Effects of Volatile Anesthetic on Channel Structure of Gramicidin A

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ABSTRACT Volatile anesthetic agent, 1-chloro-1,2,2-trifluorocyclobutane (F3), was found to alter gramicidin A channel function by enhancing Na^+ transport (Tang et al. 1999. *Biophys. J.* 77:739–746). Whether this functional change is associated with structural alternation is evaluated by circular dichroism and nuclear magnetic resonance spectroscopy. The circular dichroism and nuclear magnetic resonance results indicate that at low millimolar concentrations, 1-chloro-1,2,2-trifluorocyclobutane causes minimal changes in gramicidin A channel structure in sodium dodecyl sulfate micelles. All hydrogen bonds between channel backbones are well maintained in the presence of 1-chloro-1,2,2-trifluorocyclobutane, and the channel structure is stable. The finding supports the notion that low affinity drugs such as volatile anesthetics and alcohols can cause significant changes in protein function without necessarily producing associated changes in protein structure. To understand the molecular mechanism of general anesthesia, it is important to recognize that in addition to structural changes, other protein properties, including dynamic characteristics of channel motions, may also be of functional significance.

INTRODUCTION

There is a plethora of functional analyses demonstrating the concentration-dependent effects of volatile general anesthetics and certain short chain alcohols on the functional changes of various ion channel receptors (Eckenhoff and Johansson, 1997; Franks and Lieb, 1994). These effects are taken as evidence to support the notion that general anesthetics, albeit most of which have relatively low binding affinity to neuronal receptors, exert their actions by direct interaction with proteins. It is unclear, however, how general anesthetics modulate channel functions and whether the functional changes are associated with any significant alterations in channel structures. One of the major obstacles to unambiguously answer these questions is the lack of high-resolution structures of neuronal receptors that are sensitive to general anesthetics. Before such structures are resolved, it is difficult to determine the structural changes in the receptors due to direct interaction with volatile anesthetics.

An alternative approach to revealing the protein structural response to volatile general anesthetics is to use model proteins such as bovine serum albumin (Eckenhoff and Tanner, 1998; Johansson et al., 1999), human serum albumin (Bhattacharya et al., 2000; Eckenhoff et al., 2000), and firefly luciferase (Franks et al., 1998). For some of these model proteins, x-ray structures are already available (Carter and He, 1990; Conti et al., 1996). Although these globular proteins are unlikely to be involved in general anesthesia, lessons learned from them can be generalized to yield a better understanding of anesthetic interaction with integral neuronal receptor channels. For similar reasons, we have chosen the gramicidin A channel in a membrane-mimetic environment as a transmembrane channel model to characterize its structural responses to various volatile anesthetics (Tang et al., 1999a,b, 2000a). Gramicidin A (gA) is a polypeptide of 15 amino acids: HCO-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-$NHCH_2CH_2OH$; a head-to-head dimer forms a transmembrane channel. Previously, using magnetization inversion transfer ^{23}Na nuclear magnetic resonance (NMR) experiments (Tang et al., 1999a), we have found that the ^{23}Na transport rates in gA channel can be modulated by anesthetics; the presence of volatile anesthetic agent 1-chloro-1,2,2-trifluorocyclobutane (F3) increased both apparent efflux and influx rates. The availability of high-resolution channel structures of gramicidin (Arseniev et al., 1985; Cross, 1997; Ketchem et al., 1997) allows for evaluations at the atomic resolution of potential structural changes as a consequence of interaction with volatile anesthetics. For integral membrane proteins, to which the anesthetic-sensitive neuronal receptors belong, it is generally believed that both the hydrophobic core of the membrane and the lipid-water interface are of determinant importance for channel structures and functions. Therefore, the gA channel in a membranous environment provides a relevant model for evaluating potential anesthetic effects not only on the structures of neuronal receptor channels but also on the association of the channels with their lipid and water surroundings. Moreover, because many transmembrane domains of neuronal receptors assume α-helical structures and most of volatile anesthetic molecules are too large to penetrate into the center of the helices, a helical model system is needed to study other potential mechanisms by which anesthetics can cause structural changes without penetration into the helices. For example, it is unknown whether anesthetics can act like high-affinity ligands to produce allosteric structural changes, where binding site and effect site are remotely linked. It is also of interest to know if amphipathic anesthetic molecules

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can intercalate between turns of transmembrane helices, of which many side chains are amphipathic and hydrophobic, to produce structural changes. In the case of gA dimer, tests can also be made to determine whether volatile anesthetics, which are known to partition in the lipid tail region, can interrupt the dimerization or break the H-bonds between the two monomers.

