Cholesterol Is Not Crucial for the Existence of Microdomains in Kidney Brush-border Membrane Models*

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The external membrane leaflet plays a key role in the organization of the cell plasma membrane as a mosaic of ordered microdomains enriched in sphingolipids and cholesterol and of fluid domains. In this study, the thermotropic behavior and the topology of bilayers made of a phosphatidylcholine/sphingomyelin mixture, which mimicks the lipid composition of the external leaflet of renal brush-border membranes, were examined by differential scanning calorimetry and atomic force microscopy. In the absence of cholesterol, a broad phase separation process occurred where ordered gel phase domains of size varying from the mesoscopic to the microscopic scale, enriched in sphingomyelin, occupied half of the bilayer surface at room temperature. Increasing amounts of cholesterol progressively decreased the enthalpy of the transition and modified the topology of membranes domains up to a concentration of 33 mol % for which no membrane domains were detected. These results strongly suggest that, in membranes highly enriched in sphingolipids like renal and intestinal brush borders, there is a threshold close to the physiological concentration above which cholesterol acts as a suppressor rather than as a promoter of membrane domains. They also suggest that cholesterol depletion does not abolish the lateral heterogeneity in brush-border membranes.

According to the current view, the plasma membrane of eucaryotic cells is organized in an in-plane mosaic of microdomains (1, 2). Rafts correspond to a category of microdomains, enriched in sphingolipids (SPL)1 and cholesterol (Chl), which play a key role in the expression and regulation of the plasma membrane functions (3, 4). This conclusion was reached essentially through the use of two experimental procedures, the low temperature non-ionic detergent extraction (2) and the Chl depletion of cells (5, 6). The resistance to low temperature, non-ionic detergent extraction of numerous membrane proteins is associated to a liquid ordered (L∞) or to a gel ordered (Lg) state of membrane lipids, which strongly suggests that the physical state of these membrane lipids is of primary importance in the formation of the membrane microdomains mosaic (7, 8). Formation of the L∞ phase, or more precisely of the fluid liquid ordered L∞ phase, and gel liquid ordered Lg phases (9), depends on the presence of Chl (10, 11). SPL also appear to be determinant for the existence of eucaryotic plasma membrane rafts (3, 4), and this could be explained by the preferential interaction of Chl with SPL rather than with the other phospholipid species in natural phospholipid-Chl mixtures (10, 12, 13). Because SPL are essentially localized on the external leaflet of the plasma membrane (14), this strongly suggests that this membrane leaflet plays a crucial role in the existence of microdomains.

Renal brush-border membranes (BBM), which constitute the apical membrane of the proximal tubule epithelial cells, are highly ordered structures, as shown by fluorescence polarization and ESR data (15). Their glycerophospholipid GPL/SPL/Chl ratio (0.9:0.7:1), where sphingomyelin accounts for >95% of SPL (reviewed in Ref. 16), is close to that reported (1:1:1) by Brown and Rose (1) for the detergent-resistant membrane fragments (DRMs). As a consequence of the asymmetrical distribution of SPL in membranes, the BBM external leaflet is composed of ~75% sphingomyelin (SM) and 25% zwitterionic phospholipids, essentially phosphatidylcholine (17). To better understand the properties of these membranes, the existence, size, and in-plane distribution of microdomains in Langmuir-Blodgett films with a lipid composition mimicking that of the BBM external leaflet was recently examined (18) by atomic force microscopy (AFM). AFM is a useful tool for probing the mesoscopic lateral organization of lipid mixtures (19–23). The results of these AFM experiments suggested that the phospholipids of BBM external leaflet should be under phase separation conditions, even in the absence of Chl.

The use of Langmuir-Blodgett films provides a basic step for the understanding of the physicochemical properties of isolated membrane leaflets (24), but interactions between the two membrane leaflets could modify the properties of each leaflet. Accordingly, in this paper we have studied, by differential scanning calorimetry (DSC) and AFM, the Chl effect in lipid bilayers made of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), bovine brain SM, and Chl mixtures.

