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Phospholipid Complexation of General Anesthetics in Fluid Bilayers

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Abstract

A nearest-neighbor recogntion analysis has been performed in cholesterol-rich and cholesterol-poor liposomes derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in the presence of varying concentrations of chloroform. This analysis has yielded a funamentally new, molecular-level view of the interaction of general anesthetics with lipid bilayers, which may be relevant to their biological action; that is, DPPC forms 1:1 complexes with $CHCl_3$ in both membranes in the fluid bilayer state.

General anesthesia is widely regarded as one of the most important advances in the history of medicine. Despite numerous attempts to clarify their mechanism of action, the question of whether general anesthetics use signaling proteins or the surrounding lipids as their primary target continues to be debated.[1,2] Similarly, whether signaling takes place in regions of cell membranes that are rich in cholesterol and sphingolipids (i.e., in hypothetical microdomains that have been termed, “lipid rafts”) also remains as a matter of debate.[3] One classic view of the mechanism of action of general anesthetics has been that they merely “dissolve” in, and loosen, lipid membranes. We now wish to report our discovery that such “dissolution” is far more interesting than previously realized; that is, phospholipids are capable of forming discrete complexes with volatile anesthetic agents.

In the course of investigating the effects of chloroform on lipid mixing in cholesterol-rich (liquid-ordered phase, l_o) and cholesterol-poor (liquid-disordered phase, l_d) bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) via the nearest-neighbor recognition (NNR) method, we obtained evidence for 1:1 complex formation between chloroform and DPPC. We also found greater sensitivity of lipid mixing towards the presence of $CHCl_3$ in the l_o. This paper documents our main findings.

As discussed elsewhere, NNR measurements take molecular-level snapshots of bilayer organization by detecting and quantifying the thermodynamic tendency of exchangeable monomers to become nearest-neighbors of one another.[4-7] Typically, two lipids of interest (**A** and **B**) are converted into exchangeable dimers (homodimers **AA** and **BB,** and heterodimer **AB**), which are then allowed to undergo monomer interchange via thiolate-disulfide exchange. The resulting equilibrium that is established is governed by an equilibrium constant, $K = [\mathbf{AB}]^2/([\mathbf{AA}][\mathbf{BB}])$. When monomers **A** and **B** mix ideally, this is reflected by an equilibrium constant that equals 4.0. When homo-associations are favored, the equilibrium constant is less than 4.0; favored hetero-associations are indicated by a value that is greater than 4.0. Taking statistical considerations into account, nearest-neighbor

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SUPPORTING INFORMATION. Experimental procedures and tables of data. This material is available free of charge via the Internet at http://pubs.acs.org.

interaction free energies between **A** and **B** are then given by $\omega_{AB} = -1/2\ RT\ \ln(K/4)$.[8] When using low concentrations of the exchangeable lipids **A** and **B** (Chart 1) in host membranes made from DPPC and cholesterol, as in the present study, NNR senses changes in compactness of the host membrane.[5]

In a recent NNR investigation, we examined the effects of $CHCl_3$ on liposomal membranes (~200 nm diameter, extrusion) made from DPPC and cholesterol in the l_o and l_d phases under conditions in which the dispersions were *saturated* with $CHCl_3$ and the molar ratio of membrane-bound $CHCl_3$/phospholipid was ca. 3.[9] In brief, we found that *K* was reduced from 9.20 ± 0.33 to 6.02 ± 0.28 for l_o bilayers upon exposure to $CHCl_3$; for l_d bilayers *K* increased from 4.01 ± 0.47 to 6.04 ± 0.18. These results, by themselves, implied that the presence of $CHCl_3$ produced a new membrane phase that we termed, the "liquid-anesthetic" (l_a) phase.

In the present study, we sought to gain a deeper understanding of this l_a phase by investigating the effects of *sub-saturation* concentrations of $CHCl_3$ on nearest-neighbor interactions in cholesterol-rich (l_o) and cholesterol-poor (l_d) bilayers.[10,11] Specifically, we sought to quantify nearest-neighbor interaction free energies between **A** and **B** as a function of the molar ratio of bound $CHCl_3$/DPPC for both types of membranes.