In the present study, we used circular dichroism (CD) and NMR spectroscopy to determine the effects of anesthetic agent F3 on the structure of gA. Both CD and NMR results confirmed that the change of the channel structure was very subtle in the presence of F3 at low millimolar concentrations. A generalization of the current finding seems to suggest that functional changes of the channel due to general anesthetics might not necessarily be accompanied by structural changes, and one may need to look beyond structural changes to understand the action of low-affinity drugs. This realization will potentially shed new light on the role of protein dynamics, rather than structures alone, in the molecular mechanisms of general anesthesia.

MATERIALS AND METHODS

Materials

Volatile anesthetic F3 was purchased from PCR Inc. (Gainesville, FL). Purified gramicidin A was purchased from Calbiochem (La Jolla, CA). Deuterated sodium dodecyl sulfate (SDS_{d25}) and D_2O were obtained from Cambridge Isotope Laboratories (Andover, MA). SDS was recrystallized in ethanol before use. Other chemical and materials were purchased from Sigma (St. Louis, MO) and used without further purification.

Sample preparation

A detailed description of sample preparation was given previously (Tang et al., 1999a,b). Briefly, a 1000 mM SDS solution in H_2O and 25 mM gA solution in 2,2,2-trifluoroethanol (TFE) were prepared separately. The gramicidin solution was then titrated into the SDS solution to reach a gramicidin-to-SDS molar ratio of 1:200. The samples were mixed vigorously for 5 s after adding water to a water-to-TFE ratio of 16:1 by volume, rapidly frozen in CO_2/acetone or liquid nitrogen, and lyophilized overnight at −50°C. To ensure the complete removal of TFE, the sample was further vacuumed for at least 24 h. The dry samples were rehydrated in different solvents for different measurements: in 90% deionized water and 10% D_2O for NOESY experiments and for COSY measurements of $^3J_{HNH\alpha}$, in pure D_2O for the $^1H/^2H$ exchange NMR and for COSY measurements of $^3J_{H\alpha H\beta}$, and in pure deionized water for the CD experiments. The gA concentrations in the samples were 0.06 mM for CD, 1.9 to 2.5 mM for NOESY and COSY, and 1.9 mM for $^1H/^2H$ exchange experiments. For rapid $^1H/^2H$ exchange measurements, rehydrated samples were quickly mixed using a vortex, centrifuged for 30 s, and immediately transferred to a 5-mm tube for NMR. F3 was titrated directly into NMR tubes using a Hamilton micro syringe. After equilibration with the gas phase, the total anesthetic concentrations in the solution phase were determined by ^{19}F NMR using an external standard of 0.19 mM trifluoroacetic acid in a 10-mm NMR tube, which was coaxial to the 5-mm sample tube.

CD measurements

CD spectra were recorded on a JASCO J-715 spectropolarimeter (Jasco Inc., Easton, MD). All measurements were made at room temperature in a quartz cuvette of 0.1-cm path length. Spectra were recorded over the wavelength range of 180 to 280 nm with a time constant of 1 s, spectral steps of 1 nm, and a scan rate of 100 nm/min. Ten repeated measurements were made and averaged for each sample with solvent absorbance subtracted from the sample spectra.

NMR measurements

All NMR experiments were performed at 30°C. NOESY spectra of gA in the absence and presence of 14.8 mM F3 were recorded on a Bruker 750 MHz spectrometer with DMX console, operating at the ^1H resonance frequency of 750.13 MHz. Typical experimental parameters were 10-μs 90° pulses, a 9-kHz spectral width, 1.5-s repetition delays, 100-ms mixing time, and WATERGATE (Piotto et al., 1992) for water suppression. Two-dimensional data were acquired in 4096 complex points with 512 t_1 increments using the time proportional phase increments (Marion and Wuthrich, 1983) for quadrature detection in the t_1 dimension. For each t_1 value, 64 scans were averaged after two dummy scans. Phase-sensitive COSY (Bax et al., 1994) experiments were performed on a Bruker 500-MHz spectrometer (^1H resonance frequency of 500.13 MHz). Presaturation of water was used to ensure the resolution of the Hα peaks close to the water proton resonance. Typical experimental parameters are: 10.4-μs 90° pulse for ^1H, 5.79-kHz spectral width, 6400 complex points in the acquisition domain, and 1360 increments in t_1 domain with states quadrature detection (States et al., 1982), and 32 scans for each t_1 value with 1.6-s repetition delays. The NOESY and COSY spectra were processed using the NMRPipe program (Delaglio et al., 1995) and analyzed using the PIPP program (Garrett et al., 1991). Deuterium exchange experiments were conducted on either a Bruker 500-MHz spectrometer or a CMXW-400SLI spectrometer (Fort Collins, CO). Data were acquired in 16,384 complex points and averaged for 128 scans. The spectra were saved every 5.5 min and acquired continuously for up to 20 h. The $^1H/^2H$ exchange rate constants were derived by fitting the ^1H NMR signal decays as a function of time using the single exponential decay function $I(t) = I_0e^{-kt}$, in which k is the exchange rate constant.

