Halothane Interactions with Nicotinic Acetylcholine Receptor Membranes

Steady-state and Kinetic Studies of Intrinsic Fluorescence Quenching

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Background: Although it has been suggested that anesthetics alter protein conformational states by binding to nonpolar sites within the interior regions of proteins, the rate and extent to which anesthetics penetrate membrane proteins has not been characterized. The authors report the use of steady-state and stopped-flow spectroscopy to characterize the interactions of halothane with receptor membranes.

Methods: Steady-state and stopped-flow fluorescence spectroscopy was used to characterize halothane quenching of nicotinic acetylcholine receptor (nAChR)-rich membrane intrinsic fluorescence and the rate of isoflurane-induced nAChR desensitization.

Results: At equilibrium, halothane quenched only 54 ± 1.4% of all tryptophan fluorescence. Diethyl ether failed to reduce fluorescence quenching by halothane, suggesting that it does not bind to the same protein sites as halothane. Stopped-flow fluorescence traces defined two kinetic components of quenching: a fast component that occurred in less than 1 ms followed by a slower biphasic fluorescence decay. Protein unfolding with sodium dodecyl sulfate reduced halothane's Stern-Volmer quenching constant, eliminated the biphasic decay, and rendered fluorescence accessible to quenching by halothane within 1 ms. Functional studies indicate that anesthetic-induced desensitization of nAChR occurs in less than 2 ms.

Conclusions: Unquenchable fluorescence arises from tryptophan residues that are buried within the protein and protected from halothane. Sodium dodecyl sulfate unfolds membrane proteins and allows previously buried fluorescence protein residues to be rapidly and homogeneously quenched by halothane. Halothane quenches protein components of nAChR membranes over the same concentration range and time scale that it exerts its functional effects, a finding that is generally consistent with a protein site of action. (Key words: Anesthetics, volatile: halothane. Nicotinic acetylcholine receptors. Theories of anesthetic action.)

The mechanism by which general anesthetics inhibit membrane protein function is not known. Because the potencies with which structurally diverse anesthetic compounds act correlate remarkably well with their hydrophobicities (Meyer-Overton correlation), theories of anesthesia have traditionally focused on the interactions between anesthetics and the lipid regions of neuronal membranes.1,2 Such theories propose that anesthetics bind within the lipid region of membranes and disrupt critical interactions between membrane proteins and their lipid environment.3-5

Recently, more attention has focused on the possibility that general anesthetics bind to nonpolar sites within the interior of proteins.5-11 Such interactions may disrupt function by stabilizing inactive protein conformational states.11,12 However, the extent and rate with which general anesthetics reach the interiors of membrane proteins has not been defined.

Under the appropriate conditions, both soluble and membrane proteins that contain aromatic amino acid residues such as tryptophan exhibit intrinsic fluorescence.13 This phenomenon is useful because it allows us to characterize interactions between proteins and compounds that quench fluorescence.14-16 Such studies indicate that some compounds cannot quench all protein fluorescence because they do not gain access to tightly packed regions within the interior regions of

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proteins where tryptophan residues frequently reside. Because the general anesthetic halothane quenches tryptophan fluorescence, this approach may be used to gauge its ability to reach the interior regions of membrane proteins.

In this study, we have characterized the interactions between halothane and membranes rich in nicotinic acetylcholine receptors (nAcChORs) by measuring quenching of protein-intrinsic fluorescence using steady-state and stopped-flow spectroscopy. Because halothane and diethyl ether both stabilize the desensitized nAcChOR conformational state, we also tested the hypothesis that these anesthetics compete for discrete binding sites within these membranes. Finally, we defined a limit for the rate with which halothane and isoflurane convert the nAcChOR to the desensitized state to determine whether the functional effects of these anesthetics on receptor conformational states occur on the same time scale as do their interactions with protein components of nAcChOR membranes.

Materials and Methods

Materials

Torpedo nobiliana was obtained from Biofish Associates (Georgetown, MA). Diisopropylfluorophosphate and (dansylaminoethyl) trimethylammonium perchlorate were purchased from Sigma Chemical Company (St. Louis, MO). Halothane was purchased from Anaquest (Murray Hill, NJ), and the thymol was removed with an aluminum oxide column. Diethyl ether was purchased from Fisher Scientific (Pittsburgh, PA). The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes (Eugene, OR).

