Fluorescence quenching of gramicidin D in model membranes by halothane

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Abstract: Inhaled anesthetics were introduced in surgery over a century ago. To this day, the molecular mechanism of anesthetic action remains largely unknown. However, ion-channels of neuronal membranes are believed to be the most-likely molecular targets of inhaled anesthetics. In the study presented here, we investigated the interaction of a simplified ion-channel system, gramicidin, with halothane, a small haloalkane inhaled anesthetic in various environments. Fluorescence-quenching experiments of gramicidin D in dioleoylphosphatidylcholine (DOPC) large unilamellar vesicles (LUVS) have shown that halothane can directly interact with the ion channel (K_{SV} = 66 M^{-1}). Halothane quenched the fluorescence from tryptophan residues located at the lipid bilayer – aqueous interfaces as well as those tryptophans located deeper in the bilayer. Quenching data from gramicidin D in sodium dodecyl sulfide (SDS) micelles revealed that the tryptophan residues located at the micelle–solvent interface were preferentially quenched by halothane (K_{SV} = 22 M^{-1}). In 1-octanol, fluorescence quenching was observed, but with a lower K_{SV} value (K_{SV} = 6 M^{-1}) than in DOPC LUVS and SDS micelles. Taken together, these results indicate that halothane interactions with gramicidin, mediated by a lipid bilayer, are the strongest, and that the mechanism of anesthetic action may also be lipid-mediated.

Key words: anesthetics, lipid bilayer, membrane protein, fluorescence quenching.

Résumé : Les anesthésiants absorbés par inhalation ont été introduits en chirurgie il y a plus de cent ans. À ce jour, le mécanisme moléculaire de l’action anesthésique demeure toujours largement inconnu. Toutefois, on croit généralement que les membranes neuronales à canaux ioniques sont les cibles moléculaires les plus probables des anesthésiants absorbés par inhalation. Dans l’étude présentée ici, on a étudié l’interaction d’un système à canal ionique simplifié, la gramicidine, avec l’halothane, une petite molécule d’alcane halogéné utilisée comme anesthésiant dans divers environnements. Des expériences de piégeage de la fluorescence de la gramicidine D dans de grosses vésicules unilamellaires (GVUL) de dioéthylphosphatidylcholine (DOPC) ont permis de montrer que l’halothane peut interagir directement avec le canal ionique (K_{SV} = 66 M^{-1}). L’halothane permet de piéger la fluorescence des résidus de tryptophane situés aux interfaces de la bicouche lipide – eau ainsi que celles des tryptophanes situés dans des parties plus profondes de la bicouche. Les données du piégeage de la gramicidine D dans des micelles de dodécylsulfure de sodium (DSS) révèlent que les résidus de tryptophane situés à l’interface micelle–solvant sont piégés d’une façon préférentielle par l’halothane (K_{SV} = 22 M^{-1}). Dans le 1-octanol, on a observé du piégeage; toutefois la valeur de K_{SV} (K_{SV} = 6 M^{-1}) est beaucoup plus faible que celles observées dans les micelles de GVUL DOPC ou de DSS. Considérés dans leur ensemble, ces résultats suggèrent que les plus fortes interactions sont celles de l’halothane avec la gramicidine qui sont catalysées par une bicouche lipidique et que le mécanisme de l’action anesthésique pourrait aussi être catalysé par les lipides.

Mots-clés : anesthésiques, bicouche lipidique, membrane de protéine, piégeage de la fluorescence.

Introduction

Inhaled anesthetics were introduced in surgery over a century ago. To this day, the molecular mechanism of anesthetic action remains elusive. At the beginning of the twentieth century, Overton and Meyer noted a strong correlation between oil solubility of general anesthetics and their anesthetic potency (1, 2) (Overton–Meyer correlation). As a result of the Overton–Meyer correlation, it was hypothesized that inhaled anesthetics exert their action by targeting the lipid matrix of neuronal cell membranes (3).

