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The Role of Structured Water in Mediating General Anesthetic Action on $\alpha 4\beta 2$ nAChR

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Abstract

Water is an essential component for many biological processes. Pauling proposed that water might play a critical role in general anesthesia by forming water clathrates around anesthetic molecules. To examine potential involvement of water in general anesthesia, we analyzed water within $\alpha 4\beta 2$ nAChR, a putative protein target hypersensitive to volatile anesthetics. Experimental structure-derived closed- and open-channel nAChR systems in a fully hydrated lipid bilayer were examined using all-atom molecular dynamics simulations. At the majority of binding sites in $\alpha 4\beta 2$ nAChR, halothane replaced the slow-exchanging water molecules and caused a regional water population decrease. Only two binding sites had an increased quantity of water in the presence of halothane, where water arrangements resemble clathrate-like structures. The small number of such clathrate-like water clusters suggests that the formation of water clathrates is unlikely to be a primary cause for anesthesia. Despite the decrease in water population at most of the halothane binding sites, the number of sites that can be occupied transiently by water is actually increased in the presence of halothane. Many of these water sites were located between two subunits or in regions containing agonist binding sites or critical structural elements for transducing agonist binding to channel gating. Changes in water sites in the presence of halothane affected water-mediated protein-protein interactions and the protein dynamics, which can have direct impact on protein function. Collectively, water contributes to the action of anesthetics in proteins by mediating interactions between protein subunits and altering protein dynamics, instead of forming water clathrates around anesthetics.

Keywords

anesthesia mechanism; halothane; nicotinic acetylcholine receptor; $\alpha 4\beta 2$; water; water clathrates

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Supporting Information Available: A graph of the total quantities of transiently occupied water sites in the control and halothane systems (Figure S1).

Introduction

Water is considered one of the most fundamental components of any biomolecular process.¹ Protein motions that drive and mediate protein functions are often intimately linked to motions of the surrounding water.^{2–5} In the absence of water, even correctly folded proteins cannot function properly until the hydration level reaches a critical value.^{6, 7}

A potential role of water in general anesthesia was proposed independently by Pauling and Miller nearly 50 years ago.^{8, 9} Pauling attributed anesthesia to the formation in the brain of minute hydrate crystals of the clathrate type.⁸ Miller hypothesized that anesthetics could increase the “ice cover” at a membrane or the surface of a protein.⁹ The formation of water clathrates or ice cover in the brain affected nerve conduction and transmission, leading to an anesthesia state.^{8,9} The proposals stemmed from the fact that many volatile anesthetics form clathrate (gas hydrate) structures at low temperatures.^{10, 11} Moreover, there is a good correlation between the anesthetizing partial pressure of non-hydrogen-bonding anesthetics and the disassociation pressure of the hydrate at 0°C.⁸ A great challenge to validate Pauling and Miller’s theory, however, is the lack of evidence that anesthetic hydrates or “ice cover” exist in a biological system at a physiologically relevant temperature.

Perhaps one should not interpret the role of water in general anesthesia solely based on the face value of Pauling or Miller’s original proposal. Intuitively, water clathrates or “icebergs” could not sustain body temperature. However, even not in an ice or clathrate structure, water can be affected by anesthetics, and anesthetic-induced changes in water in a critical region or site of a biological system can impact the function of the system. To gain insights into anesthetic modulation of water that has implication in general anesthesia, we carefully inspected water in one of the molecular targets of general anesthetics, the neuronal $\alpha 4\beta 2$ nicotinic acetylcholine receptor (nAChR), in the absence and presence of the volatile anesthetic halothane. The Cryo-EM structure-derived closed- and open-channel systems in a fully hydrated ternary membrane bilayer^{12, 13} were investigated by multiple molecular dynamics simulations. The $\alpha 4\beta 2$ nAChR is a pentameric neurotransmitter-gated ion channel. Each subunit of the pentamer channel has a large extracellular (EC) domain, four transmembrane (TM) helices, and an intracellular domain. Agonist binding to the EC domain triggers the channel opening and allows cations to pass through the channel. Previous experiments indicated that the channel function of the $\alpha 4\beta 2$ nAChR could be inhibited by halothane.^{14–17} Our recent MD simulations demonstrated that halothane altered the global motion of the $\alpha 4\beta 2$ nAChR more in the open channel conformation than in the closed channel conformation,^{12, 13} suggesting that anesthetic inhibition to the $\alpha 4\beta 2$ nAChR function might result from the anesthetic perturbation to the motion of the open channel. It is possible that water facilitates the anesthetic disturbance to the protein global motions that are essential for protein function.^{12, 18–20}