Methods

Preparation and Characterization of nAcChOR Membranes. Membrane fragments were prepared from the electric organs of Torpedo nobiliana, as previously described and approved by the Massachusetts General Hospital Animal Care and Use Committee. Purified membrane fragments were alkaline extracted to reduce nonreceptor protein components. Membranes were stored in Torpedo physiological saline (TPS; 250 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 2 mM MgCl₂, 5 mM NaH₂PO₄, and 0.02% NaN₃, pH 7.0) at −80°C and used within 48 h of being thawed. The number of agonist binding sites was determined from the competition of (dansylaminoethyl) trimethylammonium perchlorate binding by acetylcholine.

Emission Spectra of nAcChOR-rich Membrane Intrinsic Fluorescence. Intrinsic fluorescence emission spectra were measured with and without halothane were made with a Fluoro-Max 2 spectrofluorimeter (Jobin Yvon-Spex, Edison, NJ). Solutions of halothane in TPS were added to membrane fragments and quickly transferred to a 10-mm-pathlength quartz cell with a Teflon stopper. The dead space above the aqueous solution was negligible. Fluorescence was excited at 295 nm to selectively excite tryptophan residues, and emission was detected between 305 and 450 nm. The sample compartment temperature was set at 20 ± 1°C. For competition studies, a control spectrum was obtained in the presence of halothane, aliquots of diethyl ether were added to achieve the desired final concentration, and additional spectra were recorded.

Stopped-flow Studies of Halothane-induced Quenching of Intrinsic Fluorescence. Receptor-rich membranes (0.2 µM in agonist binding sites in TPS) were loaded into one of the gas-tight mixing syringes of a DX.17MV stopped-flow spectrofluorimeter (Applied Photophysics, Leatherhead, UK), and an aqueous solution of halothane in TPS was loaded into the other. The spectrofluorimeter was set at 20 ± 0.5°C. The contents of the syringes were mixed rapidly (1:1 vol:vol), yielding final concentrations of 0.1 µM agonist binding sites and the desired concentration of halothane. Tryptophan fluorescence was excited at 295 nm using a 150-watt xenon arc lamp and monochromator. The time-dependent change in fluorescence intensity above 335 nm was recorded through a high-pass filter (4,000 points typically over 200–500 ms). Unless otherwise indicated, the reported fluorescence intensity at equilibrium was determined using our stopped-flow spectrofluorimeter, which allowed both kinetic and equilibrium data to be obtained from a single experimental trace. For each experiment, approximately 10 individual shots were signal averaged to improve the signal-to-noise ratio. Signal-averaged fluorescence traces were transferred to a personal computer (Macintosh; Apple Computers, Cupertino, CA) for analysis.

Preparation of Extracted Torpedo Lipid Vesicles and Stopped-flow Studies of Halothane-induced Quenching of Lipid-Incorporated DPH. Approximately 5 mg of receptor-rich membranes were solubilized in 2 ml chloroform:methanol (2:1 vol:vol) and filtered through glass wool. The filtrate was added to 0.6%
sodium chloride and gently mixed. The organic phase was removed and combined with 2 ml hexane:chloroform (3:1 vol:vol). The supernatant was removed and dried under a stream of nitrogen, yielding 1 mg extracted lipid. The fluorescent probe DPH was added to the extracted lipid in chloroform to achieve a final ratio of DPH/lipid of 1:100 (wt:wt). The mixture was dried under a stream of nitrogen for 1 h, resuspended in 1 ml TPS, and intermittently sonicated on ice under a stream of nitrogen for 15 min to form vesicles.

The extracted Torpedo lipid/DPH vesicles were loaded into one of the gas-tight mixing syringes of the stopped-flow spectrofluorimeter, and an aqueous solution of halothane in TPS was loaded into the other. The spectrofluorimeter was thermostatted at 20 ± 0.3°C. The contents of the syringes were rapidly mixed (1:1 vol:vol). Fluorescence with DPH was excited at 350 nm, and fluorescence was recorded (3,000 points over 200 ns) through a 440 - 480-nm bandpass filter.