With this aim in mind, we carried out a series of $CHCl_3$-binding and NNR measurements with aqueous concentrations of $CHCl_3$ that ranged between 2 and 40 mM and liposomes (~200 nm) that were prepared by extrusion methods. For this purpose, a specially-designed reaction vessel was used in which the liposome dispersion and a fraction of the buffer were physically separated (Figure 1). Determination of excess chloroform in the dispersion then afforded molar ratios of bound $CHCl_3$/phospholipid. In addition, a separate compartment (i.e., an empty test tube) was used to volatilize $CHCl_3$ for those experiments requiring relatively high concentrations of the anesthetic. Direct injection of neat $CHCl_3$ into these dispersions was avoided because it resulted in partial precipitation of the lipids. For all of the experiments reported herein, the total volume of the aqueous phase and the total volume of the gas phase were held constant. All NNR measurements were made using liposomes containing 5 mol% of an equimolar mixture of **A** and **B** (i.e., 2.5 mol% **AB**) plus 95 mol% of a mixture of DPPC/cholesterol (57.5/37.5, mol/mol) or pure DPPC at 45°C. Binding measurements were made, independently, using 2.5 mol% of cholesterol and DPPG in place of **AB**. Our principal results are summarized in Figure 2 and Tables 1 and 2.

In accordance with our previous results that were obtained under saturation conditions, sterolphospholipid association in cholesterol-rich membranes was weakened in the presence of $CHCl_3$ (Figure 1).[9] For cholesterol-poor membranes, the exact opposite was observed; that is, sterol-phospholipid association was strengthened by $CHCl_3$. Plots of *K* and ω_{AB} as a function of the molar ratio of membrane-bound $CHCl_3$/phospholipid clearly show this dichotomy (Figure 2). In addition, changes in ω_{AB} in the l_o phase showed a greater sensitivity toward the presence of $CHCl_3$, especially at low $CHCl_3$/phospholipid ratios. This greater sensitivity can also be seen from the changes in ω_{AB} (i.e., $\Delta\omega_{AB}$), which are given in Tables 1 and 2. The fact that the values of ω_{AB} from both phases converge at a $CHCl_3$/phospholipid ratio of ca. 1 provides strong evidence that $CHCl_3$ preferentially binds to DPPC with a 1:1 stoichiometry. In addition, the ability of both liposomes to bind excess $CHCl_3$ is fully consistent with saturable and unsaturable binding behavior that has previously been reported for enflurane, where unsaturable binding appears to occur on the surface of the membrane.[12]

The greater sensitivity of ω_{AB} in the l_o phase toward $CHCl_3$ and the 1:1 $CHCl_3$-phospholipid stoichiometry required to achieve a maximum change in ω_{AB} for both the l_o

and l_d phases, together with previous Raman measurements showing an increase in gauche conformers in the presence of $CHCl_3$, lead us to propose a "drilling and filling" mechanism for the conversion of the l_o to the l_a phase (Figure 2).[9] Specifically, these results support a model in which a $CHCl_3$ molecule first inserts into the bilayer (i.e., drilling) creating void space due to a mismatch in geometry between the sphere-like anesthetic and the extended acyl chains of neighboring phospholipids. To maximize hydrophobic interactions, these acyl chains then fill in this space by forming gauche conformers, thereby triggering a cascade of additional filling events among neighboring lipids. In this stylized illustration, it should be noted that the $CHCl_3$ molecules that are complexed to the phospholipids have been arbitrarily positioned in the middle of the monolayer leaflet; their actual depth of penetration, however, remains to be established.

The concentrations of $CHCl_3$ that we have used in this study extend down to 2 mM, which is close to an EC_{50} value of ca. 1 mM that has been determined for $CHCl_3$ in humans, dogs, and mice; that is, the anesthetic concentration that is required to induce anesthesia in 50% of a population of humans, dogs, and mice.[13] At this concentration, the mole ratio of membrane bound $CHCl_3$/phospholipid in cholesterol-rich bilayers is 0.026, corresponding to a net reduction in ω_{AB} of 24.5 ± 5.5 cal/mole of lipid. Although theis energy change is very small, it should be noted that values of this magnitude are sufficient to cause a significant change in the lateral organization of lipid membranes.[8]