The implication of water in the action of general anesthesia is only one of many theories that deserve close attention. The classical view of non-specific lipid perturbation theory has become disfavored, but anesthetic induced lateral pressure changes in membrane is still viewed as a possible mechanism of anesthetic action.²¹ Overwhelming evidence suggests that ion channels, particularly the post-synaptic neurotransmitter-gated receptors like $\alpha 4\beta 2$ nAChR, can be modulated by general anesthetics. Three schools of thought prevail in the development of protein theories: the first believes that a very small number of targets in the central nervous system and a finite number of anesthetic binding pockets in these targets are responsible for general anesthesia.^{22, 23} The second argues that a structurally diverse array of general anesthetic molecules necessitate a multiple-site, multi-mechanism viewpoint.²⁴ The third theorizes that the unifying action of all general anesthetics on all proteins is the modulation of protein global dynamics on the timescale that matches the timescale of the

particular protein function under investigation.^{12, 18, 20, 25, 26} We believe that water plays a central role in mediating the general anesthetic action on protein global dynamics.

In this study, our focus was placed on how halothane affected the distribution and dynamics of water within the protein, and how changes in water properties disturbed the structure and dynamics of the $\alpha 4\beta 2$ nAChR. For a fully hydrated channel protein, such as the $\alpha 4\beta 2$ nAChR in Figure 1, water can be classified into at least four categories (bulk, surface, channel, and internal) based on their locations in the system, albeit no clear boundaries can be defined between the categories. The majority of water molecules belong to the bulk solvent, which has nearly uniform physical properties and little or no interaction with the protein. The surface water is defined as a water layer (or shell) of 7–10 Å thick, interacting directly with the surface of the protein.^{27, 28} The surface water has distinct physical properties from the bulk water.^{29, 30} It has been the subject of investigations related to protein diffusion³¹ and dynamics.^{32, 33} The water within the pore of the protein, or in short, the channel water, has smaller self-diffusion coefficients and rotational reorientation rates than the bulk water.³⁴ The geometry and flexibility of the pore can have a strong influence on the permeation of ions and the channel water.³⁵ The internal water, also referred to as structural water, has direct contacts with the protein and exchanges with bulk water slower than any other class of water. The internal water molecules can be an integral part of the protein structure and are often as conserved as the amino acids themselves.³⁶ Sometimes, the internal water molecules can even be captured by X-ray crystallography.² Because of their integration with the protein structure and their proximity to the halothane binding sites in the $\alpha 4\beta 2$ nAChR, the internal water was the primary focus in the current study.

Results and Discussion

Three scenarios of water distribution at halothane binding sites

The halothane binding sites and the corresponding sites in the control system are numbered and highlighted in Figure 2. Within the immediate vicinity of the halothane-binding sites, water behaved differently between the control and halothane systems. Among a total of 12 halothane sites in the open- and closed-channel $\alpha 4\beta 2$ nAChRs, there were three scenarios in terms of halothane effect on local water. First, because of its hydrophobic nature, site 4_{closed} had a negligible number of water molecules within a 7-Å radius of the binding site before and after halothane binding. It is not surprising to see a relatively low probability (1/12, 8%) for this scenario, considering that anesthetics prefer an amphiphilic environment.^{20, 37–39} Second, the number of water molecules within 7 Å from the center of site 6_{closed} and site 2_{open} (2/12, 17%) increased slightly. A close examination at these two sites revealed some rearrangement of side chains and backbone atoms of the residues involved in halothane binding. Such local structural rearrangements provided more space for both halothane and additional water. Third, halothane displaced or repelled water near the binding sites, causing a significant reduction in local water population. The majority (9/12, 75%) of halothane sites fell into this scenario.

Clathrate hydrates at halothane sites

In each of the two halothane sites where water population increased, structured water clusters were observed. As shown in Figure 3, a small cluster of water, held together by hydrogen bonding, was observed for 10 ps in the MD simulation of the control system. For the same site in the halothane system, a larger cluster of water was formed around hal-2_{open} and remained in place for as long as 40 ps in the simulation. Although the hydrogen-bonding pattern of the water surrounding hal-2_{open} appeared similar to the fused rings of a clathrate, it is far from complete when compared to the clathrate structures proposed by Pauling and Miller.^{8, 9} The lifetime of these water clusters also seemed too transient to offer a

sustainable effect as expected by Pauling and Miller.^{8, 9} In addition, the probability to form a water cluster as that observed around hal-2_{open} is low. Because most halothane sites experienced local water reduction, there were not enough water molecules around halothane to form an ordered water structure. Overall, clathrate water structures are unlikely to exist at a physiologically relevant temperature and they cannot play a significant role in anesthetic action.