**Stopped-flow Studies of Halothane-induced nAChOR Desensitization.** The fraction of all nAChORs preexisting in the desensitized state before agonist-induced desensitization was determined using a fluorescence binding assay, as previously described. Briefly, receptor-rich membranes (0.8 μM in agonist binding sites in TPS) were loaded into one of the gastight mixing syringes of a DX.17MV stopped-flow spectrofluorimeter, and a solution containing the fluorescent acetylcholine analog Dns-C₆₅-chol in TPS was loaded into the other. In these studies, we used a concentration of Dns-C₆₅-chol that was higher than that in our previous reports (8 μM) to improve the time resolution of our assay, which is limited by the rate of agonist binding. The spectrofluorimeter was thermostatted at 20 ± 0.3°C. The contents of the syringes were rapidly mixed (1:1 vol:vol), yielding final concentrations of 0.4 μM agonist binding sites and 1 μM Dns-C₆₅-chol. An excitation wavelength of 290 nm was used and the desensitization kinetics were followed by recording the time-dependent change in fluorescence intensity through a 560-nm high-pass filter. Such traces are characterized by a rapid increase in fluorescence intensity, reflecting binding of the fluorescent agonist to receptors preexisting in the high-affinity desensitized state before agonist-induced desensitization. The fraction of all nAChORs that preexist in the desensitized state is equal to the fluorescence amplitude of the rapid component divided by the change in fluorescence intensity observed between mixing and equilibrium.

**Statistical Presentation.** All data are the means of at least three separate determinations, and the error bars indicate the standard deviation.

**Results**

**Equilibrium Quenching of nAChOR Intrinsic Fluorescence by Halothane**

Receptor membranes exhibit an intrinsic fluorescence emission maximum of 355 nm (fig. 1). Equilibration of halothane with membranes reduces intrinsic fluorescence in a concentration-dependent manner without producing a detectable shift in the emission maximum. Figure 1B shows a difference spectrum, derived by subtracting the emission spectrum in the presence of halothane from that in its absence. Figure 2A is a plot of the intrinsic fluorescence intensity of nAChOR membranes at equilibrium with various concentrations of halothane. Figure 2B shows this data in the form of a Stern-Volmer plot in which F₀ is the fluorescence intensity in the absence of halothane and F is the fluorescence intensity in the presence of halothane. When a homogeneous class of tryptophan residues exists, collisional quenching will produce a linear Stern-Volmer plot described by the following equation:

\[
\frac{F_0}{F} = 1 + K_{sv}[\text{halothane}] \tag{1}
\]

where \(K_{sv}\) is the Stern-Volmer quenching constant. However, as seen in figure 2B, a Stern-Volmer plot of our data was not linear. Nonlinear Stern-Volmer plots with curvature toward the concentration axis are obtained with globular proteins that contain multiple tryptophan residues having different accessibility to quencher. The classical Stern-Volmer equation may be modified to account for this heterogeneity by assuming a range of accessibility. In the simplest case, a tryptophan may be considered to be either accessible or inaccessible to quencher. This is described by the following equation:

\[
\frac{F_0}{F} = f_0 + f_i(1 + K_{sv}(\text{eff})[\text{halothane}]) + f_i \tag{2}
\]

where \(f_0\) is the fraction of all fluorescence emanating from tryptophan residues that are accessible to quenching by halothane and \(f_i\) is the fraction of fluorescence from inaccessible tryptophan residues (\(1 - f_0\)). \(K_{sv}(\text{eff})\) is the effective Stern-Volmer constant. The fraction of quenchable fluorescence does not necessarily equal the fraction of quenchable tryptophan.
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Fig. 1. (A) Fluorescence spectra obtained after equilibrating nicotinic acetylcholine receptor membranes with halothane at the indicated concentrations. (B) The solid line is the difference spectrum, derived by subtracting the fluorescence spectrum obtained in the presence of 8.5 mM halothane from the control spectrum (no halothane). The amplitude of the difference spectrum was normalized to that of the control spectrum to facilitate comparison. The excitation wavelength was 295 nm.

residues, because all tryptophan residues in a multi-tryptophan protein may not be equally fluorescent. In the case of the nAChR's 51 tryptophans, those that fluoresce most will contribute a relatively greater fraction of the total protein fluorescence. The fraction of tryptophan fluorescence accessible to quenching at equilibrium determined using equation 2 was 0.54 ± 0.014, with an effective Stern-Volmer quenching constant of 240 ± 13 M⁻¹.