Structural calculations

The intensities of the cross-peaks in NOESY spectra were determined by volume integration using the PIPP program (Garrett et al., 1991) and converted into loose approximate interproton distance restraints (e.g., 1.8–2.7 Å, 1.8–3.3 Å, 1.8–5.0 Å, and 1.8–6.0 Å for strong, medium, weak, and very weak NOEs, respectively) with the lower bounds given by the sum of the van der Waals radii of the two protons (Clore and Gronenborn, 1998). The hydrogen bonds of NH(i) to CO($i + 5$) for even i and of NH(i) to CO($i - 7$) for odd i were included in the structure calculations. Each hydrogen bond was converted into two distance constraints $r_{NH\text{-}O}$ (1.8–2.2 Å) and $r_{N\text{-}O}$ (2.2–3.3 Å) (Mitchell and Price, 1990). The initial structures were calculated with the standard three-stage distance geometry and simulated annealing protocol (Nilges et al., 1988) using X-PLOR (Brünger 1992). The resulting structures with no violations above the threshold conditions of 5° for angle, improper, and dihedral angles, and 0.05 Å and 0.5 Å for bonds and NOEs, respectively, were taken for the refinement by imposing the dihedral terms, the standard Lennard-Jones function, and the electrostatic interactions. Thirty of the lowest energy structures were used for data analysis. The orientations of side-chains in the initial structural calculations were defined by long-range (i, $i \pm 6$) NOE spatial constraints from the NOESY spectra. The side-chain orientations, especially those of the tryptophan side-chains, were measured by angles χ_1 (HN-C_α-C_β−C_γ) and χ_2 (C_α-C_β−C_γ−$C_{\delta 1}$) from the energy-refined conformations, and the orientation difference in the presence and absence of F3 was evaluated using SPSS program.

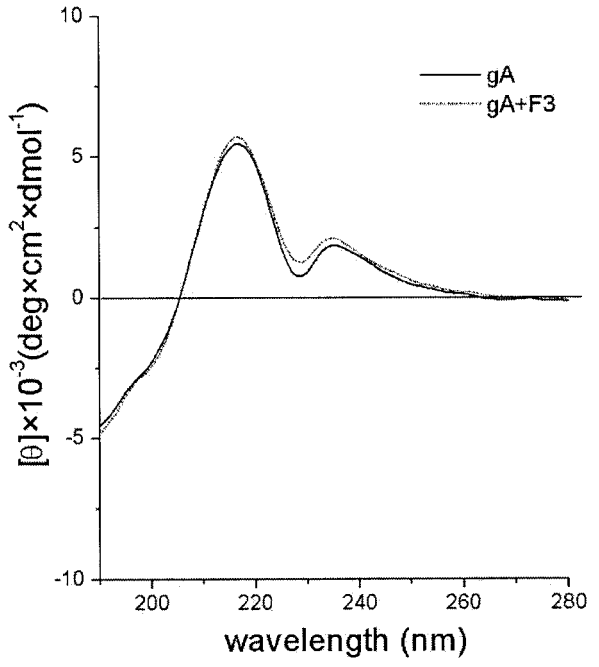

FIGURE 1 Representative CD spectra of 60 μM gA channel in the membrane-mimicking micelles in the absence (*solid line*) and presence (*dotted line*) of 5 mM F3.