In a separate simulation where halothane was placed in a small water box, representing bulk water, we noticed (by visual inspection) that water molecules surrounding halothane were not as well ordered as those around hal-2_{open} over the same 10-ps simulation (Figure 3D). The protein certainly provided a protection for the order and lifetime of the observed cluster structures.

Water reduction at halothane binding sites

Water reduction was observed for the majority halothane-binding sites. This comes as no surprise, as displacement of water from a polyamphiphilic binding site has been proposed as a favorable step in ligand binding.⁴⁰ As an example, Figure 4 provides quantitative comparisons of water at site 3_{closed} in the absence and presence of halothane over the last 5-ns MD simulations. The total number of water molecules at site 3_{closed} was reduced by ~60% after halothane binding (Figure 4A). The residence time of water molecules also decreased substantially (Figure 4B). Both data in the halothane and control systems could be fit well with a triple exponential function, resulting in three characteristic water residence times and relative populations of each water type for each system. As summarized in Table 1, the population of long residence water and the residence time are considerable lower in the halothane system than in the control system, suggesting that the replacement of long residence water molecules by hal-3_{closed} was the major cause of local water reduction at site 3_{closed}.

The water molecules with long residence times often participated in coordinating local structures. Figure 5 shows two snapshots toward the end of the 20-ns simulation, exhibiting different water population and residence time at site 3_{closed} in the absence and presence of halothane. Water-mediated interactions between a POPC lipid molecule and the pre-TM1 linker or the Cys loop in the EC domain can be found in Figure 5A. There were also multiple water molecules making connections between the TM2–TM3 linker and the Cys loop. In contrast, water-mediated hydrogen bonding along with total number of water at this site became much fewer in the presence of halothane, which is consistent with a previous experimental finding that halothane can disrupt hydrogen bonding in model proteins.⁴¹

Number of water sites increased in the halothane systems

The evidence is compelling that the overall water population and residence time in the areas adjacent to halothane sites were decreased, and water-mediated protein-protein or protein-lipid interactions were perturbed by halothane. A more complete picture of this perturbation requires an evaluation of the coupling between water modulation and the global effects of halothane on protein structure and dynamics. The changes on a local scale often propagate onto a global scale in order to make a functional impact.^{12, 13} Examining global water distributions may be more informative than focusing on specific molecules.⁴² Therefore, we evaluated global changes in the context of water dynamics using a recently developed software tool, named HOP.⁴³

HOP analysis revealed a significant disparity between the total quantities of transiently occupied water sites in the control and halothane systems. Over the course of last 5-ns simulations (from 15 to 20-ns simulations), there were ~900 and ~700 water sites (defined

by the composite water density being 2.71 times larger than the bulk water density) for the closed conformation in the presence and absence of halothane, respectively. The same trend was also observed for the open-channel system, in which the number of water sites was ~1000 and ~850 in the presence and absence of halothane, respectively (Figure S1). Clearly, the open systems contained more water sites than the closed systems. More interestingly, the halothane systems had more water sites than the control systems during this time period. As observed previously,¹³ the open-channel system is also more susceptible to modulation by halothane, and the difference in the number of water sites became apparent in an earlier stage of the simulation. At first glance, the increase in the number water sites in the halothane systems contradicts the observed reduction in the number of water molecules around the halothane binding sites. It is important to recognize that the number of transiently occupied water sites is not a measure of the amount of water, but instead represents the integrated positions of the water found within and around the protein. The transiently occupied water sites are strongly coupled with the motions (or dynamics) of water molecules over the course of the simulation.

Where were those extra water sites located in the halothane systems? Because of the inherent flexibility of the $\alpha 4\beta 2$ nAChR, it is difficult to compare precise geometric positions of transiently occupied water sites between the halothane and control systems. Thus, we compared the density of water sites as a function of the Z-coordinate (parallel to the channel axis) of the systems. As illustrated in Figure 6, if dismissing the variations in the particularly flexible tip regions, the section between Z= 0 to 20 Å for the open system or slightly lower Z coordinates for the closed system stands out and exhibits a substantial increase in transiently occupied water sites in the halothane systems.

