J|A|C|S
COMMUNICATIONS

Published on Web 12/06/2003

Unraveling the Mystery Surrounding Cholesterol's Condensing Effect

Honghua Cao, Nobuya Tokutake, and Steven L. Regen*

Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015

Received October 22, 2003; E-mail: slr0@lehigh.edu

Cholesterol's ability to condense fluid phospholipid membranes has been known for almost 80 years.[1] Despite numerous studies of cholesterol−phospholipid interactions, the mechanism by which this sterol uncoils phospholipids in monolayer and bilayer assemblies has remained a mystery.[2−18] In a recent publication, we hypothesized that the cholesterol's condensing action is a direct consequence of the hydrophobic effect.[19] Specifically, we postulated that the flexible acyl chains of phospholipids are able to complement, perfectly, the shape of neighboring cholesterol molecules, resulting in a high number of hydrophobic contacts and tight packing. In a sense, the sterol acts as a template for unraveling neighboring phospholipids. Here, we report the results of a structure−activity investigation that provide compelling evidence for such a mechanism.

Previous nearest-neighbor recognition (NNR) experiments have shown that cholesterol induces homophospholipid association in fluid bilayers made from exchangeable dimers, **AA**, **AB**, and **BB** (Chart 1).[20,21] Related studies, involving exchangeable forms of cholesterol, have shown that this effect is due to the sterol becoming a favored nearest-neighbor of two or more of the longer phospholipids, which leads to a liquid-ordered phase.[22,23] Stronger sterol−phospholipid interactions, therefore, are expected to result in a higher degree of homophospholipid association. In the present work, we have tested our condensing mechanism by means of a structure−activity approach. Specifically, we have examined the degree of homophospholipid association in bilayers containing **AA**, **AB**, and **BB** in the presence of a series of natural sterols whose hydrophobicity, shape, and ability to migrate within the hydrocarbon core of the bilayer have been varied.

Chart 1

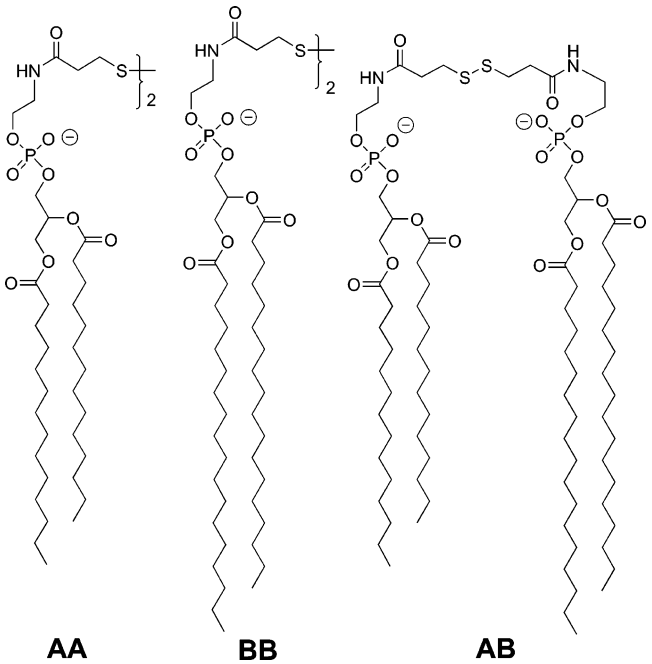
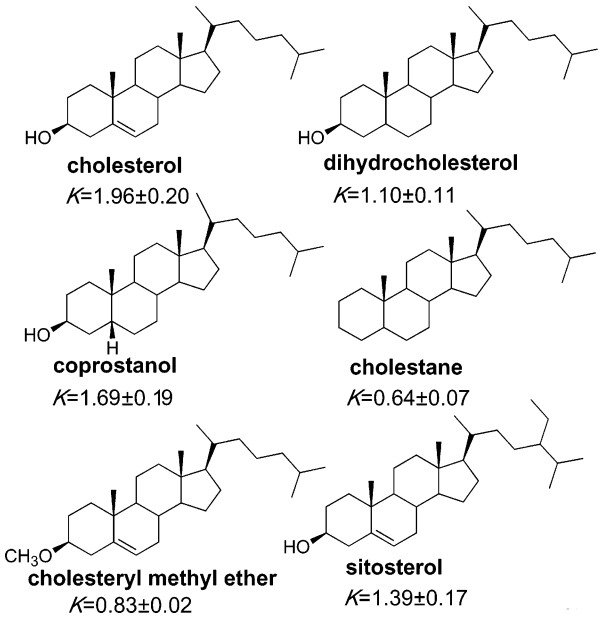


