathic domains at the peptide-lipid-water interface.

INTRODUCTION

The molecular targets for general anesthetic action have proved peculiarly difficult to determine. A superfamily of ligand-gated synaptic ion channels, including the γ-aminobutyric $acid_A$ ($GABA_A$) receptor, glycine receptor, neuronal nicotinic acetylcholine receptor, and 5-hydroxytryptamine$_3$ receptor, has been considered the top candidates because of their supersensitivity to general anesthetics. Recent studies (Forman et al., 1995; Mihic et al., 1997) showed that a simple substitution of a single amino acid in some of these ligand-gated ion channels can greatly change the sensitivity to general anesthetics. Although sensitivity alone cannot serve as a criterion for unequivocal identification of the sites of action, these mutagenesis findings nevertheless support the idea that general anesthetics exert their primary action by interacting with proteins (Franks and Lieb, 1994). It remains unclear, however, whether these residues constitute part of the anesthetic-binding sites, or they are involved only in allosteric linkage (Franks and Lieb, 1997). A specific structural requirement for anesthetic binding on membrane proteins has not been elucidated (Eckenhoff and Johansson, 1997).

Three-dimensional (3D) structural analysis is not yet possible for the authentic ligand-gated ion channels because of their size and structural complexity. We recently showed (Xu et al., 1998) that gramicidin A (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}-$NHCH_2CH_2OH$), a simple cation channel with well-resolved 3D structure (Arseniev et al., 1985; Lomize et al., 1992; Cross, 1997), can serve as a model for the study of interaction of general anesthetics with transmembrane proteins. We showed that a volatile anesthetic, 1-chloro-1,2,2-trifluorocyclobutane (F3), interacted specifically with the tryptophan residues of gramicidin A near the channel entrances, whereas a structurally similar nonimmobilizer (nonanesthetic), 1,2-dichlorohexafluorocyclobutane (F6), had no specific interaction with these regions. The direct functional consequence of this was that F3 could increase the unidirectional rates of Na^+ transport across the gramicidin A channel, whereas F6 had no effects on Na^+ transport.

In the present study, we use high-resolution NMR spectroscopy to investigate possible structural changes in the gramicidin A channel after interaction with F3 or F6 takes place. We show that although neither F3 nor F6 at pharmacological concentrations can produce measurable changes in the secondary structure of the gramicidin A channel, F3, but not F6, can significantly alter the tryptophan side-chain association with the interfacial water or with the lipid headgroup.

MATERIALS AND METHODS

Materials

Purified gramicidin A was purchased from Calbiochem (La Jolla, CA). Deuterated sodium dodecyl sulfate (SDS-d_{25}) and D_2O were obtained from Cambridge Isotope Laboratories (Andover, MA). F3 and F6 were purchased from PCR Inc. (Gainesville, FL). 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.

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Sample preparation

To determine anesthetic and nonanesthetic effects on channel conformation, it is critically important to minimize the amount of organic solvents in the peptide samples, for many of the solvents are general anesthetics themselves. To achieve high NMR spectral resolution in the liquid state, gramicidin A was incorporated in SDS micelles rather than in lipid bilayers. The structure of gramicidin A in the channel conformation is known to be very similar in these two environments (Cross, 1994, 1997; Weinstein et al., 1979; Killian et al., 1994; Ketchem et al., 1997; Mobashery et al., 1997). To prepare gramicidin A channel in SDS micelles, the procedure developed by Killian et al. (1994) was modified and used. Briefly, a 25 mM solution of gramicidin A in 2,2,2-trifluoroethanol (TFE) and 1000 mM SDS in H_2O were prepared separately. Aliquots of gramicidin solution were added to SDS solution to reach a gramicidin-to-SDS molar ratio of 1:200. Water was then added to yield a water-to-TFE ratio of 16:1 by volume. The samples were mixed vigorously for 5 s, rapidly frozen in CO_2/acetone, and lyophilized overnight at −50°C. The lyophilized samples were further vacuumed for at least 24 h to ensure nearly complete removal of TFE. The amount of TFE remaining in the samples was less than 100 μM, as determined by GC in selected samples and confirmed by the nonexistence of any ^{19}F resonance in ^{19}F-NMR spectra before the addition of fluorinated anesthetics or nonimmobilizers. For NMR measurement, the dry samples were rehydrated with deionized water (90% H_2O and 10% D_2O for field-lock purposes). In each NMR sample, the gramicidin A concentration ranged from 1.9 to 2.5 mM, 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 2.0-ml vapor space above the solution.