Several features about the water sites are worth mentioning. First, the region having a significant increase in water sites contains agonist binding sites and critical structural elements for transducing agonist binding to channel gating.⁴⁴ Disrupting the communication between the agonist binding sites and the hydrophobic gate of the channel, located around z= 35 Å (Figure 6)^{45–48} may be sufficient to disrupt the normal function of the channel. Second, any halothane effect on water sites must be allosteric because several halothane molecules, except dehydrated site 4_{closed}, were near but not in the region having large changes in water sites. Third, many water sites existed between different subunits rather than within a subunit, indicating potential water modulation of quaternary structure of the $\alpha 4\beta 2$ nAChR. Fourth, small but significant decrease in backbone flexibility, as measured by root-mean-squared fluctuation (RMSF), was observed for the β -sheets in the same region of the open system.

Figure 7 illustrates most of these features. The aforementioned region at the interface of two $\beta 2$ subunits near halothane site 6_{open} had almost twice as many water sites as in the same region of the control system (22 vs. 14). It appeared that rearrangement of the interfacial loops in the halothane system reduced the space between the two subunits. Although the total number of water molecules was ~ 20% less in a smaller gap between the subunits, water-mediated polar interactions made water more stationary, creating more water sites and water-mediated protein interactions between two subunits. Such water-mediated protein-protein interactions are believed more common than direct hydrogen bonding in soluble proteins.⁴⁹ Changes in water sites and water-mediated protein-protein interactions certainly affected the protein dynamics. As shown in Figure 7, the backbone RMSF reduction was observed for many residues at this interface of the halothane system, demonstrating that protein flexibility is correlated with the behavior of the surrounding water.⁵⁰

Systems and Methods

Our MD simulations on the open- and closed-channel $\alpha 4\beta 2$ nAChR with and without anesthetic halothane^{12, 13} were used for the water analysis in study. Details of system preparations and MD simulations were provided in our previous publications.^{12, 13, 45} In short, the open- and closed-channel structures of the $\alpha 4\beta 2$ nAChR were individually embedded in a pre-equilibrated ternary lipid bilayer⁵¹ and TIP3P water. Each system also contained sodium and chloride ions to neutralize the overall charge and to obtain a final ion concentration of 200 mM. For halothane systems, six halothane molecules were positioned in the prepared open- or closed-channel control systems based on the experimental suggestions, docking, and MD simulations. These four systems were each simulated for over 20 ns using NAMD2.6⁵² at the Pittsburgh Supercomputing Center. The CHARMM-27 force field⁵³ was used for all molecules except halothane, for which we used our previously developed parameters.⁵⁴

VMD⁵⁵ and its associated TCL scripts were used for analyzing local water and protein dynamics and making protein figures. For local water analysis, a sphere of 7-Å radius was used to select the water molecules at each halothane site. The size of the sphere was chosen to encompass all water that contacted a freely rotating halothane molecule. This sphere was located at the center of each halothane-binding site. The center of a binding site was recalculated every frame based on the center of mass of all protein residues within 8 Å from the halothane molecule in the halothane system. Using these criteria to define a site allows for the same selection of protein residues to be used for the control system where halothane was absent. For every unique water molecule within the 7-Å sphere, the residence time was determined based on how many frames that particular water molecule was within the sphere. Gnuplot 4.3 and two of its smoothing algorithms, kernel density (kdensity) and natural smoothing spline (acsplines), were utilized for generating graphs and plots in water analysis. Water dynamics was analyzed using the software tool HOP (<http://sbcb.bioch.ox.ac.uk/oliver/software/#Hop>)⁴³ that read the MD trajectory files using MD-Analysis python framework.⁵⁶ HOP analysis provides average positions of transiently occupied water sites. For each of the four systems examined, all frames in the last 5 ns of the simulation trajectories were aligned by the protein backbone. The composite water densities within the protein were calculated based on the oxygen atom of water molecules no more than 3.5 Å away from the protein. Based on work by Beckstein, et al., water sites were considered only if the water density was at least 2.71 times greater than the density of the bulk water in the system.⁴³

Conclusions

At least two major conclusions can be drawn from the current study. First, long-lived water clathrates could not form around halothane in the $\alpha 4\beta 2$ nAChR system. A few hydrogen-bound water clusters were found near halothane, but they resembled only portions of the clathrate structures and had only transient lifetime (< nanosecond). Our results discourage the conviction of Pauling and Miller that the formation of hydrated crystals of the clathrate structures is the cause of general anesthesia.^{8, 9}

Secondly, water plays an important role in halothane-induced changes in protein global dynamics. As shown in our study, most halothane-binding sites exhibited reduction of local water population, shortening of water residence time, and reorientations of protein side chains or adjacent lipid molecules. These local changes are often allosteric. Water has the ability to propagate these local changes to a more global scale through altering water-mediated protein-protein interactions, especially at the interface between two subunits.