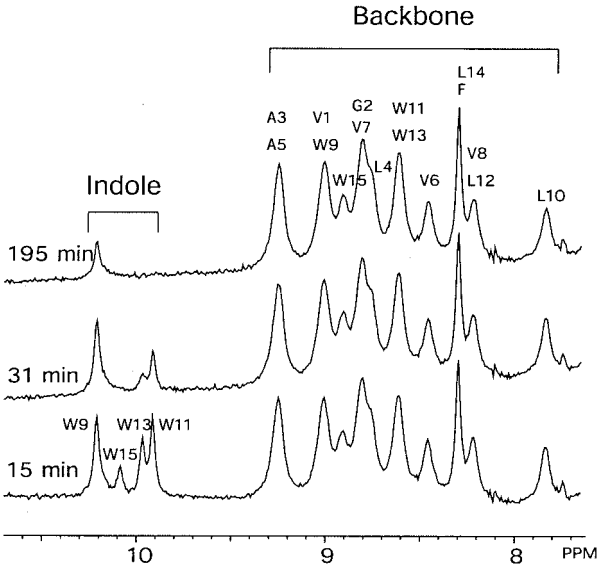

FIGURE 2 Stack plot of representative high-resolution ^{1}H-NMR spectra (amide proton region) of gA channel in deuterated SDS micelles taken at different time points after rehydration in D_2O. The backbone amide protons showed no significant exchange with D_2O, suggesting that the structures were stable. In contrast, the tryptophan indole amide protons exchange with D_2O at different rates depending on their depths in the micelles.

RESULTS

Circular dichroism spectroscopy

In general, the CD spectra do not provide information about dimerization. In the case of gramicidin, however, the dimer channel conformer can be inferred based on the CD spectra because distinctly different CD patterns of various gA conformations have been well documented in the literature (Greathouse et al., 1994; Masotti et al., 1980; Wallace et al., 1981). The CD pattern of gA in lipids with positive maximums near 218 and 235 nm, a minimum at 230 nm, and negative ellipticity below 208 nm has been used as an indicator of the channel conformation. The CD spectra of gA in SDS micelles exhibited the same general features as in lipid bilayers, indicating that two monomers of single-stranded, right-handed, $\beta^{6.3}$ helical gA join at their N termini to assume a channel conformation (Abdul-Manan and Hinton, 1994; Arseniev et al., 1985). As shown in Fig. 1, the unique CD pattern of the gA channel was virtually unaffected by the addition of 5 mM F3. The remarkable wavelength matching at maximal and minimal ellipticity is a strong indication that backbone structure of the gA channel is the same in the presence and absence of 5 mM F3. The slight difference in magnitude of the ellipticity is within the variation of sample preparations (Arseniev et al., 1985; Wallace et al., 1981).

^{1}H/^{2}H exchange NMR spectroscopy

The intramolecular and intermolecular NH$\cdots$O═C hydrogen bonds are the key elements to stabilize the gA channel structure. If the channel structure is stable and the backbone amide protons are strongly involved in the hydrogen bonding, these amide protons are expected not to exchange easily with hydrogen in water or deuteriums in D_2O, and the proton NMR signals from the amide protons can sustain for long time in D_2O. The backbone amide proton NMR spectra of the gA channel in Fig. 2 demonstrate that the hydrogen bonds of the channel backbone remain strong in the presence of 14.8 mM F3. The amide protons of Val-1, Ala-3, and Ala-5 are involved in hydrogen bonding between two gA monomers in a channel dimer. The nearly constant intensities of these signals in the ^{1}H/^{2}H exchange experiments suggest that the effect of 14.8 mM F3 on the tertiary structure of the gA channel is also minimal.

The tryptophan indole amide protons may form hydrogen bonds with oxygen atoms of the micelle head groups, but these hydrogen bonds are not as strong as those formed by backbone amide protons. In contrast to the backbone amide protons, tryptophan indole amide protons exchange with deuterium of D_2O. The rate of the exchange varies depending on the locations of the indoles along the channel and on solvent accessibility. W15, W13, and W11 are located near the two ends of the channel and well exposed to the aqueous phase. W9 is in the second helical turn from the end of the channel and relatively less exposed to the aqueous phase. The time needed to completely remove the indole amide proton signals in a ^{1}H/^{2}H exchange experiment follows the order of W15 $<$ W13 $\sim$ W11 $\ll$ W9. This order remains the same after the addition of F3. Fig. 3 shows the depen-

