J|A|C|S COMMUNICATIONS

Published on Web 03/23/2009

Loosening and Reorganization of Fluid Phospholipid Bilayers by Chloroform

Serhan Turkyilmaz, Wen-Hua Chen, Hideyuki Mitomo, and Steven L. Regen*

Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015

Received February 13, 2009; E-mail: slr0@lehigh.edu

The mechanism of action of general anesthetics is still open to debate.[1] One long-standing hypothesis is that they loosen cell membranes, resulting in "relaxed" and dysfunctional transmembrane proteins.[2] A second hypothesis is that they bind to receptors in transmembrane proteins.[2] Here we show that chloroform not only loosens cholesterol-rich and cholesterol-poor phospholipid membranes but also rearranges them. This finding suggests a fundamentally new mechanism of anesthesia, where the anesthetic, by solvating the lipid components, *profoundly changes the lateral organization of the lipid framework*.

To probe the influence of an anesthetic on lipid mixing, we employed the nearest-neighbor recognition (NNR) technique.[3,4] By virtue of its high sensitivity toward lipid−lipid associations, this method is ideally suited for quantifying such effects. As a first anesthetic, we selected chloroform because it is a moderately lipophilic molecule that should readily insert itself into the lipid bilayer. Given the prevalence of cholesterol and high-melting lipids in mammalian cell membranes and the strong effects that cholesterol is known to have on the structure of phospholipid bilayers, we examined the mixing of **A** with **B** in host bilayers made from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol (Figure 1).[3,5,6]

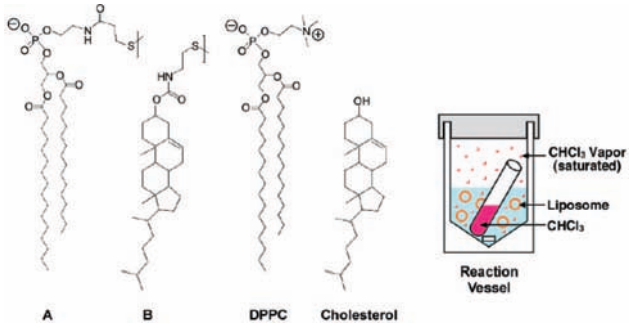


Figure 1. Lipids and reaction vessel used for NNR experiments.

As discussed elsewhere, NNR experiments 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] Typically, two lipids of interest (**A** and **B**) are converted into exchangeable dimers (**AA**, **AB**, and **BB**), which are then allowed to undergo monomer interchange via thiolate−disulfide exchange. The resulting equilibrium that is established, whereby one molecule of **AA** reacts with one molecule of **BB** to give two molecules of **AB**, is then governed by an equilibrium constant, $K = [\mathbf{AB}]^2/([\mathbf{AA}][\mathbf{BB}])$. Ideal mixing of monomers **A** and **B** is reflected by an equilibrium constant that equals 4.0. When homolipid associations are favored, the equilibrium constant is less than 4.0; favored heterolipid associations are indicated by a K value that is greater than 4.0. As shown previously, nearest-neighbor association between **A** and **B** in host membranes made from DPPC and cholesterol reflects the phase of the bilayer and becomes increasingly favored as one moves from the liquid-disordered (l_d) to the liquid-ordered (l_o) phase.[3a]

Mechanistic studies involving general anesthetics and lipid membranes are inherently complex because of partitioning of the anesthetic among the solution, gas, and membrane phases. To simplify our experiments, NNR exchange reactions were carried out under saturation conditions using sealed reaction vessels that contained an open tube of chloroform (Figure 1).

Through the use of procedures similar to those previously described, NNR reactions were carried out at 45 °C in the absence and in the presence of chloroform. All of the host liposomes (200 nm, extrusion) included 2.5 mol % **A**, 2.5 mol % **B**, and varying concentrations of cholesterol. Those bilayers having low (2.5 mol %) and high (32 or 40 mol %) sterol concentrations were in the l_d and l_o states, respectively; at intermediate concentrations (e.g., 20 mol %) they were in the l_o/l_d coexistence region.[7] To ensure that equilibrium product mixtures were obtained in each case, one set of experiments was carried out using pure heterodimer **AB** as the source of the exchangeable lipids and a second set used liposomes containing an equimolar mixture of the corresponding homodimers **AA** and **BB**.[8] Values of K were then calculated from the average dimer composition from both sets of experiments. Examination of particle sizes by dynamic light scattering (Nicomp) confirmed that these liposomes were stable under the conditions used for thiolate−disulfide interchange (see the Supporting Information).