In recent years, a protein-mediated mechanism of general anesthesia has been favored over the solely lipid-mediated mechanism. Ion channels of neuronal membranes are believed to be the most-likely molecular targets of inhaled anesthetics (4, 5). Emerging evidence suggests that anesthetics
might also interact directly with G-protein-coupled receptors (GPCRs) (6, 7). However, a specific receptor for general anesthetics has yet to be identified.

A molecular understanding of the mechanism of anesthesia will require detailed structural information regarding anesthetic–protein complexes. This presents a challenge when working with membrane proteins, since structural information is scarce. To overcome this problem, novel approaches have included using well-defined water-soluble model systems, such as synthetic four α-helix bundles (8) and the naturally occurring serum albumin proteins. These studies have indicated that inhaled anesthetics bind to pre-existing hydrophobic cavities or defects found within water-soluble protein model systems. Within these cavities, dipole – induced dipole interactions between anesthetics and hydrophobic side chains, such as tryptophan and phenylalanine, have been found to further stabilize and increase binding affinities (9, 10). The current consensus is that inhaled anesthetics interact with proteins via weak hydrogen and van der Waals bonding. Recent work by Liu et al. has shown that in the case of ferritin, a natural 4-helix bundle containing protein, weak polar interactions can in fact confer significant anesthetic selectivity (11).

Despite the significant insights into inhaled anesthetic – protein interactions obtained from water-soluble protein studies, the structural requirements for optimal anesthetic binding in membrane proteins is unclear. In the study presented here, we investigate the interaction of a simplified ion-channel system, gramicidin, with halothane, a small haloalkane-inhaled anesthetic. Halothane, although seldom used in clinical work today, is frequently used in veterinary medicine and in drug–protein binding studies. Gramicidin is a well-characterized small polypeptide composed of alternating D- and L-amino acids (12, 13). Gramicidin D is a mixture of three main isoforms, with the sequence of the most abundant (~85%) gramicidin A being HCO–L-Val1–Gly2–L-Ala3–D-Leu4–L-Ala5–L-Val6–L-Ala7–D-Val8–L-Trp9–D-Leu10–L-Trp11–D-Leu12–L-Trp13–D-Leu14–L-Trp15–NHCH2CH2OH. The lower-abundance isoforms, gramicidin B and C, differ at residue 11 (Trp11(gA):Phe11(gB):Tyr11(gC) is 85:15:5 mol%, respectively). Gramicidin’s secondary structure is solvent-dependent (14, 15). In lipid bilayers, gramicidin forms dimeric ion channels by the association of two right-handed, single stranded beta-helical monomers, each of which spans a single-bilayer leaflet (16). In organic solvents, gramicidin can adopt a variety of conformers: monomer or left-handed and right-handed double helices. In SDS micelles, gramicidin has been shown to adopt the single-stranded beta-helical monomer conformation (17). The structures of gramicidin are depicted in Fig. 1, which also includes a space-filling model of halothane for comparison. Gramicidin has been previously used as a model in anesthetic – ion-channel studies using NMR (17), photo-affinity (18) labeling, and molecular dynamics simulations (19, 20).

Presently, we have investigated the gramicidin-inhaled anesthetic interaction in a DOPC lipid bilayer in sodium dodecyl sulphate (SDS) micelles and in octanol by steady-state fluorescence quenching. Fluorescence-quenching experiments have been previously used to determine the binding of halothane to serum albumin (21). Halothane quenching of aromatic side chains (tryptophan, tyrosine, and phenylalanine) fluorescence is thought to occur as a result of an increase in intersystem crossing, which arises by spin–orbit coupling between the excited singlet state of the aromatic ring and the heavy atoms of chlorine and bromine (22). Our results indicate that the strongest interaction between gramicidin and halothane occurs in the lipid-bilayer environment. This suggests that although anesthetics may directly bind with membrane proteins, it is likely they do so mediated by lipids in the cell membrane.