F3 or F6 was titrated directly into the samples in the NMR tube with a Hamilton microsyringe. After equilibrating with the vapor phase, the total F3 or F6 concentrations in the SDS solution were estimated by ^{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 with the 5-mm sample tube.

NMR spectroscopy

High-resolution ^{1}H NMR spectra of the rehydrated micelles containing gramicidin A were recorded on Bruker 600 and 750 spectrometers with DMX consoles, operating at the ^{1}H resonance frequencies of 600.33 and 750.13 MHz, respectively. The sample temperature was maintained at 30°C. Typical experimental parameters were 10–17-μs 90° pulses, 1.5-s repetition delays, a 9-kHz spectral width, and WATERGATE for water suppression. For one-dimensional spectra, 64 scans were accumulated in 8192 complex points. The data were zero-filled once before Fourier transformation. For NOESY experiments, spectra were acquired using a mixing time of 100 ms, 64 averages for each t_1 value after two dummy scans, a datum set of 4096 complex points with 512 t_1 increments, and the time proportional phase incrementation (TPPI) or States method for quadrature detection in the t_1 dimension. The 2D NMR spectra were processed using the NMRPipe program developed at the National Institutes of Health. The 2D peak intensities were calculated by volume integration, using the Sparky program from University of California at San Francisco.

RESULTS

At pharmacologically relevant concentrations, neither F3 nor F6 significantly altered the secondary structure of the gramicidin A channel. Fig. 1 shows an overlay of the fingerprint region of NOESY spectra before and after addition of 14.8 mM F3 to a sample containing 1.9 mM gramicidin A in SDS micelles. Similar results were obtained for F6. Resonance assignments of the spectra were performed based on the NOE connectivity and by comparison with the literature (Lomize et al., 1992; Arseniev et al., 1985). Except for peak Val^{7}-Val^{6} (V7-V6), which showed a 0.017-