MATERIALS AND METHODS

POPC, bovine brain SM, and Chl were purchased from Sigma-Aldrich (Saint Quentin, France). Lipids were dissolved in a chloroform/methanol solution (2/1, v/v) at concentrations of 10 mM. Phospholipids and Chl concentrations in solutions were determined as described previously (17). Multilamellar vesicles (MLVs) were prepared at 60 °C in multimamellar vesicle; SUV, small unilamellar vesicle; Chl, cholesterol; SM, bovine brain sphingomyelin; POPC, 1-hexadecanoyl-2-[(cis9-octadecenoyl)-sn-glycero-3-phosphocholine; GPL, glycerophospholipids; PBS, phosphate-buffered saline; L∞, liquid ordered state; Lg, gel ordered state.
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The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The SM concentration in the calorimeter cell was 7 mM for pure SM, 15 mM for all samples. A heating scan rate of 10 °C/h was used. Sample runs were repeated at least three times to ensure reproducibility. The Smith et al. (28) study the thermotropic properties of POPC/SM 1:3 MLVs in PBS also undergo a broad transition with two distinct maxima at 8.9 ± 0.6 °C and 29.0 ± 0.2 °C (Fig. 1, curve b). The proximity of the ice to water transition prevented an accurate determination of the onset of the transition, located around 5 °C, and of the enthalpy variation. As compared with pure SM, the upper end of the transition was lowered by ~6 °C.

The Chl of renal BBM varies between 0.3 and 0.45 mol % (reviewed in Ref/16), but about one-third of the BBM Chl interacts poorly with the other membrane lipids (29). We therefore examined the thermotropic properties of POPC/SM 1:3 MLVs containing 20, 25, and 33 mol % Chl. Addition of 20 mol % Chl to POPC/SM liposomes practically suppressed the lower endothermic peak and markedly reduced the intensity of the upper peak, shifting downward to 27.0 ± 0.4 °C (Fig. 1, curve c). The thermogram was asymmetrical, and completion of the transition was obtained for a temperature comparable with that of pure SM. Although for the same reason as before the enthalpy of the transition could not be determined accurately, comparison of curves b and c indicated that, even without taking into account the higher phospholipid concentration of sample c (see “Materials and Methods”), Chl markedly reduced this enthalpy. Raising the Chl concentration to 25 and 33 mol % (Fig. 1, curves d and e, respectively) resulted in more symmetrical curves with a maximum at 24.8 ± 0.3 °C. The temperature of the upper end of the transition was not significantly modified and the change in slope around 5 °C (arrow) strongly suggests that it extended over a temperature range of 40 °C. Determination of the corresponding ΔH gave values of 1.48 and 1.03 Kcal/mol of phospholipid. These DSC data were compatible with, but did not prove, the existence of phase separations phenomena in the bilayers. To address this question, the topology of bilayers was investigated by AFM.

AFM Study of BBM Model Bilayers—The topology of bilayers made from POPC/SM 1:3 was characteristic of a bilayer under phase separation (Fig. 2A). The brighter (thicker) gel phase domains of a size up to 3 μm protruded from the darker liquid crystalline matrix by an apparent height (Δh) of 0.7 ± 0.1 nm. They occupied 55 ± 4% of the bilayer surface. Few vesicles, which had not fused with the bilayer, appeared as brighter dots. The height difference between the bilayer surface in the fluid phase and the mica determined either at the edge of bilayers that did not completely cover the substrate or from the depth of holes in the bilayers was 9.0 ± 0.3 nm. Addition of 20 mol % Chl did not suppress the phase separation (Fig. 2B). Lighter domains were connected, forming an extended network, which accounted for 62 ± 4% of the total surface. The size of the darker domains was, for most of them, below 300 nm. The significant reduction in the height difference between the lighter and the darker domains (Δh = 0.4 ± 0.1 nm) rendered more difficult the visualization of the phase separation. Increasing the Chl concentration to 25 mol % resulted in a disconnection of the lighter domains which formed patches of various shape occupying 40 ± 6% of the bilayer surface (Fig. 2C). The Δh was not further decreased (0.3 ± 0.1 nm). The size range of these patches varied from below 100 nm to a few micrometers. Finally, upon addition of 33 mol % Chl, numerous holes pierced the bilayer, and domains were no longer observed (Fig. 2D). In these samples, the height difference between the surface of the bilayer and the mica support was 9.1 ± 0.5 nm (Fig. 2D).