Materials and methodology

1,2-Dioleoyl-sn-3-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL). 2-Bromo-2-chloro-1,1,1-trifluoroethane (Halothane) 99% (0.01% thymol), sodium dodecyl sulfate (SDS), and gramicidin D were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All reagents were used as delivered. Ultrapure water was used in the preparation of buffers and aqueous solutions using a Mega Pure™ System ACS (Corning, NY).

Preparation of gramicidin-containing large unilamellar DOPC vesicles (LUVS) and SDS micelles

Solutions of large unilamellar vesicles (LUVS) were prepared from a chloroform lipid stock (2.5 mg/mL). An appropriate aliquot of chloroform–lipid stock was transferred to a 10 mL volumetric flask to give a final lipid concentration of 0.1 mmol/L (0.25 mg/mL lipid). Stock solutions of gramicidin D were prepared in methanol (1 mmol/L final concentration). An aliquot of the protein stock was mixed with an aliquot of the lipid–chloroform stock. The organic solvents were evaporated using nitrogen. The final gramicidin concentration was 1 µmol/L, giving a DOPC:gramicidin molar ratio of 100:1. The lipid – gramicidin D film was resuspended in 10 mmol/L phosphate buffer (pH = 7.0) by stirring for 1 h. Under very similar conditions, gramicidin was found to adopt a right-handed, single-stranded beta-helical conformation (23). Large unilamellar vesicles were prepared by sonication using a sonicator bath (Laboratory Supplies Co., Inc.) followed by extrusion through a 100 nm polycarbonate membrane using an Avanti MiniExtruder. Alternatively, DOPC LUVS were prepared using a probe sonicator. The lipid solutions were kept on ice to prevent overheating because of the sonication energy. The solutions were sonicated in 10 s pulses to a total of five sonication cycles at which point the lipid solution was clear.

SDS micelles were prepared by dissolving the detergent in ultrapure water to a final concentration of 80 mmol/L. Solutions were allowed to stir for 1.5 h and then sonicated up to 30 min. Aliquots of gramicidin stock in methanol were added to a volumetric flask, and the organic solvent was evaporated with nitrogen gas. The protein film was resuspended with the micelle solution by stirring for an hour and sonicating for 5 min.

Acquisition of spectra and fluorescence-quenching analysis

The four tryptophan residues that gramicidin contains are the only fluorescent moieties in the polypeptide. Fluorescence experiments were performed using a Cary-Eclipse fluorimeter. Protein emission spectra were collected by exciting samples...
with 280 nm, and emission and excitation slit widths were set at 5 nm. Fluorescence-quenching data for 1-octanol solution was analyzed using the Stern–Volmer equation

\[
\frac{F_0}{F} = 1 + K_{SV}[Q]
\]

where \(F_0\) and \(F\) are the integrated fluorescence intensities of the main tryptophan fluorescence peak in the absence and presence, respectively, of the quencher, \(Q\); and \(K_{SV}\) is the Stern–Volmer quenching constant.

Fluorescence-quenching data in LUV and micelle media were analyzed using a modified Stern–Volmer equation, which accounts for the partitioning of halothane into the LUVs and micelles (24)

\[
\frac{F_0}{F} = \left(1 + \frac{K_{SV}K_{part}[Q]_{tot}}{1 + K_{part}\gamma_L[L]}\right)
\]

where \(K_{part}\) is the water–lipid partition constant, \([Q]_{tot}\) is the total quencher concentration for the entire solution, \([L]\) is the lipid (or surfactant) concentration, and \(\gamma_L\) is the partial molar volume of the lipid (or surfactant).

Results

Since a large amount of evidence suggests a protein-mediated mechanism of general anesthesia, we investigated the interaction of halothane with the peptide ion channel, gramicidin, within bilayers and bilayer-mimetic environments. This was done to ascertain the effect of the local environment on halothane–gramicidin interactions. In previous work, we examined the location of inhaled anesthetic in a fluorescently labeled DOPC lipid bilayer (25). We found that halothane locates on the acyl chain side, near the headgroup region of the bilayer (25). Therefore, we might anticipate a heterogeneous distribution of halothane around gramicidin in bilayer environments.