Sodium dodecyl sulfate may be used to unfold proteins and expose otherwise buried tryptophan residues.²⁴ Figure 3A is a plot of the intrinsic fluorescence intensity of membranes that were preequilibrated with 1% sodium dodecyl sulfate at 37°C before mixing with various concentrations of halothane. A Stern-Volmer plot of this data was linear, indicating homogeneous quenching of tryptophan residues. The Stern-Volmer quenching constant was determined to be 92 ± 7.8 M⁻¹ using equation 1 (fig. 3B).

Competition Studies with Diethyl Ether
To determine whether the nonquenching general anesthetic diethyl ether competes with halothane for binding sites within nAChR membranes, we tested its ability to reduce the intrinsic fluorescence quenching induced by 1 mM halothane. We observed no increase in fluorescence even at concentrations of diethyl ether as high as 75 mM (fig. 4).

Kinetic Analysis of nAChR Membrane Intrinsic Fluorescence Quenching
Stopped-flow traces obtained after mixing nAChR-rich membranes with halothane defined two major components of quenching. There was a large, fast com-

Fig. 2. (A) Equilibrium fluorescence intensity obtained after mixing nicotinic acetylcholine receptor membranes with various concentrations of halothane. The excitation wavelength was 295 nm and fluorescence was recorded through a 335-nm highpass filter. (B) Stern-Volmer plot of the data in A. The curve is a fit to equation 2 with f₀ and Kₚₚₑ,eff equal to 0.54 ± 0.014 and 240 ± 13 M⁻¹, respectively. The dashed line in B is a linear fit of the data up to 2 mM halothane and emphasizes the nonlinearity of the Stern-Volmer plot.
ponent of fluorescence quenching that occurred within the 1-ms mixing time of the spectrophotometer (fig. 5A). This component was detected as a reduction in the fluorescence intensity observed immediately on mixing. After this fast component, a fluorescence decay was observed that could be eliminated completely by pre-equilibrating membranes with 1% sodium dodecyl sulfate for 30 min at 37°C (fig. 5B). The fluorescence decay was not a simple first-order process as determined from nonrandom residuals typically obtained when fitting traces to an exponential function (i.e., a plot of the residual versus time typically oscillated around zero in a regular manner). Rather, the decay was biphasic, having a larger, faster phase that was essentially complete within 10 ms of mixing membranes with halothane and a smaller, slower phase that was complete only after approximately 100 ms. The fluorescence amplitudes and time constants of the biphasic fluorescence decays were, therefore, determined by fitting fluorescence traces to the sum of two exponentials. For a range of halothane concentrations, such fits had residuals that were random. The time constants of both the fast and slow phases of the fluorescence decay, which did not vary systematically with halothane concentration, were 2.8 ± 0.62 ms and 30 ± 12 ms, respectively (fig. 6A). These rates were not significantly altered by pre-equilibration of membranes with 1 ms of the nicotinic agonist carbamylcholine chloride (2.3 ± 0.17 ms and 32 ± 5.6 ms for the fast and slow phases, respectively), which converts the receptor to the desensitized conformational state.