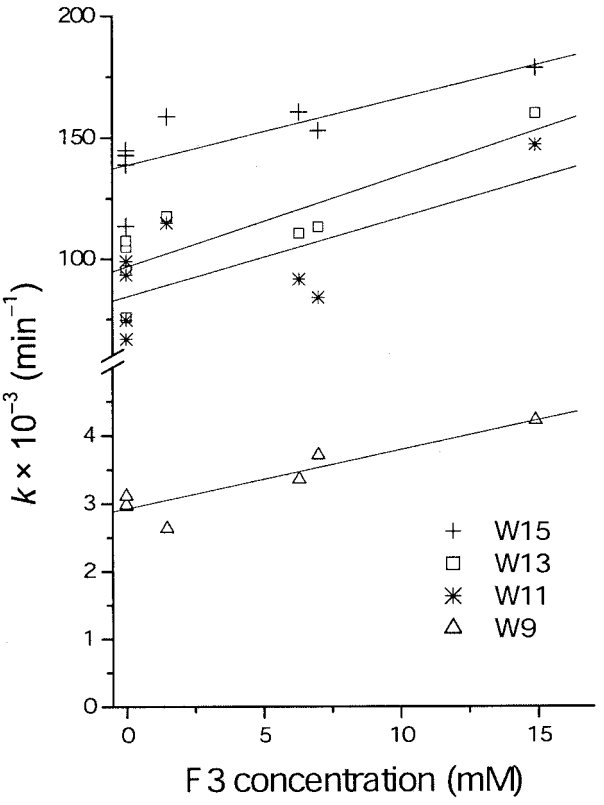

FIGURE 3 Dependence of the rate constant (k) of tryptophan indole amide proton exchange with deuterated water on F3 concentration in SDS micelles. In the studied concentration range, F3 accelerates indole amide proton exchange with water. All slopes are significantly different from zero (p = 0.001, 0.05, 0.007, and 0.02 for W9, W11, W13, and W15, respectively).

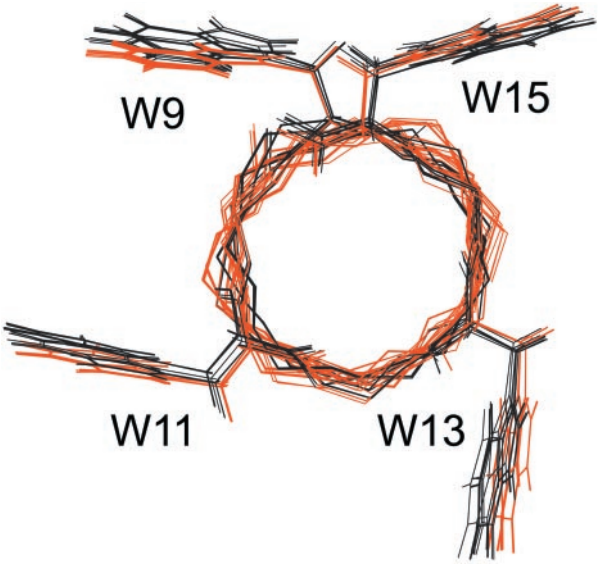

FIGURE 4 Superposition of the backbone (N, C_α, and C) atoms of 20 refined structures of gramicidin A channel in the presence (*red*) and absence (*black*) of 14.8 mM F3. The sidechains of the tryptophans in top segment of gramicidin were also depicted. At pharmacologically relevant concentrations, F3 produces no significant effects on the channel backbone structure and the side-chain orientations.

dence of the $^1H/^2H$ exchange rate on F3 concentration (n = 8). Solid lines are linear least squares fit to the data. The slopes are significantly different from zero for all indole amide protons (p = 0.001, 0.05, 0.007, and 0.02 for W9, W11, W13, and W15, respectively), suggesting that F3 in the studied concentration range can accelerate indole amide proton exchange with water significantly.

^{1}H NOESY and COSY NMR and structural calculations

The assignment of the ^{1}H NOESY spectra of gramicidin A in SDS micelle has been done previously (Arseniev et al., 1985; Tang et al., 1999b). The chemical shifts of most backbone amide and α protons are essentially the same in the presence and absence of F3. For those that showed small chemical shift change, the difference is less than 0.006 ppm. The patterns of short-range and long-range NOEs remained the same and exhibited unique NOE connectivity between $N_iH\cdots C_jH$ protons (in which $j = i + 6$ for even i indices and $j = i - 6$ for odd i indices), which are consistent with a right-handed, single-stranded $\beta^{6.3}$ helical dimer structure (Arseniev et al., 1985) in the presence and absence of F3.