Fluorescence quenching in DOPC unilamellar vesicles

The quenching of gramicidin D by halothane in a lipid bilayer was studied using DOPC LUVS (50 nm radius). Analysis of the emission spectra indicated that halothane preferentially quenches the long-wavelength-emitting tryptophans. Figure 2 shows the emission spectrum of tryptophan in the presence of halothane over a range of concentrations (0.1 mmol/L to 20 mmol/L). A large fraction of the accessible tryptophan residues is extensively quenched at halothane concentrations as low as 0.1 mmol/L. At 10 mmol/L halothane, the fluorescent signal was almost entirely quenched. The emission maximum of tryptophan in DOPC LUV occurred at 335 nm, indicative of a partial hydrophobic-residue environment. The quenching of tryptophan in DOPC LUVS resulted in a shift of the \(\lambda_{max}\) to longer wavelengths. Normalization of the spectra shows that the \(\lambda_{max}\) shifted by 20 nm to longer wavelengths (335 nm to 355 nm) (inset of Fig. 2). The small feature at 450 nm is likely a halogenated photoproduct. The integrated emission intensities of the \(\lambda_{max}\) feature were plotted vs. the quencher concentration (Fig. 3) and analyzed according to eq. [2]. From the value of the slope, the Stern–Volmer constant, \(K_{SV}\), was calculated to be 66 M\(^{-1}\).

Fluorescence quenching in SDS micelles

Since the LUV work showed that direct quenching of the ion channel by halothane is possible, we decided to investigate the direct halothane–peptide interaction in SDS micelles. Halothane concentrations ranged from 0.1 mmol/L to 20 mmol/L. The emission spectrum of gramicidin D in the presence of halothane at different concentrations is shown in Fig. 4. The emission maximum of gramicidin D occurred at 334 nm in the absence of halothane. At 20 mmol/L halothane concentration, 56% of tryptophan emission was quenched. Quenching of the fluorescence by higher halothane concentrations was accompanied by a significant shift of the emission maximum to shorter wavelengths. Normalization of the emission spectra, as shown in the inset of Fig. 4, revealed that greatest effects on the position of the
Fig. 2. Quenching of gramicidin D by halothane present at different concentrations in DOPC LUVs. The tryptophan $\lambda_{\text{max}}$ in DOPC LUVs was at 335 nm in the absence of the anesthetic ($\lambda_{\text{ex}} = 280$ nm). Halothane was used at 0.1, 0.5, 1, 10, and 20 mmol/L. The normalized spectra are shown in the inset. Note the shift in $\lambda_{\text{max}}$ to longer wavelengths. The prominent shoulder at ~450 nm is likely a photoproduc.

Fig. 3. Stern–Volmer plot for the quenching of gramicidin in DOPC LUVs. The calculated $K_{SV}$ was 66 M$^{-1}$, using eq. [2].
λ\textsubscript{max} was observed at 10 mmol/L and higher halothane concentration. At these concentrations, λ\textsubscript{max} shifted 22 nm to shorter wavelength (334 nm to 312 nm) because of an increased influence of the SDS micelle CH Raman-scattering peak (26). At 5 mmol/L halothane, λ\textsubscript{max} shifted only 5 nm to shorter wavelengths, whereas at lower concentrations (0.1 mmol/L to 1 mmol/L), the emission maximum remained unchanged at 334 nm. Similar to the study in LUVS, the Stern–Volmer plot was linear (Fig. 5). The K\textsubscript{SV} calculated using eq. [2] was 22 M\textsuperscript{-1}.

Quenching of gramicidin D in octanol
To ascertain if conformation can affect halothane–gramicidin interactions, we investigated halothane quenching in 1-octanol. Gramicidin can be present in different conformers, depending on the organic solvent used (14). An equilibrium mixture of both parallel and antiparallel double helices is present in octanol, and above the dimerization constant for gramicidin, the predominant conformer is expected to be the left-handed antiparallel double helix (14). In our 1-octanol study, gramicidin concentration was below the peptide-dimerization constant (2 \(\mu\)mol/L). As a result, gramicidin monomers were almost exclusively present in solution.