DISCUSSION

According to the current view, the cell plasma membrane is organized as a mosaic of ordered microdomains enriched in sphingolipids and Chl and of fluid domains. Upon Chl depletion in the bilayer, the height of the transition progressively decreases, and the phase separation becomes less evident. After a transition range of 40 °C, the Chl concentration in the bilayer could be estimated to 55 ± 4% of the total membrane phospholipid, in agreement with previous reports (28).
The ordered microdomains vanish, and the diluted sphingolipids become miscible with the fluid domains. Using a lipid bilayer model close to biological membranes, we provide here strong evidence that, in cells rich in membrane sphingolipids like the renal epithelial cells, Chl depletion might not suppress strong evidence that, in cells rich in membrane sphingolipids like the renal epithelial cells, Chl depletion might not suppress phase separation in POPC/SM bilayers (30, 31, 32). For the different binary and ternary phospholipid mixtures (19, 21, 23), the observation that gel phase action between SM and Chl (10, 12, 13). Addition of 20 mol % Chl to the bilayers reduced the enthalpy of the transition was further decreased at 25 mol % Chl and the DSC scan became more symmetrical. Thus, in 1:3 POPC/SM bilayers, Chl interacted with both SM and POPC. Both the complexity of the thermogram and the operating range of the calorimeter (0–115 °C), which limits the accuracy of the detection of the onset of the transition, did not allow to conclude about the existence of the reported preferential interaction between SM and Chl (10, 12, 13). Addition of 20 mol % Chl to the bilayers reduced the δh from 0.7 to 0.4 nm. Such a reduction likely involved both the decrease in bilayer thickness at the level of SM enriched domains (35) and the increase in the bilayer thickness of the POPC enriched domains (36) promoted by Chl. At 33 mol % Chl, no membrane domains were visualized by AFM, either at the microscopic or at the mesoscopic scales, which strongly suggests that the bilayer was in the liquid ordered phase. These AFM data differ from those obtained on monolayers, where microdomains 20–70 nm in size forming a branched network were observed (18). This suggests that coupling between membrane leaflets might affect the interactions between Chl and phospholipids in each monolayer. Such qualitative differences in the behavior of monolayers versus bilayers was previously reported for POPC/Chl mixtures (37, 38). Taking into account this limitation we cannot ascertain if the DSC thermogram at 33 mol % Chl is associated with the presence of a single (Lq) or multiple (Lq + Lq + Lq) ordered phases. Our AFM data on a model of the renal BBM lipids

Phase Separation in POPC/SM Bilayers—The presence of a broad endothermic transition with two maxima at 9 and 29 °C and the AFM images showing the presence at room temperature of domains from mesoscopic to microscopic sizes indicate that POPC/SM 1:3 mixtures form bilayers in which gel domains enriched in SM coexist with liquid crystalline domains enriched in POPC. The present calorimetric data compare with those obtained for mixtures of egg PC/SM at the same molar ratio (26), which can be explained by considering the fatty acid composition of egg PC. AFM detection of lipid-lipid immiscibility in supported bilayers essentially depends on the apparent difference in thickness (δh) between the lipid domains. The δh is the sum of the absolute height difference between the domains plus a variable height contribution, which depends on the local mechanical properties of the lipid and on the scanning force applied (19, 21, 23). For the different binary and ternary lipid mixtures under phase separation made by vesicle fusion so far examined (19, 23, 30), the observation that gel phase phospholipid domains have a constant δh above fluid phase domains likely corresponds to a superimposition of gel-gel and fluid-fluid domains of each membrane leaflet (23). This interpretation is strongly supported by the existence of domains of intermediate height when the bilayers are made by Langmuir-Blodgett successive transfers (31). Accordingly, the protruding domains observed here in the POPC/SM bilayers can be attributed to Lq SM-enriched domains present in the external leaflet, accessible to the AFM tip, superimposed on SM-enriched domains of the same size and shape facing the mica substrate. The darker matrix is made of POPC Lq-enriched domains. This phase coupling between the two leaflets has also been observed in giant liposomes using fluorescence techniques (32). It is worth noting that the height of the buffer-bathed surface of the bilayer above the mica substrate was ∼9 nm. Compared with X-ray diffraction data on SM multi-bilayers (33), this suggests that the buffer layer between the mica and the bilayer has a thickness between 3 and 4 nm, a value close to that estimated by neutron diffraction of supported bilayers (34). Shape and size of the domains varied from small (150 nm) disc shaped to large (3 μm) elongated structures. This variety, already reported for supported bilayers made of a binary mixture of synthetic phospholipids under phase separation, illustrates the complexity of the phase separation process when observed at the mesoscopic scale (23).
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differ from those reported recently on 1:1 DOPC/SM bilayers containing variable amounts of Chl, where large domains still persist at 50 mol % Chl (39). Besides the difference in the PC/SM molar ratio used, this different finding can be explained by the fact that Chl tends to be segregated out in unsaturated PC membranes and poorly interacts with DOPC in DOPC/SM mixtures (12). Using two-photon fluorescence imaging, Dietrich et al. (32) reported the existence of a L\textsubscript{o}-L\textsubscript{o} phase separation in GUVs made of renal BBM total lipid extracts. Our experiments suggest that this liquid disordered-liquid ordered phase separation is due to the loss in the asymmetrical distribution of SM and of the other phospholipids upon lipid extraction and GUVs preparation, which results in the mixing of the inner and external membrane leaflets components.