We want to emphasize that changing water-mediated protein-protein interactions initiated by anesthetics is unlikely a sole mechanism for anesthetic action in relevant molecular targets. Anesthetics in soluble phase of proteins, such as the extracellular domains of $\alpha 4\beta 2$ nAChR, have more weight on water-mediated anesthetic effects. However, this should not be the reason to negate possible functional role of anesthetics bound to the protein sites where water is scarce. Anesthetic disruptions to protein dynamics, ultimately to protein functions, do not always involve water mediation.^{12, 13, 20} Nevertheless, the work presented here may increase awareness of water-mediated anesthetic effects in the mechanistic underpinnings of anesthetic action in biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Four types of water in a typical ion channel system: (1) Bulk water (purple) is far from the protein and lipid; (2) surface water (green) separates protein and lipid from bulk water; (3) channel water (yellow) is within the pore; and (4) structural water (red) is non-channel water within the protein. Structural water is the focus of the present study. For clarity, two front most subunits of the protein (gray cartoon representation) are removed, and only an 8-Å thick slice of the membrane (gray lines) and water are displayed.

Figure 2. Three scenarios of halothane-induced changes in local water population in the (A) closed- and (B) open-channel $\alpha 4 \beta 2$ nAChR are color coded on halothane molecules: decreased (green); increased (red); and dehydrated (blue). Halothane binding sites are labeled and referred to in the text by the numbers shown in this figure. Local water population was reduced at the majority of sites in the halothane system compared with the control system. Two nicotine agonists in the open-channel system are in yellow.

Figure 3.

Details of water clustering at site 2 in the open-channel system. (A) Water positions at site 2_{open} of the control system integrated for 10 frames; (B) within 4 Å of hal-2_{open} (red) in the halothane system integrated for 10 frames; (C) the same as B but integrated for 40 frames; and (D) within 4 Å of halothane (gray) in bulk water integrated for 10 frames. Time interval between adjacent frames is 1 ps. To track water movement, the same color was used for each individual water in all frames. For clarity, a narrower selection criterion, 4 Å from halothane rather than 7 Å from the center of the site, was used for B-D.

Figure 4.

(A) Local water population at site 3_{closed} was less in the halothane system (dashed line) than in the control (solid line) system. The data were smoothed using acsplines with a uniform smoothing weight of 500. (B) Water molecules were ranked by their residence time at site 3_{closed} over the course of the last 5-ns simulations in the presence (dashed line) and absence (solid line) of halothane. The thin solid lines are the best fit to the data by a triple exponential function and yielded characteristic residence times of water and the relative populations as tabulated in Table 1.

Figure 5. Water molecules at site 3_{closed} in the control (A) and halothane (B) systems. Purple and cyan colors represent water being present at the site for more or less than half of the last 5 ns of the simulation, respectively and the gray wire frame indicates time-average positions of water. Hydrogen bonds between select side chains, lipids, and water are shown by dotted lines in magenta. Halothane is shown in green.

Figure 6.

Density of water sites along the channel axis, quantified by HOP analysis on the data collected over the last 5 ns of simulations, in (A) the closed- and (B) open-channel systems in the presence (dashed lines) and absence (solid lines) of halothane. The data in (A) and (B) were smoothed using the k-density smoothing algorithm with a uniform bandwidth of 3 for each water site. (C) A structure snapshot of $\alpha 4\beta 2$ nAChR on the same scale is provided for Z-coordinate reference with vertical boundaries for the lipid bilayer marked by thin horizontal lines.

Figure 7.

Water sites at a selected region of the interface between two $\beta 2$ subunits in the control (in yellow surface representation, 14 sites) and halothane (in cyan surface representation, 22 sites) systems of the open-channel $\alpha 4\beta 2$ nAChR. Note that $\text{hal-6}_{\text{open}}$ (green) is close to the interface. Compared to the control system (transparent cartoon structure in yellow), A loop and $\beta 6$ strand in the halothane system (opaque cartoon structure) moved closer to each other and reduced the gap between the two subunits. The backbone RMSF in the halothane system is reduced as color-coded in blue on the structure.

Table 1

Characteristic residence times, τ_c , of different water populations in the control and halothane systems. Values obtained by fitting a triple exponential function to the data in Figure 4B.

Control		Halothane	
Population (%)	τ_c (ps)	Population (%)	τ_c (ps)
40	7.5	50	1.4
35	159.3	31	19.2
25	1941.0	19	611.8