The amplitudes of the fast and slow phase of this decay increased along with halothane concentration before plateauing (fig. 6B). The concentration of halothane producing a half-maximal increase in each phase of the decay was determined by fitting a plot of the amplitude of the decay versus halothane concentration to the following logistic equation:

$$\text{Amplitude} = \text{Amp}_{\text{max}} \left(1 + \frac{[\text{halothane}]}{[\text{halothane}] + EC_{50}}\right)$$

(3)

where \(\text{Amp}_{\text{max}}\) is the maximum amplitude at high halothane concentrations, and \(EC_{50}\) is the halothane concen-

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Fig. 3. (A) Equilibrium fluorescence intensity obtained after mixing denatured nicotinic acetylcholine receptor membranes with various concentrations of halothane. The excitation wavelength was 295 nm. For concentrations up to and including 8.5 mM halothane, fluorescence was recorded through a 335-nm highpass filter using the stopped-flow instrument. The fluorescence in the presence of 12 mM halothane was recorded at the emission maximum in the presence of sodium dodecyl sulfate (339 nm) using a Fluoro-Max 2 spectrofluorometer. (B) Stern-Volmer plot of the data in A. The line is a fit to equation 1, yielding a Stern-Volmer quenching constant of 92 ± 7.8 M⁻¹.

Fig. 4. Plot of the change in the equilibrium fluorescence intensity with the addition of diethyl ether to solutions containing both nicotinic acetylcholine receptor membranes and 1 mM halothane. Excitation was 295 nm and emission was recorded at 335 nm.

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Fig. 5. (A) Stopped-flow fluorescence traces obtained after rapidly mixing nicotinic acetylcholine receptor (nAChR) membranes with various concentrations of halothane. There was a fast component of quenching that occurred within the mixing time of the spectrofluorimeter. This can be seen as a decrease in the fluorescence intensity recorded immediately after mixing nAChR membranes with halothane. This is followed by a slower component of quenching that is resolved as a fluorescence decay which in the 8.5 mM halothane trace is indicated by the arrow. (B) Stopped-flow fluorescence traces obtained after rapidly mixing denatured nAChR membranes with various concentrations of halothane. All quenching occurs within the mixing time of the spectrofluorimeter (1 ms), and the fluorescence decay is not observed.

Fig. 6. Plot of the time constants (A) and amplitudes (B) of the fast and slow phases of the slow fluorescence decay derived by fitting experimental fluorescence traces to an equation describing the sum of two exponentials. The curves in B are the fits of the data to equation 5, yielding median effective concentrations of 2.4 ± 0.28 mM and 0.8 ± 0.24 mM for the fast and slow phases of the decay, respectively.

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**Stopped-flow Studies of Anesthetic-induced nAcChoR Desensitization**

When Dns-C₆-chol was rapidly mixed with nAcChoR membranes, there was a rapid component of fluorescence reflecting binding of the fluorescent agonist to receptors preexisting in the high-affinity state. This was followed by slower components reflecting conversion of resting state receptors to the high-affinity state. When a final concentration of 4 μM Dns-C₆-chol was used, agonist binding to the population of nAcChoRs preexisting in the desensitized state was essentially complete within 5–10 ms of mixing (fig. 8). Preequilibration for 5 min with millimolar concentrations of either halothane or isoflurane produced an increase in the fraction of preexisting desensitized nAcChoRs, as indicated by the relative increase in the amplitude of the rapid fluorescence component. Because pilot studies using preequilibration times as short as 15 ms (using our spectrofluorimeter’s double-mixing capability) before assaying for the fraction of desensitized receptors showed that anesthetic stabilization of the desensitized state was complete within this short preequilibrium time, we resorted to exposing membranes simultaneously to both anesthetics and agonist to increase the time resolution of the assay. We saw no significant difference between fluorescence traces obtained using membranes that had been preequilibrated with anesthetics for 5 min and those that had been exposed to anesthetics simultaneously with agonist (fig. 8). Although this assay has a time resolution of 5–10 ms, close inspection of the

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**Fig. 7.** Stopped-flow fluorescence traces obtained after rapidly mixing nicotinic acetylcholine receptor membranes with halothane and isoflurane. The final concentrations were 8.5 mM halothane and 7.5 mM isoflurane. These concentrations are equal to half-saturated aqueous solutions. Only the first 20 ms is shown to facilitate comparison of the fast phase of the fluorescence decay. Fluorescence has been normalized to 1 in the absence of anesthetic.