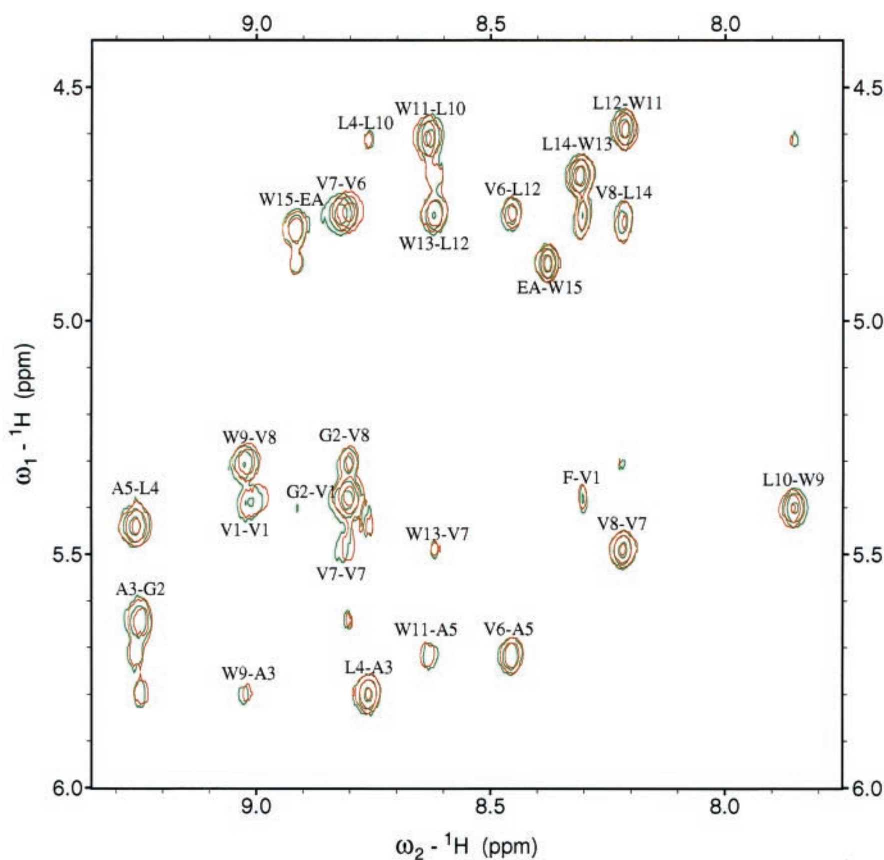

FIGURE 1 Overlay of the fingerprint region of two 750-MHz ^{1}H NOESY spectra, acquired at 30°C before (*green*) and after (*red*) addition of 14.8 mM F3 to 1.9 mM gramicidin A in 380 mM SDS micelles. Cross-peaks are labeled as "amide-α proton," using the one-letter notation for amino acids and the sequence number in the primary structure. The mixing time was 100 ms, and the experiment time needed to acquire each NOESY spectrum was 18.5 h. Except for V7-V6, no significant changes in chemical shifts and cross-peak intensities were found in this region.

ppm shift in the Val[7] amide proton resonance, no significant changes in chemical shift or cross-peak intensity were found in this region. However, the resonance of all indole N-H protons in the four tryptophan side chains were significantly shifted by F3 in a concentration-dependent manner. As shown in Fig. 2, all shifts are in the up-field direction. In particular, the Trp[9] indole N-H proton, which is located farthest from the surface, showed the largest shift. Fig. 3 depicts the chemical shift changes in Trp[9] indole N-H resonance as a function of F3 or F6 concentration. Clearly, F6 in the similar concentration range showed much less perturbation to the chemical shifts in this region.

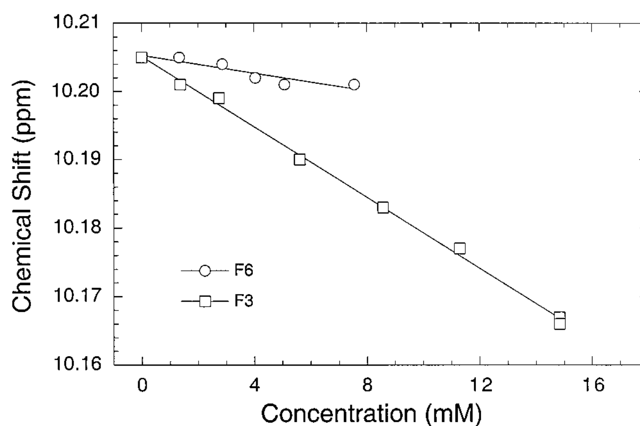

FIGURE 3 Changes in Trp[9] indole N-H chemical shift are plotted as a function of F3 or F6 concentration in 380 mM SDS micelles. The chemical shifts of indole N-H protons are more sensitive to F3 than to F6.