That $CHCl_3$ is not unique in its ability to form the l_a phase is evidenced by the fact that halothane (i.e., 2-bromo-2-chloro-1,1,1-trifluoroethane, a general anesthetic that is currently in use) showed very similar effects under saturation conditions; that is, it reduced *K* from 9.37 ± 0.04 (ω_{AB} = −268.8 ± 1.5 cal/mol) in cholesterol-rich membranes to 6.06 ± 0.06 (ω_{AB} = −131.1 ± 2.9 cal/mol); it also raised *K* in cholesterol-poor membranes from 4.16 ± 0 .04 (ω_{AB} = −12.7 ± 3.0 cal/mol) to a value of 5.84 ± 0.06 (ω_{AB} = −119.4 ± 3.2 cal/mol).

The mechanism by which general anesthetics act on neurons at the molecular level continues to be a subject of intense debate. At present, researchers in this field are divided among three groups: (i) those who favor classic lipid theory (i.e., where an anesthetic changes the structure of the lipid framework surrounding membrane proteins, and this change then leads to an alteration in the structure and activity of these proteins), (ii) those who favor protein theory (i.e., where the anesthetic changes the structure and activity of membrane proteins by interacting, directly, with them), and (iii) those who are waiting for more compelling evidence to appear before either of these theories can be accepted.[1,2,14-16] If the lipid theory of general anesthetics is ultimately proven to be correct, then the present findings indicate that phospholipid complexation is likely to play a significant role in their biological action. In this regard, it should be noted that the greater sensitivity of ω_{AB} that we have observed in the liquid-ordered phase is consistent with the notion that signaling occurs in cholesterol-rich regions of neural membranes; e.g., in putative lipid rafts. At a minimum, the present findings offer a fundamentally new, molecular-level perspective of the interaction of general anesthetics with lipid bilayers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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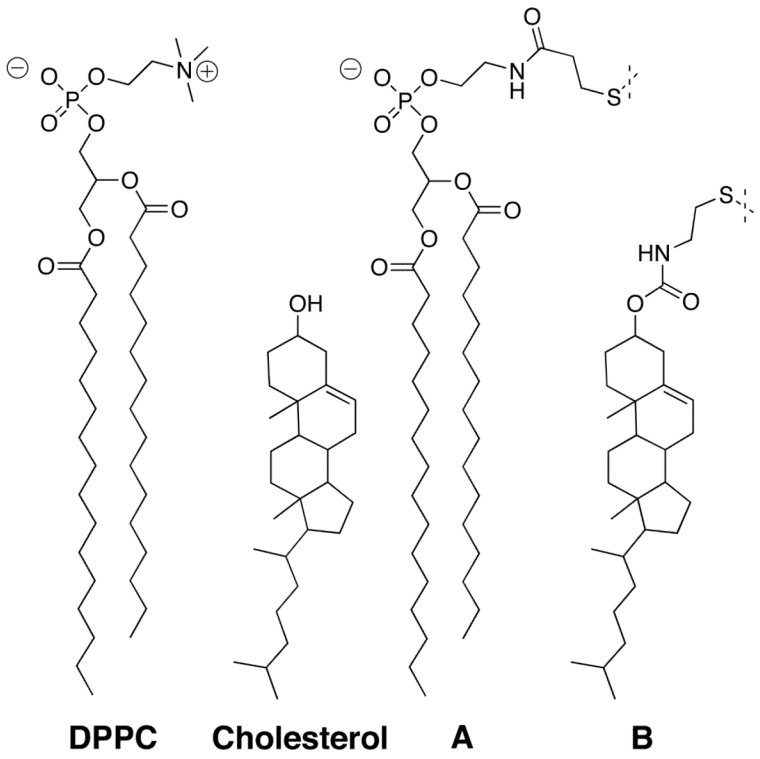

Chart 1.