**Fig. 8.** Stopped-flow fluorescence traces obtained after rapidly mixing nicotinic acetylcholine receptor (nAcChoR) membranes with the fluorescence agonist Dns-C₆-chol. The trace labeled “preequibrated” was obtained using membranes that were preequilibrated with 4 mM isoflurane for 5 min before rapidly mixing with a solution containing 8 μM Dns-C₆-chol and 4 mM isoflurane. The trace labeled “simultaneously added” was obtained by rapidly mixing membranes without anesthetic with a solution containing both 8 μM Dns-C₆-chol and 8 mM isoflurane. Thus, for both preequilibrated and simultaneously added experiments, the final concentrations were 4 mM isoflurane and 4 μM Dns-C₆-chol. The control trace was obtained by rapidly mixing nAcChoR membranes with 8 μM Dns-C₆-chol. The same experiments are shown in A and B but on different time scales. Excitation was 290 nm and emission was recorded above 560 nm.
kinetic traces in figure 8 revealed that isoflurane enhances agonist binding within 2 ms of mixing.

Discussion

At equilibrium, halothane quenches only one half of all protein intrinsic fluorescence from nACChOR-rich membranes. We conclude that the fluorescence that cannot be quenched by halothane emanates from tryptophans that are buried within the protein matrix and thus shielded from anesthetic. This conclusion is supported by the observation that when sodium dodecyl sulfate is used to induce protein unfolding, the Stern-Volmer plot becomes linear, suggesting that all (or nearly all) fluorescence becomes accessible to quenching by halothane. Because the Stern-Volmer quenching constant of quenchable tryptophan decreases substantially with protein denaturation, the protein structure must also provide an environment that facilitates anesthetic interactions with those regions that are accessible. Although the specific requirements for anesthetic binding sites on proteins is not well defined, studies using poly(L-lysine) of varying lengths to simulate proteins suggest that the supersecondary structure is a critical feature of such sites. 26 The more traditional hydrophobic quencher acrylamide was used previously to characterize the accessibility of tryptophan residues to hydrophobic quenchers and yielded analogous results in other protein systems. For example, acrylamide quenches only 22—57% of all tryptophan fluorescence in Na,K-ATPase (depending on the buffer used) and 30% of all fluorescence in both horse liver alcohol dehydrogenase and alkaline phosphatase. 24,27

The fluorescence emission maximum of a tryptophan residue is very sensitive to the polarity of its environment. When tryptophan is exposed to a nonpolar environment, the emission maximum occurs at a shorter wavelength than when exposed to a polar one such as water. 13 Thus a protein tryptophan can serve as a probe of its own environment. In proteins that contain more than one tryptophan residue, the observed emission maximum is an intensity-weighted average of all tryptophan residues. The 335-nm fluorescence emission maximum of nACChOR membranes is significantly shorter than the 355 nm reported for free tryptophan in water, indicating that, on average, tryptophan residues in nACChOR membranes are exposed to an environment that is substantially less polar than water, despite results of

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nated with protein unfolding, we considered the possibility that this decay could reflect either slow diffusive penetration of halothane to incompletely shielded protein domains or an anesthetic-induced conformational isomerization. These two possible models are discussed in the sections that follow.

**Protein Penetration Model**
Current models of protein structure emphasize the concept that proteins are dynamic entities subject to a wide range of structural fluctuations.39 Fluorescence and phosphorescence quenching studies suggest that such fluctuations may lead to transient holes or channels within some regions of the protein matrix through which small molecules can diffuse to quench tryptophans that are not exposed to the bulk solvent.27 The movement of small molecules such as anesthetics through the protein matrix may be orders of magnitude slower than that through water because channel formation limits the rate with which molecules move. Because channel formation is generally a property of the protein and not the quencher, a quencher’s penetration rate is predicted to be relatively insensitive to its size. This has been shown experimentally using phosphorescence lifetime as a gauge of quencher penetration rate and is consistent with our observation that the fluorescence decay is similar for halothane and isoflurane.27 When channel formation rather than anesthetic diffusion is the rate-limiting step, the penetration rate is also predicted to be insensitive to anesthetic concentration. This is consistent with our data because we found no halothane concentration-dependence on the rate of quenching. Within the context of the protein penetration model, our observation that approximately one half of all fluorescence is inaccessible to halothane, even at equilibrium, suggests that although some protein domains may be slowly accessible to the anesthetic, other domains are packed so tightly that anesthetic molecules are completely excluded. Such sites are logically excluded as being relevant binding sites for anesthetics.