DISCUSSION

In the channel conformation, gramicidin A forms head-to-head $\beta^{6.3}$ helical dimers (Arseniev et al., 1985). The 3D structures of this channel are well documented from high-resolution solution-state (Arseniev et al., 1985) and solid-state (Cross, 1997) NMR. Based on the known structures of the channel, the changes in chemical shift found in this study can be interpreted by considering changes in the hydrogen bonding between the observed protons and their environments. The backbone amide proton of Val[7] is oriented toward the middle section of the channel (i.e., deep in the tail region of the micelle) to form a hydrogen bond with the N-terminal carbonyl group. Thus the major changes in the Val[7] amide proton resonance are likely caused by the F3 or F6 perturbation to this hydrogen bond. The perturbation can be specific through direct interaction of F3 or F6 with the peptide in this region, or nonspecific through possible changes in the micelle shape or diameter, which in turn place strain on the hydrogen bonding. Based on the up-field direction of the shift, it is believed that the perturbation weakens the hydrogen bonding in this region (Wagner et al., 1983). Earlier studies by others have shown that a large number of anesthetics containing the so-called acidic hydrogens have hydrogen bond-breaking effects (for a review, see Urry and Sandorfy, 1991). It has also been suggested that a good relationship may exist between hydrogen bond-breaking ability and the potency of halogenated anesthetics (Trudeau et al., 1978). Our result with F6 indicates that the fluorinated nonimmobilizer seems to have a similar ability to perturb the hydrogen bonding near the tail region of the micelles. This perturbation is in the same direction as that caused by the anesthetic F3. Thus destabilization of the dimer state by weakening of hydrogen bonding in the deep tail region of the micelle, or in the core of the lipid by analogy, seems unlikely to represent an action that is of importance to general anesthesia.

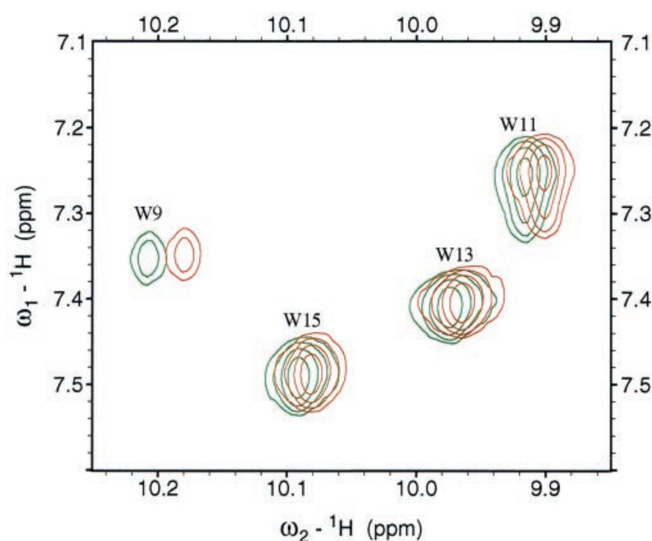

FIGURE 2 Overlay of the indole N-H region of two 750-MHz ^{1}H NOESY spectra, acquired at 30°C before (*green*) and after (*red*) addition of 14.8 mM F3 to 1.9 mM gramicidin A in 380 mM SDS micelles. The experiment time for each NOESY spectrum was 18.5 h. All resonance peaks shifted to lower frequencies; Trp[9] was most sensitive to F3.

The different effects of F3 and F6 on the tryptophan side chains, however, may reveal some important characteristics associated with anesthetic modulation of transmembrane channel peptide. It has been suggested (Hu et al., 1993; Hu and Cross, 1995) that the tryptophan side chains play a critical role in anchoring the channel in the lipid membrane. The indole rings are oriented in a unique way that favors hydrogen bonding between indole N-H protons and the water molecules that either are at the surface of the membrane or penetrate into the interfacial region (Hu et al., 1993; Woolf and Roux, 1997). From the direction of changes in chemical shifts of the indole amide proton, it appears that the anesthetic F3 facilitates indole-water interaction. This is shown most profoundly for the Trp[9] indole N-H proton, which is farthest (~4 Å) from the surface of the micelles. It is conceivable that the amphipathic property of the anesthetic may help to reduce the energy barrier to the interaction of the Trp[9] side chain with the micelle-water interface. This can be achieved either by weakening any possible hydrogen bonding of Trp[9] indole N-H with micelle headgroups or by mediating more interfacial water mole-