Figure 6 shows the emission spectrum of gramicidin D in 1-octanol in the presence of 0.2 mol/L to 1.2 mol/L halothane. Quenching of the emission maximum at 340 nm increased with increasing halothane concentration. A blue-shifted peak in octanol appears after halothane is added as evidenced by an additional feature at 0.2 mol/L halothane (Fig. 6). By 0.4 mol/L halothane, the emission spectrum has shifted entirely over to the feature at 310 nm, which then decreases in intensity with increasing halothane. This feature is Raman-scattering peak for OH stretch (3300 cm\textsuperscript{-1}) of 1-octanol (26, 27), which was revealed as the fluorescence of tryptophan was quenched. Plotting of the integrated emission intensity vs. quencher concentrations yielded a linear Stern–Volmer plot with a K\textsubscript{SV} value of 6 M\textsuperscript{-1} determined using eq. [1] (Fig. 7).

Discussion
To elucidate some of the molecular details of anesthetic–membrane protein interactions, we studied the halothane-induced quenching of tryptophan fluorescence in gramicidin. Gramicidin has been shown to create an ion channel through the association of two monomers, each of which spans one leaflet in a lipid bilayer. Each of the four tryptophan residues per monomer has a unique orientation in the lipid bilayer as indicated by several studies (28–31) (see Fig. 1). Tryptophan at position 9 is buried deepest in the membrane, and the remaining three tryptophan residues are located closer to the lipid–aqueous phase interface. The quenching experiments in LUV environments revealed that most tryptophans were equally accessible to halothane, independent of the anesthetic concentrations used, otherwise a sublinear Stern–Volmer plot would result. This suggests that the lipid bilayer mediates anesthetic–gramicidin interactions. The quenching was accompanied by a slight shift of λ\textsubscript{max} to longer wavelengths. This shift may be the result of halothane increasing the net polarity within the bilayer such that the remaining unquenched tryptophans experience a slightly more-polar environment.

The previous observations agree with findings from recent molecular-dynamics-simulations studies of the gramicidin–halothane system in a fully hydrated DMPC bilayer (20). In this study, Tang et al. showed that halothane targeted the anchoring residues at the channel–lipid interface. Halothane molecules (6 out of 10) were found to migrate towards the
membrane interface. It has been previously shown that this interaction requires gramicidin to be in the channel conformation promoted by the lipid environment (18, 32, 33). Tang et al. (18) found that tryptophan 9 and 15 create an amphiphilic pocket in the space between the side chain and lipid headgroups. Such an amphiphilic pocket is an optimal binding site for the slightly polar anesthetic molecule, halothane. Since we saw evidence of a direct peptide–anesthetic interaction, we were compelled to investigate the halothane–gramicidin system in SDS micelles. As in the case of DOPC LUVS, halothane was found to interact with tryptophan when brought in close proximity in SDS micelles, as indicated by tryptophan fluorescence quenching. Preferential partitioning of halothane at the solvent–micelle interface has been previously reported (25), which suggests that there may be a selective quenching of the tryptophans. Indeed, there appears to be enhanced quenching of the red portion of fluorescence spectrum at lower halothane concentrations in Fig. 3.

Significantly, Tang et al. observed a similar tryptophan-residue arrangement in SDS micelles (18). Moreover, our observation that the preferred interaction between halothane and the tryptophan in gramicidin occurs near the micelle–solvent interface region is corroborated by earlier studies by others (34, 35). Using $^{19}$F NMR, Yoshida et al. (34) showed that halothane interacted with SDS micelles at the interfacial region. A later study by Yoshino et al. (35), using the $^{19}$F NMR, $^1$H NMR, and NOE, further confirmed that halothane interacted with the hydrophilic moieties of the SDS micelles.