Chart 2



On the basis of our mechanism, replacement of cholesterol by dihydrocholesterol should lead to stronger sterol−phospholipid association due to an increase in the sterol's hydrophobicity and more effective acyl chain contact with the B-ring of the sterol; this, in turn, should lead to a higher degree of homophospholipid association (Chart 2). By similar reasoning, coprostanol (a stereoisomer of dihydrocholesterol, having a curved shape due to cis fusion of its A and B rings) would be expected to show a lower degree of homophospholipid association relative to dihydrocholesterol, since fewer hydrophobic contacts with neighboring acyl chains are possible. Finally, removal of the 3β-hydroxyl group of dihydrocholesterol (to give cholestane) would free the sterol from the headgroup region of the bilayer, allowing it to find an optimum location within the hydrocarbon core for maximum hydrophobic interactions. In such a case, homophospholipid association should be relatively high. Here, we report NNR evidence that verifies each of these predictions.

As discussed elsewhere, equilibrium mixtures of exchangeable lipid dimers that are generated via thiolate−disulfide interchange reactions are defined by an equilibrium constant, K, which governs the monomer interchange among **AA**, **BB**, and **AB** (eqs 1 and 2).[22] When **A** and **B** mix ideally, this is reflected by an equilibrium constant that equals 4.0. When homophosopholipid associations are favored, the equilibrium constant is less than 4.0; favored heterophospholipid associations are indicated by a value that is greater than 4.0.

10.1021/ja039172x CCC: $25.00

$$\mathbf{AA} + \mathbf{BB} \rightarrow [\mathbf{AA} + \mathbf{AB} + \mathbf{BB}] \leftarrow \mathbf{AB}$$
equilibrium mixture

$$\mathbf{AA} + \mathbf{BB} \overset{K}{\rightleftharpoons} 2\mathbf{AB} \quad (1)$$

$$K = [\mathbf{AB}]^2/([\mathbf{AA}][\mathbf{BB}]) \quad (2)$$

Dimers **AA**, **BB**, and **AB** were synthesized using methods previously described.[24] Experimental procedures that were used in forming liposomes (reverse-phase evaporation), carrying out monomer interchange reactions, and analyzing dimer distributions (HPLC) were also similar to those previously described.[22] To ensure that product mixtures were thermodynamically controlled, liposomes were prepared from two different compositions that had identical sterol and phospholipid monomer content, that is, from an equimolar mixture of **AA** and **BB** plus 29 mol % of a given sterol, and also from **AB** plus 29 mol % of the same sterol. All interchange reactions were carried out at 60 °C, which is in excess of the gel to liquid-crystalline-phase transition temperature of the phospholipids, to maintain the physiologically relevant fluid phase.[25] Equilibrium constants, *K*, were calculated from averaged dimer compositions from both sets of experiments.

Table 1 summarizes our principal findings. As expected, the relative efficiency of these four sterols in promoting homophospholipid association was: cholestane > dihydrocholesterol > coprostanol > cholesterol (entries 1−4). A control experiment that was carried out in the absence of sterol showed ideal mixing, as has been previously reported (entry 5).[24]

To test for the possibility that hydrogen bonding contributes to cholesterol−phospholipid association, we performed an additional set of NNR experiments in D_2O.[26] In principle, replacement of H_2O by D_2O should lead to stronger hydrogen bonding, stronger cholesterol−phospholipid interactions, and a greater preference for homophospholipid association. As shown in Table 1, the value of *K* that was observed for cholesterol-containing bilayers in D_2O was the same as that found in H_2O (entries 1 and 6). These results indicate that hydrogen bonding does not contribute significantly to cholesterol−phosholipid associations.

To obtain further evidence that cholesterol−phospholipid interactions are dominated by hydrophobic forces, we examined the influence of cholesteryl methyl ether on the mixing behavior of these phospholipids. By "capping" the sterol's hydroxyl group with a methyl group, the possibility of direct hydrogen bonding between the sterol's 3β-hydroxyl group and the carbonyl oxygen of a neighboring phospholipid is eliminated.[26] The fact that cholesteryl methyl ether induces a *greater* degree of NNR (lower value of *K*) than cholesterol supports our conclusion that hydrogen bonding effects are negligible (entries 1 and 7).

One final issue that we sought to clarify in this study was the influence that hydrophobicity within the pendant alkyl chain of the sterol has on phospholipid mixing. For this purpose, we compared the effect of cholesterol with that of sitosterol. As seen in Table 1, the introduction of a single ethyl group at the C-24 position (i.e., on going from cholesterol to sitosterol) significantly increases the sterol's ability to induce homophospholipid association (entries 1 and 8). Thus, sterol−phospholipid interactions are sensitive to the hydrophobicity of the alkyl side chain as well as the sterol nucleus.