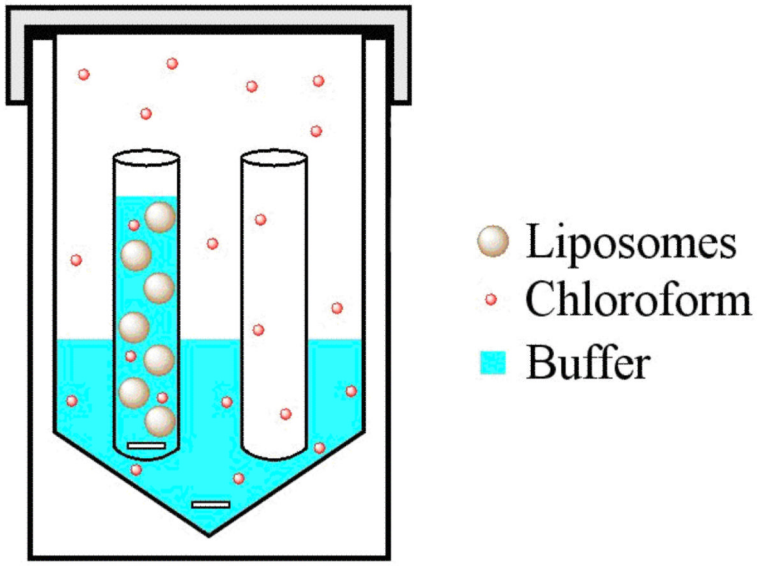

Figure 1.
Reaction vessel used for carrying out NNR and binding measurements.

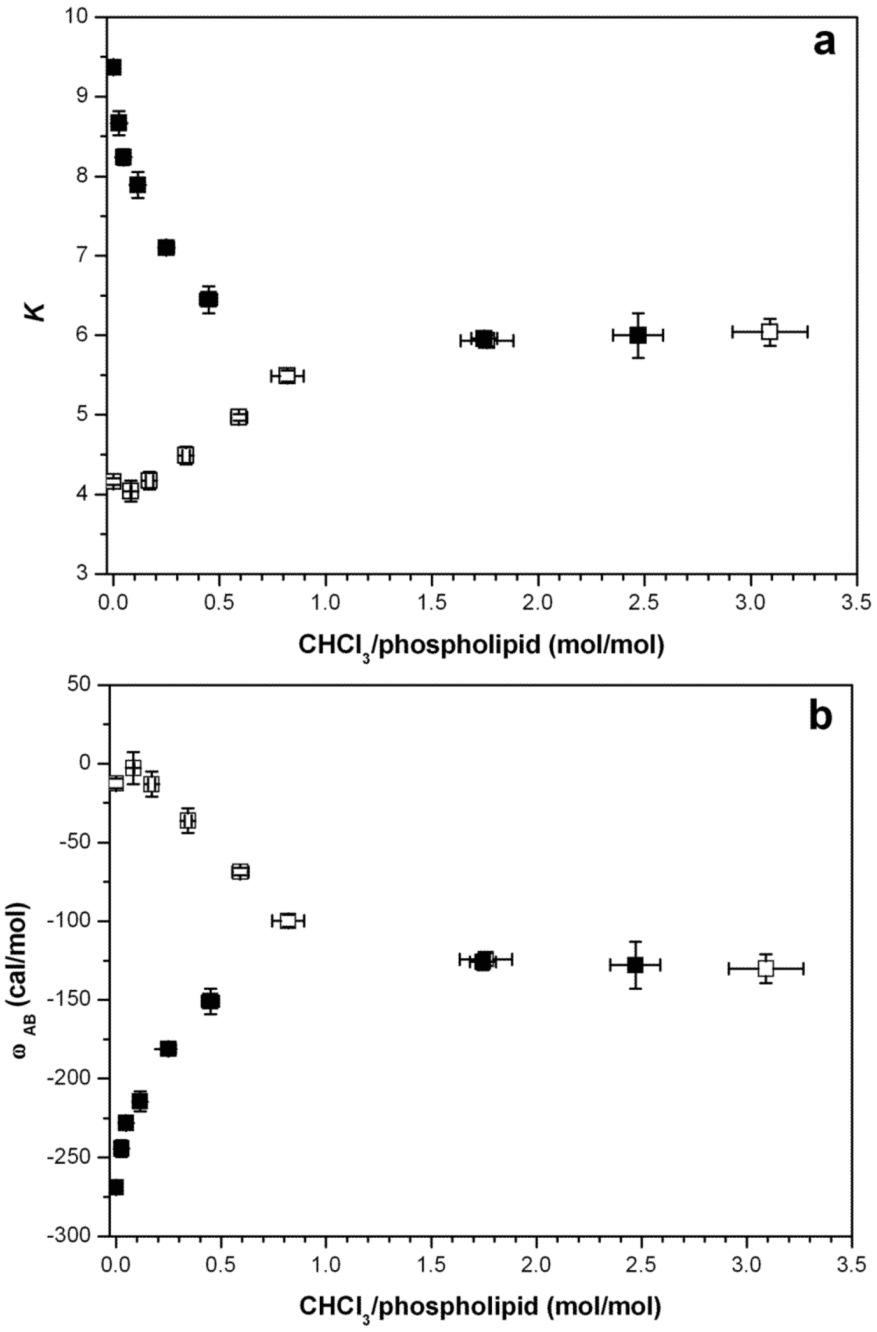

Figure 2.