Values of K for the four types of membranes in the absence and in the presence of chloroform are plotted in Figure 2. As is apparent, the presence of chloroform has a leveling effect on sterol−phospholipid mixing; that is, it increases K in cholesterol-poor membranes and decreases K in cholesterol-rich membranes. These results imply that the presence of chlorofom creates a common state that is characterized by moderate sterol−phospholipid affinity.

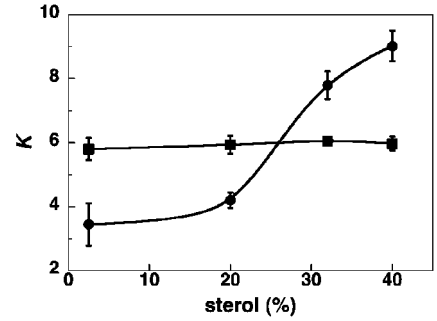


Figure 2. Plot of K versus sterol concentration in the (●) absence and (■) presence of a saturated chloroform atmosphere.

Further evidence for a common state was obtained by measuring the amount of chloroform that was absorbed by the liposomes under our experimental conditions. Thus, whereas 3.06, 2.42, and 1.48 molecules of chloroform were absorbed per lipid (DPPC + cholesterol) in liposomes containing 2.5, 20, and 40 mol %

10.1021/ja9011468 CCC: $40.75

cholesterol, respectively, the number per DPPC molecule (3.09, 3.03, and 2.47 chloroform molecules per phospholipid, respectively) was very similar for all three membranes. The fact that this number is practically constant implies that chloroform favors the solvation of DPPC and that this state of solvation is independent of the concentration of cholesterol that is present.[9]

To gain insight into the effects of chloroform on the compactness of these membranes, we measured the generalized polarization (GP) of a bound fluorescent probe (i.e., Laurdan) as a function of temperature.[10] As shown previously, Laurdan detects changes in membrane-phase properties by sensing the polarity of its microenvironment in the bilayer.[10] Specifically, variations in membrane water content induce shifts in the Laurdan emission spectrum that can be quantified by calculating its GP, which is given by GP = $(I_{440} - I_{490})/(I_{440} + I_{490})$, where I_{440} and I_{490} are emission intensities at 440 and 490 nm, respectively (λ_{ex} = 350 nm).

As shown in Figure 3A, in the absence of chloroform, cholesterol-poor membranes show a well-defined gel- to fluid-phase transition, with a transition temperature, T_m, of 41 °C. In sharp contrast, exposure to chloroform results in a fluidlike state that extends from above to below its T_m. It should be noted that the GP for Laurdan dissolved in chloroform is 0.66. For cholesterol-rich membranes, a relatively high level of compactness was found between 30 and 55 °C. When exposed to chloroform, the membrane's compactness shifted toward an *intermediate* level at all temperatures.

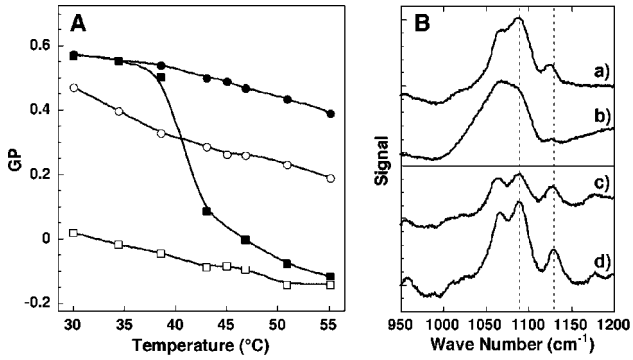


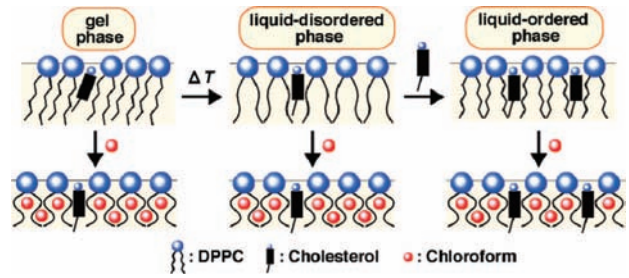
Figure 3. (A) Plots of GP versus temperature in liposomes made from DPPC/DPPG/cholesterol (97.5/2.5/2.5, mol %) without (■) and with (□) $CHCl_3$ and from DPPC/DPPG/cholesterol (57.5/2.5/40, mol %) without (●) and with (○) $CHCl_3$. (B) Raman spectra at 45 °C for liposomes made from (top frame) DPPC/DPPG/cholesterol (97.5/2.5/2.5, mol %) and (bottom frame) DPPC/DPPG/cholesterol (57.5/2.5/40, mol %) without (a and c) and with (b and d) $CHCl_3$.