Table 1. Equilibrium Constants for Dimer Interchange

entry	sterol[a]	aqueous phase	K[b]
1	cholesterol	H_2O	1.96 ± 0.20
2	dihydrocholesterol	H_2O	1.10 ± 0.11
3	coprostanol	H_2O	1.69 ± 0.19
4	cholestane	H_2O	0.64 ± 0.07
5		H_2O	3.92 ± 0.16
6	cholesterol	D_2O	1.99 ± 0.20
7	cholesteryl methyl ether	H_2O	0.83 ± 0.02
8	sitosterol	H_2O	1.39 ± 0.17

[a] Mol % of sterol (29%) is based on the total lipid that is present, where each phospholipid counts as two lipid molecules. [b] Equilibrium constants calculated from eq 2 (±1 SD); equilibrium was reached in all cases within 6 h at 60 °C.

In summary, the results of this structure−activity investigation have provided compelling evidence that cholesterol unravels neighboring phospholipids by acting as a rigid hydrophobic template, which helps maximize hydrophobic interactions within the membrane.

Acknowledgment. We are grateful to the National Institutes of Health (PHS GM56149) for support of this research.

Supporting Information Available: Procedures for carrying out NNR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Leathes, J. B. *Lancet* **1925**, *208*, 853.
(2) Hinz, H.; Sturtevant, J. M. *J. Biol. Chem.* **1972**, *247*, 3697.
(3) Subramaniam, S.; McConnell, H. M. *J. Phys. Chem.* **1987**, *91*, 1715.
(4) Ipsen, J. H.; Karlstrom, G.; Mouritsen, O. G.; Wennerstrom, H.; Zuckermann, M. J. *Biochim. Biophys. Acta* **1987**, *905*, 162.
(5) Needham, D.; McIntosh, T. J.; Evans, E. *Biochemistry* **1988**, *27*, 4668.
(6) Ipsen, J. H.; Mouritsen, O. G. *J. Chem. Phys.* **1989**, *91*, 1855.
(7) Vist, M. R.; Davis, J. H. *Biochemistry* **1989**, *29*, 451.
(8) Sankaram, M. B.; Thompson, T. E. *Biochemistry* **1990**, *29*, 10676.
(9) Huang, J.; Feigenson, G. W. *Biophys. J.* **1999**, *76*, 2142.
(10) Radakrishnan, A.; McConnell, H. M. *J. Am. Chem. Soc.* **1999**, *121*, 486.
(11) Keller, S. L.; Radhakrishnan, A.; McConnell, H. M. *J. Phys. Chem.* **2000**, *104*, 7522.
(12) Anderson, R. G.; Jacobson, K. *Science* **2002**, *296*, 1821
(13) Veatch, S. L.; Keller, S. L. *Phys. Rev. Lett.* **2002**, *89*, 268101.
(14) McConnell, H. M.; Radhakrishnan, A. *Biochim. Biophys. Acta* **2003**, *1610*, 159.
(15) Gennis, R. B. *Biomembranes*: *Molecular Structure and Function*; Springer-Verlag: New York, 1989.
(16) Simons, K.; Ikonen, E. *Science* **2000**, *290*, 1721.
(17) Czech, M. P. *Nature* **2000**, *407*, 147.
(18) Feingold, L. *Cholesterol in Membrane Models*; CRC Press: Ann Arbor, 1993.
(19) Sugahara, M.; Uragami, M.; Yan, X.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 7939.
(20) Krisovitch, S.; Liu, Y.; Regen, S. L. *J. Am. Chem Soc.* **1993**, *115*, 10104.
(21) For a review of the NNR method, see: Davidson, S. K. M.; Regen, S. L. *Chem. Rev.* **1997**, *97*, 1269.
(22) Sugahara, M.; Uragami, M.; Regen, S. L. *J. Am. Chem. Soc.* **2002**, *124*, 4253.
(23) Sugahara, M.; Uragami, M.; Regen, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 13040.
(24) Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 9828.
(25) Gel- to liquid-crystalline-phase transition temperatures of **AA**, **AB**, and **BB** are 22.7, 33.9, and 55.4 °C, respectively.[24]
(26) Cholesterol−phospholipid association via hydrogen bonding has been the subject of considerable controversy: (a) Presti, F. T.; Pace, R. J.; Chan, S. I. *Biochemistry* **1982**, *21*, 3831. (b) Bush, S. F.; Adams, R. G.; Levin, I. W. *Biochemistry* **1980**, *19*, 4429.

JA039172X