There are 256 and 253 measurable cross-peaks in NOESY spectra with and without F3, respectively. The distance restraints generated from these cross-peaks provided the basis for the structural determination. Fig. 4 shows two sets of backbone structures of gA obtained in the absence and presence of 14.8 mM F3. Clearly, the presence of F3 had no effect on the channel structure. The RMSD (backbone) of the structures with and without F3 were 0.22 ± 0.09 Å and 0.23 ± 0.06 Å, respectively. If all structures were pooled together, the RMSD of all structures shown in Fig. 4 was only 0.32 ± 0.03 Å, suggesting again that there is no significant difference between two sets of structures.

There are at least 48 long-range (i, $i \pm 6$) NOEs contributing to constraining tryptophan side-chain orientations. The position of W9 side chain is unambiguously determined based on the intensity of the cross-peaks between W9 indole NH and C-terminal ethanolamine Hαs and Hβs and of the cross-peaks between W9 Hδ and W15 Hβs. The relative side-chain positions of tryptophans in our refined gramicidin structures are in agreement with those previously reported for gramicidin in SDS (Arseniev et al., 1985; Townsley et al., 2001). The similar cross-peak intensity in both sets of spectra with and without F3 indicated that the effects of F3 on overall channel structure and the tryptophan side-chain orientation were minute, as revealed in Fig. 4. The COSY experiments support the same notion. Fig. 5 shows

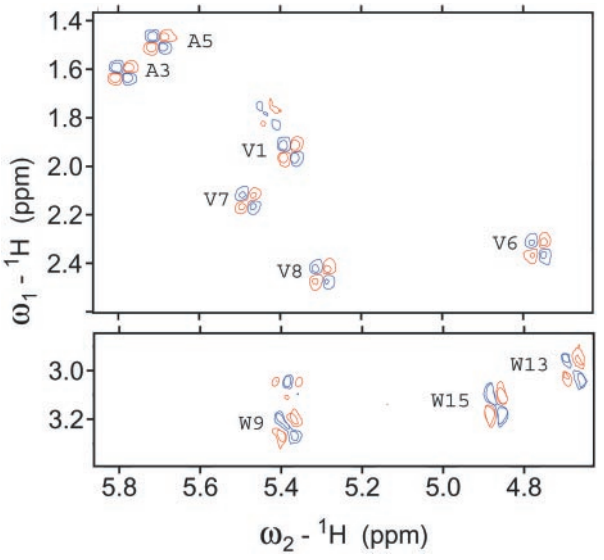

FIGURE 5 Overlay of the Hα-Hβ regions of two 500-MHz phase-sensitive COSY spectra of gramicidin in SDS micelles, recorded in D_2O at 30°C, in the absence (*upper contour level*) and presence (*lower contour level*) of F3. For clarity, only one contour level was plotted for each spectrum; the blue and red were used to differentiate negative and positive phase of cross-peaks, respectively. The spectra were acquired as a 1360 × 6400 data matrix with identical spectral widths in the two dimensions. Data were apodized with Gaussian windows, before zero filling to 2000 × 9600. The excellent overlap of COSY cross-peaks in the absence and presence of 14.8 mM F3 suggested that the F3 effects on side-chain orientations were minute.

the overlay of well-resolved COSY Hα-Hβ cross-peak regions of gramicidin in SDS with and without F3 and demonstrates minor orientation change of tryptophan side-chain in the presence and absence of F3. The effects of 14.8 mM F3 on dihedral angles χ_1 and χ_2 are summarized in the Fig. 6.

DISCUSSION

Although the present study is the first attempt to combine CD and NMR to evaluate general anesthetic effects on the structures of transmembrane channels, similar efforts have been made previously using globular protein models (Franks et al., 1998; Johansson et al., 1999). The crystal structure of the low-ATP form of luciferase in the presence of bromoform, an anesthetic agent, was found to be essentially the same at a 2.2-Å resolution as that in the absence of bromoform. The two bromoform molecules bound within the pockets in the large N-terminal domain caused minimal perturbations to the overall structure of the luciferase (Franks et al., 1998). The CD spectra of bovine serum albumin also showed that addition of either halothane or isoflurane to albumin resulted in only minor changes in the spectra, suggesting the absence of any major structural alteration (Johansson et al., 1999). The findings in the present study are consistent with the observation in globular proteins. The similarity in CD spectra of gA in the presence and absence of F3 indicated that general anesthetics had minimal effects on channel structure; high-resolution NMR results further confirmed that the channel structure was stable and unaltered in the presence of F3 at the concentration range studied. Compared with CD, NMR is more sensitive to the subtle changes in the side chain structures. Our earlier NMR studies (Tang et al., 1999a,b, 2000a) showed that the most profound changes in resonance fre-