Plots of (a) *K* and (b) ω_{AB} as a function of membrane-bound $CHCl_3$/phospholipid (mol/mol) in cholesterol-rich, l_o (■) and cholesterol-poor, l_d (□) bilayers. Error bars that are not visible lie within the symbols themselves. The highest ratio in each series were obtained under saturation conditions.[9]

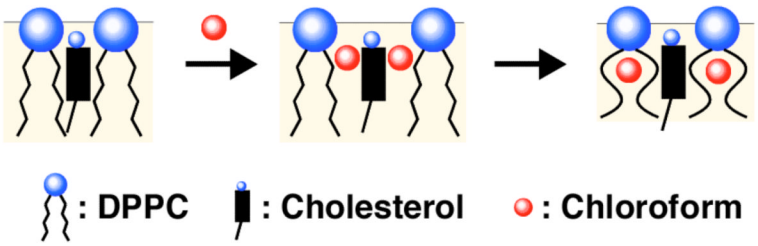

Figure 3.
A stylized illustration showing the insertion (i.e., "drilling") of two $CHCl_3$ molecules into a segment of a cholesterol-rich monolayer and simultaneous filling in of void space.

Table 1

$CHCl_3$-binding, *K*, and $\Delta\omega_{AB}$ values for cholesterol-rich liposomes.

$[CHCl_3]_{Buffer}$ (mM)	$n_{CHCl3}/n_{phospholipid}$ (×100)[a]	*K*	$\Delta\omega_{AB}$ (cal/mol)[b]
0.00	0.00	9.37 ± 0.04	---
2.04 ± 0.02	2.61 ± 0.23	8.67 ± 0.15	24.5 ± 5.5
4.16 ± 0.02	4.83 ± 0.38	8.24 ± 0.10	40.6 ± 4.1
8.24 ± 0.07	11.50 ± 1.52	7.89 ± 0.16	54.4 ± 6.6
15.92 ± 0.32	24.90 ± 3.56	7.10 ± 0.08	87.6 ± 3.7
22.40 ± 0.30	44.89 ± 3.98	6.45 ± 0.17	118.0 ± 8.3
39.51 ± 0.17	174.52 ± 6.08	5.96 ± 0.09	142.9 ± 5.2

[a] Molar ratio of membrane-bound CHCl3/phospholipid multiplied by 100.

[b] Change in ω_{AB} upon exposure to $CHCl_3$.

Table 2

$CHCl_3$-binding, *K*, and $\Delta\omega_{AB}$ values for cholesterol-poor liposomes.

$[CHCl_3]_{Buffer}$ (mM)	$n_{CHCl3}/n_{phospholipid}$ (×100)[a]	*K*	$\Delta\omega_{AB}$ (cal/mol)[b]
0.00	0.00	4.16 ± 0.04	---
1.82 ± 0.01	8.37 ± 0.20	4.04 ± 0.13	9.8 ± 10.7
3.97± 0.03	17.02 ± 1.73	4.17 ± 0.11	−0.4 ± 8.6
7.90 ± 0.16	34.31 ± 1.67	4.49 ± 0.11	−23.5 ± 8.3
14.84 ± 0.34	59.23 ± 3.81	4.97 ± 0.04	−56.0 ± 3.8
20.05 ± 0.69	81.97 ± 7.61	5.49 ± 0.07	−87.1 ± 4.8
37.56 ± 1.20	175.94 ± 12.44	5.93 ± 0.07	−111.6 ± 5.0

[a] Molar ratio of membrane-bound CHCl3/phospholipid multiplied by 100.

[b] Change in ω_{AB} upon exposure to $CHCl_3$.