The quenching of gramicidin in octanol also resulted in a blue shift of the emission maximum. However, in this case the blue-shifted peak arises from Raman scattering.

To gain further insight into the anesthetic binding affinity to gramicidin in its ion channel, we analyzed the quenching data using Stern–Volmer plots. In DOPC LUVS, the Stern–Volmer plot revealed a linear relationship between halothane concentration and the amount of fluorescence quenching. From the slope, the $K_{SV}$ value for the anesthetic–peptide interaction was determined to be 66 M$^{-1}$. The Stern–Volmer plot for the SDS experiments was also linear. In SDS micelles, the $K_{SV}$ value was found to be 22 M$^{-1}$.

The presence of the lipid bilayer increased the interaction of halothane by approximately 3-fold (66 M$^{-1}$ in DOPC LUVS vs. 22 M$^{-1}$ in SDS micelles). This increased quenching may lay in different accessibilities of the tryptophans to halothane in the different environments (lipid bilayer vs. micelle).

In organic solvents, gramicidin is present in its non-channel conformation. The different conformation of gramicidin did not prevent halothane quenching from occurring. In the case of 1-octanol, relatively high halothane concentrations (0.1 mol/L to 1.2 mol/L) were required to see an effect on tryptophan emission. The $K_{SV}$ value in 1-octanol is 6 M$^{-1}$, which is one order of magnitude lower than the value obtained in DOPC LUVs.

The Stern–Volmer quenching constants measured in the current study are similar to those measured for halothane interactions with plasma membrane Ca$^{2+}$-ATPase (36). However, in contrast to the study by Lopez and Kosk-Kosicka (36), we observe a 3-fold difference between lipid and detergent environments. The fact that the lowest $K_{SV}$ was observed in octanol, which also has the lowest viscosity ($\eta_{oct} = 0.07$ P vs. $\eta_{DOPC} = 0.8$ P; 1 P = 1 dyn s/cm$^2 = 0.1$ Pa s), suggests that the quenching is not collisional. Interestingly, the quenching of gramicidin in POPC liposomes by acrylamide produced a $K_{SV}$ of 0.95 M$^{-1}$ (28). Because acrylamide is an aqueous quencher, it is not expected to partition significantly into the bilayer. Thus, the quenching from the study of Rawat et al. (28) was suggested to be collisional, and therefore the Stern–Volmer constant may not be directly comparable to the one determined in the present study.

The value of $K_{SV}$ for halothane–gramicidin interactions in DOPC found in the present work suggests a free energy of binding $\Delta G_{association}$ $\leq -$10 kJ/mol, which is in the range of
van der Waals bonding. The differences between the $K_{SV}$'s measured here for different environments should reflect the changes in free energy between free and bound halothane in DOPC vs. SDS vs. octanol.

Conclusions

Fluorescence-quenching experiments of gramicidin D in DOPC LUVS have shown that halothane can directly interact with gramicidin. Halothane quenched the fluorescence from tryptophan residues located at the lipid bilayer – aqueous interfaces as good as those located deeper in the bilayer. The partition of halothane in the vesicles may cause an increase in the local polarity as shown by the shift in the tryptophan emission maximum to longer wavelengths. SDS-quenching data revealed that the tryptophan residues located at the micelle–solvent interface were preferentially quenched by halothane. Tryptophan residues located deeper in the bilayer remained partly inaccessible to halothane. The effect of the non-channel conformation on halothane binding to the peptide was investigated in 1-octanol. In 1-octanol, higher anesthetic concentrations were required to detect changes in tryptophan fluorescence emission. Fluorescence quenching was observed, but with a lower $K_{SV}$ value than in DOPC LUVS and SDS micelles.

Taken together, our data suggest that halothane can interact more strongly with gramicidin in the conformation promoted by the lipid bilayer. In turn, this suggests that the lipid matrix may be an important determinant in creating the optimal motif for inhaled-anesthetics binding to other membrane proteins.

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References