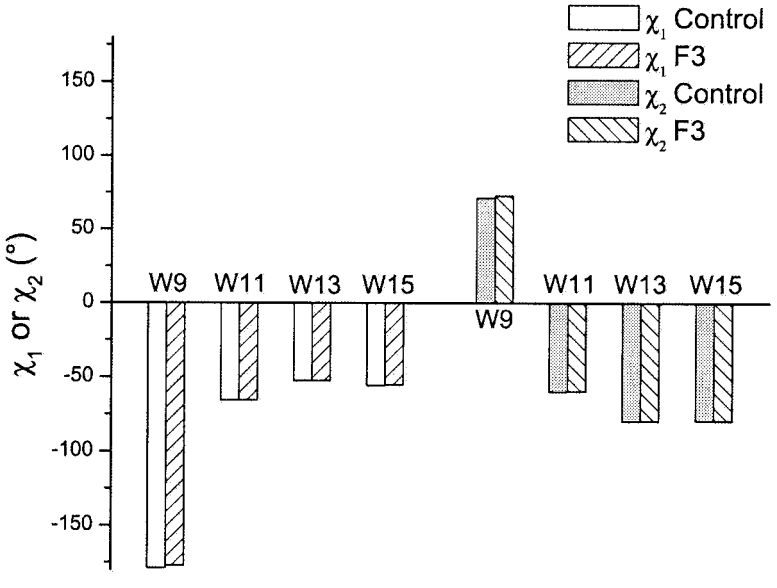

FIGURE 6 Comparison of tryptophan side chain orientations in the absence and presence of 14.8 mM F3. The dihedral angles χ_1 (HN-C_α-C_β−C_γ) and χ_2 (C_α-C_β−C_γ−$C_{\delta 1}$) were the averaged results from 30 refined structures.

quencies due to anesthetics occurred at the tryptophan side chain indole protons near the two ends of the channel. We found in the present study that even at the side chain level, the average tryptophan orientation is not significantly affected by F3 at concentrations up to 14.8 mM. Thus, the functional change observed in our previous study (Tang et al., 1999a) is unlikely to be a direct consequence of a structural change in the gA channel. This raises the question as to whether the volatile anesthetic interacts specifically with the gA channel or the functional change is due to alterations in the membranous surroundings. We have used the combination of NMR and photoaffinity labeling (Tang et al., 2000a) to show that volatile anesthetics do interact specifically with gA, but the interaction requires the channel conformation in a membranous environment. The micellar system used in this study should adequately mimic the lipid bilayers. Indeed, the NMR structure of gA in micelles is essentially the same as that in lipid bilayers (Arseniev et al., 1985; Ketchem et al., 1993; Townsley et al., 2001) except the dispute on the relative positions of W9 to W15; the radial profile of SDS micelles has been confirmed to be compatible with the conducting conformer of the gA channel. It is known, however, that considerable lateral diffusion of lipids occurs in the bilayers, whereas in comparison such diffusion is limited in micelles. As a consequence, the lower entropy effects in the micelle interior might counteract the disordering effects of general anesthetics in the tail region, albeit the current consensus does not consider lipid disordering is the primary action of general anesthetics. Nevertheless, our available data cannot separate with certainty the possible contributions to the functional change from the specific anesthetic interaction with the channel and from the indirect membrane modulation. It is clear, however, that neither can result in changes in the gA structure.

It should be emphasized that the conclusion of no anesthetic-induced structural change holds true only in the anesthetic concentration range studied. Previous CD studies (Abdul-Manan and Hinton, 1994; Veatch et al., 1974; Wallace, 1986) have shown that the conformation of gA varies with the organic solvent environment. In 10% TFE, which is a volatile anesthetic, the CD spectrum of gA shows a negative ellipticity at ~230 nm and a strong positive ellipticity at ~197 nm, resembling the CD spectra obtained in high-concentration alcohol and ethyl acetate. In these solvents, gA exists as a mixture of different forms of intertwined double-stranded helices. It is conceivable that the channel structure will undergo conformational changes if the concentration of anesthetic agents increases beyond certain limits.