Additional evidence for chloroform's strong fluidizing effect on the gel phase was obtained by NNR measurements. Thus, mixing of **A** with **B** in gel-phase liposomes (35 °C) derived from DPPC containing 2.5 mol % sterol strongly favored homolipid associations (i.e., K = 0.78). Upon exposure to chloroform, however, **A** and **B** became favored nearest neighbors at the same temperature (K = 5.3).

As a final confirmation that chloroform fluidizes cholesterol-rich and cholesterol-poor membranes, we measured the Raman spectrum of both types of membranes in the absence and in the presence of chloroform at 48 °C (Figure 3B). Here, the ratio of the 1130 and 1090 cm^{-1} band intensities reflects the conformation of the acyl chains of the phospholipids.[11] Specifically, the lower the ratio, the greater the number of gauche conformers. As is apparent, the presence of chloroform increases the number of gauche conformers in both types of membranes.

A simple model that takes all of these observations into account is illustrated in Scheme 1. Thus, when chloroform is absorbed either by the l_d or the l_o phase, the extent of solvation of the phospholipids is essentially the same. This constancy then leads to moderate sterol−phospholipid affinity, which is also constant and independent of the concentration of cholesterol in the membrane. The insertion of spherical-like chloroform molecules into the lipid bilayers loosens the assembly by disrupting chain packing and introducing additional "kinks" into the acyl chains of the phospholipids.

Scheme 1



In view of cholesterol's strong condensing and fluidizing effects on phospholipid bilayers, it is reasonable to expect that any perturbation of its time-averaged lateral distribution could result in altered structure and functioning of neighboring proteins. Thus, the significant influence that chloroform has on sterol−phospholipid mixing as seen here suggests that inhalation anesthetics could operate by a mechanism involving the rearrangement of the lipid framework, that is, by increasing the concentration of non-phospholipid-complexed cholesterol. In fact, we believe that such a mechanism would be the simplest one that takes the two-dimensional structure of biological membranes into account.

Acknowledgment. We are grateful to the National Institutes of Health (PHS GM56149) for support of this research and to Julie Molinari and Kamalakanta Routray for valuable technical assistance.

Supporting Information Available: Experimental procedures and raw data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Bondarenko, V.; Yushmanov, V. E.; Xu, Y.; Tang, P. *Biophys. J.* **2008**, *94*, 1681–1688.
(2) Peoples, R. W.; Li, C.; Weight, F. W. *Annu. Rev. Pharmacol. Toxicol.* **1996**, *36*, 185–201.
(3) (a) Cao, H.; Zhang, J.; Jing, B.; Regen, S. L. *J. Am. Chem. Soc.* **2005**, *127*, 8813–8816. (b) Sugahara, M.; Uragami, M.; Regen, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 13040–13041. (c) Sugahara, M.; Uragami, M.; Yan, X.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 7939–7940.
(4) Regen, S. L. *Curr. Opin. Chem. Biol.* **2002**, *6*, 729–735.
(5) (a) *Lipid Rafts*; McIntosh, T. J., Ed.; Springer-Verlag: New York, 2007. (b) Edidin, M. *Annu. Rev. Biomol. Struct.* **2003**, *32*, 257–283.
(6) Veatch, S. L.; Keller, S. L. *Biochim. Biophys. Acta* **2005**, *1746*, 172–185.
(7) Chiang, Y.-W.; Costa-Filho, J.; Freed, J. H. *J. Phys. Chem. B* **2007**, *111*, 11260–11270.
(8) Lipids **AA**, **BB**, and **AB** were synthesized using methods similar to those reported previously.[3a,c]
(9) Absorption measurements on isoflurane in bilayers made from egg PC, egg PG, and cholesterol strongly suggest that this inhalant also favors solvation of phospholipids over cholesterol. See: Dickinson, R.; Franks, N. P.; Lieb, W. R. *Biophys. J.* **1994**, *66*, 2019–2023.
(10) Parasassi, T.; DiStefano, M.; Loiero, M.; Ravagnan, G.; Gratton, E. *Biophys. J.* **1994**, *66*, 120–132.
(11) Gaber, B.; Peticolas, W. L. *Biochim. Biophys. Acta* **1977**, *465*, 260–274.

JA9011468