cules into the Trp^9 indole N-H location. Hydrogen bonding of indole N-H protons with water has been shown to stabilize the cation binding at the channel entrance (Hu and Cross, 1995), a critical step in cation transport across gramicidin A channel. Indeed, our studies of Na^+ transport in large unilamellar vesicles showed that F3, but not F6, can significantly increase ($p < 0.001$) the unidirectional rates of Na^+ transport across the gramicidin A channel (Xu et al., 1998). Using intermolecular truncated driven nuclear Overhauser effects (TNOE), we also confirmed that F3 did interact specifically with the tryptophan side chains. F6, in contrast, showed no measured TNOE build-up with the indole N-H protons.

The anesthetic and nonimmobilizer effects on channel dynamics may also account for some of the chemical shift changes observed. Although no attempts were made in this study to quantify the fluctuations in the channel structure, it is conceivable that by facilitating the interaction with water at the interface, where the channel is anchored, F3 may affect the channel function by altering the motion of the channel. Further studies aimed at characterizing the channel dynamics will certainly help to address this issue.

The concentrations used in this study are within the pharmacological range. We have found that the partition coefficient of F3 in SDS solution versus gas increases with increasing SDS concentration (unpublished results). At 380 mM SDS, the SDS_{380}/gas partition coefficient at 37°C is ~13.4. Because the saline/gas partition coefficient of F3 at 37°C is 1.56 (Kendig et al., 1994), it can be estimated that the hypothetical SDS_{380}/saline partition coefficient would be 8.6. Thus the highest F3 concentration used in this study (i.e., 14.8 mM in 380 mM SDS solution) is equivalent to ~1.7 mM in saline, which is comparable to the minimum alveolar concentration (1.47 mM in saline at 27°C) of the agent (Kendig et al., 1994).

The secondary structure of the channel is not significantly affected by either the anesthetic or the nonimmobilizer. This conclusion is true only at the anesthetic or nonimmobolizer concentrations studied. At higher concentrations, anesthetics and nonimmobilizers may exert solvent effects on the peptide, which can possibly alter the secondary structure of the channel. Moreover, gramicidin A consists of alternating L- and D-amino acids in its sequence, with the polar peptide groups lining the pore of the channel and the nonpolar side chains projecting from the exterior surface. Such an arrangement is unlikely to be found in neuronal receptor channels. Therefore, our conclusion does not rule out the possibility that structural changes may be involved in the action of general anesthetics on neuronal receptors.

It is interesting to note that in the ligand-gated ion channels, the anesthetic-sensitive sites identified by the point-mutation experiments are either within the aqueous pore (Forman et al., 1995) or at interfacial locations near the extracellular regions of the transmembrane domains on the channels (Mihic et al., 1997). At first glance, these results are rather unexpected, given the excellent correlation between the potency of general anesthetics and their solubility in olive oil (the Meyer-Overton rule). However, as others and we have shown recently, the difference between anesthetics and nonimmobilizers lies in their ability to distribute to regions with constant access to the aqueous phase (Tang et al., 1997; North and Cafiso, 1997). Anesthetics, but not the nonimmobilizers, have the tendency to distribute to and interact with amphipathic regions in the model membranes (Xu and Tang, 1997). Thus the ability of F3 to modulate the tryptophan side chain of gramicidin channel at the amphiphilic interfacial region, and the inability of F6 to do the same, may reflect the common characteristics of anesthetic interaction with the transmembrane channel proteins. Such characteristics may be directly related to the sensitivity of the protein to general anesthetics.

In conclusion, although F3 and F6 at pharmacologically relevant concentrations did not affect the secondary structure of the gramicidin A channel, they caused distinctly different modulations of the tryptophan side chains at the amphipathic domains near the lipid interface. This difference parallels the different functional changes in the channel caused by the same anesthetic and nonimmobilizer pair.

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