**Conformational Isomerization Model**
General anesthetics can alter protein conformational states. The anesthetic-induced nAChR conformational state (desensitized state) is characterized by a high affinity for both agonists and noncompetitive inhibitors. However, because desensitizing nAChRs with carbamylcholine chloride before mixing with halothane failed to eliminate the biphasic decay, this decay cannot reflect anesthetic-induced nAChR desensitization. In addition, the lack of a significant shift in the fluorescence emission maximum with the addition of anesthetic argues against a substantial change in protein structure. However, our data do not allow us to preclude entirely the possibility that anesthetics induce some protein unfolding.31

The halothane concentrations used in this study were generally greater than those used clinically. This allowed us to more easily measure anesthetic-induced quenching and to conclusively determine that many tryptophan residues were inaccessible to the anesthetic. At 0.5 mM (a concentration that is approximately twice the median effective concentration [EC₅₀] for anesthesia), halothane quenches 5.5% of all of the intrinsic fluorescence. Because the concentration-dependence of quenching was essentially linear up to 2.0 mM halothane, we estimate that approximately 3% of intrinsic fluorescence would be quenched at the EC₅₀ for anesthesia. Although these values may seem insignificant, it must be recognized that the nAChR’s 51 tryptophans are being used as intrinsic probes that are distributed over the entire multiple-subunit protein. A lower limit for the number of amino acids that may be interacting with halothane can be estimated by assuming that (1) the 5.5% reduction in fluorescence induced by 0.5 mM halothane represents complete quenching of 5.5% of the protein tryptophan, and (2) halothane’s interactions with tryptophan are representative of its interactions with other amino acids. The second assumption seems reasonable because tryptophan falls near the middle of the Kyte-Doolittle scale of amino acid hydrophobicity.32 Because *Torpedo* nAChRs contain 2333 amino acids, 128 nAChR amino acids are thus estimated to be interacting with halothane at a concentration of 0.5 mM. However, if the 5.5% reduction in fluorescence intensity induced by 0.5 mM halothane reflects less-than-complete quenching of more than 5.5% of the protein tryptophan, then the number of amino acids interacting with halothane would be even greater.

There have been no previous studies examining the rate with which anesthetics induce nAChR desensitization. The fluorescence agonist binding assay indicates that this rate is rather fast. We place an upper limit on the time required for halogenated anesthetics to desensitize nAChRs at 2 ms. The actual rate might well be much faster, but this is the limit of resolution of the assay. This may be compared with the 10–100 ms time scale required for saturating concentrations of agonists.
to induce desensitization. Thus within the resolution of the stopped-flow technique, the time scale of anesthetic-induced desensitization is on the same microsecond to millisecond time scale required for anesthetic-induced quenching of protein fluorescence.

We have characterized the interactions between halothane and protein components of nAChR-rich membranes by measuring quenching of intrinsic fluorescence with steady-state and stopped-flow spectroscopy. We have determined that only one half of all nAChR membrane intrinsic fluorescence is accessible to quenching by halothane at equilibrium and that the remaining unquenchable fluorescence emanates from tryptophan residues that are buried within tightly packed protein domains that exclude anesthetic molecules. Our denaturing experiments indicate that the protein structure not only excludes anesthetics from some domains but also facilitates anesthetic interactions with others. Stopped-flow studies show that anesthetic-protein interactions in these membranes occur on the same time scale as halothane-induced alterations in nAChR’s conformational state. Although our results do not prove that anesthetics act by binding directly to protein components of these membranes, they are generally consistent with this hypothesis. With the development of better algorithms to predict membrane protein structure, it should be possible to identify protein domains that are likely to be accessible to anesthetics and to predict the location of halothane binding sites within membrane proteins.

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