Although the overall structure of gA was unaltered at F3 concentrations used in the present study, the association of gA with its membrane surrounding is strongly affected. This is most profoundly reflected in the exchange of indole amide protons of tryptophan side chains with water. For each gA channel, there are eight tryptophan residues (four from each monomer) whose outwardly extending side chains are believed to anchor the gA in a transmembrane orientation. Substitutions of these amphipathic tryptophan residues with hydrophobic phenylalanine are found to reduce the single-channel conductance for cations by 25% to 60% depending on the position that is modified (Becker et al., 1991). Given that the orientations of the tryptophan side chains did not change due to anesthetic binding (Figs. 5 and 6) but the chemical shifts of the indole amide proton resonance vary as a function of anesthetic concentration (Tang et al., 2000a), it can be inferred that anesthetics can facilitate the accessibility of the anchoring residues to water in a concentration-dependent manner. In the fast-exchange regime, the resonance frequency of an exchanging proton is the weighed average of the two limiting shifts: that in the indole ring and that in water. Because the water sites are dominant, the increase in the exchange rates of the tryptophan indole amide protons with water is consistent with the direction of the indole proton resonance frequency shifts (i.e., up-field toward water), suggesting that the anesthetic-induced chemical shifts detailed in our earlier studies (Tang et al., 2000a) may be partially or even entirely attributable to the increase of proton exchange with water. Thus, although F3 at concentrations as high as 14.8 mM did not alter gA channel structure, the ability of F3 to alter the exchange rate of the indole amide protons with water strongly indicates that the functional association of the anchoring tryptophan residues with its surroundings at the membrane interface can be modulated by volatile anesthetics, which have been shown to preferentially target the amphipathic lipid-water interface (North and Cafiso, 1997; Tang et al., 1997; Xu and Tang, 1997). The modulation can be brought about by disrupting the weak hydrogen bonds of indole amide protons with lipid head groups, by increasing tryptophan side chain motion, and thereby increasing the side chain exposure to water or by both. It is worth noting that structurally similar nonanesthetics (nonimmobilizers) distinguish themselves from anesthetics in that they do not have access to the aqueous phase (Tang et al., 1997).

Considering that the entire superfamily of neurotransmitter-gated ion channels responsible for fast synaptic transmission is sensitive to general anesthetics, the results of the present study with the gA channel have far reaching implications. Unlike neurotransmitters, whose high-affinity binding to neuronal receptors are believed to cause allosteric changes in channel structures, volatile anesthetics exert their effects on neuronal receptors through low affinity binding, which are often characterized by fast binding kinetics (Xu et al., 2000). Although the uniqueness of L- and D-amino acid alteration in gA sequence, which is not found in any neuronal receptor proteins, may limit the extent to which the conclusion of the present study can be generalized, our results nevertheless suggest that in addition to structural consequences, other processes, such as anesthetic-

induced changes in channel dynamics, should be considered for functional significance.

It should be noted that the structures measured by NMR are an ensemble of structures averaged on the NMR acquisition time scale of a few hundred milliseconds. The channel dynamics, which is intimately related to structure and function, should be evaluated at a much faster time scale. For example, we have recently studied the dynamics of gA channel in a fully hydrated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine membrane in the picosecond to nanosecond time range, using large-scale, all-atom, molecular dynamics simulations (Tang et al., 2000b). Two parallel 2.2-ns simulations with and without anesthetic halothane (data not shown) revealed that anesthetics enhanced gA channel dynamics in the pico- to nanosecond time scale without changing the time-averaged channel structures. Experimental confirmation of this theoretical finding by NMR relaxation measurements is underway.

The dimerization in gA is achieved by hydrogen bonding between structured backbone atoms deep in the tail region of the lipid bilayer. This situation is unique to gA and may not be generalized to neuronal channels that are formed by oligomerization of multiple subunits. In the latter case, hydrogen bonds among subunits are more likely to be formed between atoms in the flexible side chains. This type of intersubunit hydrogen bonds between side chains may be more susceptible to anesthetic perturbation. Moreover, because general anesthetics are amphiphilic in nature and prefer lipid-water interface, the likelihood for anesthetic molecules to disrupt hydrogen bonds between domains and subunits is higher near the lipid-water interface than in the lipid tail region. Therefore, although fast anesthetic binding might not be able to sustain significant changes in the secondary structures, our results with gA do not rule out the possibility that general anesthetics may interfere with the side chain association between subunits, particularly when potential anesthetic binding sites exist inside the channel pore (Forman et al., 1995) or between subunits (Mihic et al., 1997). This interference is likely to affect the dynamics of oligomization as well.

In conclusion, although current high-resolution structural information about neurotransmitter-gated channels is scarce, an emerging body of evidence from model protein studies suggests that in addition to structural changes, other protein processes, particularly dynamical changes, may be significantly involved in the molecular mechanism of general anesthesia.

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