Plasma Membranes Implications—The present AFM data strongly suggest that the phospholipids constituting the renal BBM external leaflet phase separate to form domains, and then Chl is not necessary to observe a mesoscopic scale membrane lateral heterogeneity linked to a L\textsubscript{o} phase. The differences in bilayer topography recorded between 25 and 33 mol % Chl also suggest that limited variation in the Chl content of BBM can have a marked effect on their membrane organization. Purified DRMs in a L\textsubscript{o} phase have a 1:1:1 GPL/SPL/Chl ratio (1) giving a 0.8:3:2:2 GPL/SPL/Chl ratio for the composition of the DRMs external leaflet if one considers that ~80% SPL are on the external DRMs leaflet and that Chl is asymmetrically distributed across the membrane. This lipid composition compares well with the 1:3:2 POPC/SM/Chl ratio chosen here to mimic a renal BBM external leaflet containing 33 mol % Chl. Thus, the external leaflets of renal BBM and isolated DRMs have a comparable composition in terms of lipid classes, which most likely results in a comparable physical state. Accordingly, our data suggest that Chl depletion of purified DRMs should result in a GPL/SM phase separation. It seems that such a behavior is probably not restricted to renal cells, since a similar 1:1 GPL/SM/Chl ratio was found for the apical membrane of intestinal epithelial cells (40) and that, in accordance with this view, lipid rafts can exist as Chl-independent microdomains in intestinal BBM (41).

REFERENCES

1. Brown, D. A., and Rose, J. K. (1992) Cell 68, 533–544
2. Jacobson, K., Sheets, E. D., and Simons, R. (1995) Science 268, 1441–1442
3. Brown, D. A., and London, E. (2000) J. Biol. Chem. 275, 17221–17224
4. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572
5. Hanada, K., Nishijima, M., Akamatsu, Y., and Pagano, R. E. (1995) J. Biol. Chem. 270, 6254–6260
6. Kim, U., Gimpf, G., and Fahrenholz, F. (1995) Biochemistry 34, 13784–13793
7. Ahmed, S. N., Brown, D. A., and London, E. (1997) Biochemistry 36, 10944–10953
8. Schroeder, R., London, E., and Brown, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12110–12114
9. McMullen, T., and McElhaney, R. N. (1995) Biochim. Biophys. Acta 1234, 90–98
10. Singh, M. B., and Thompson, T. E. (1990) Biochemistry 29, 10676–10684
11. Ipsen, J. H., Karlstrom, G., Mouritsen, O. G., Wennerstrom, H., and Zuckermann, M. J. (1987) Biochim. Biophys. Acta 905, 162–172
12. Demel, R. A., Kalsbeek, R., Wurtz, K. W., and Van Deenen, L. L. (1977) Biochim. Biophys. Acta 466, 10–22
13. Slotte, J. P. (1999) Chem. Phys. Lipids 102, 13–27
14. Op den Kamp, J. A. F. (1981) in New Comprehensive Biochemistry (Finean, J. B., and Michel, R. H., eds) Vol. 1, pp. 83–126, Elsevier Science Publishers B. V., Amsterdam
15. Le Grimezellec, C., Carriere, S., Cardinal, J., and Giocondi, M. C. (1983) Am. J. Physiol. 245, F227–F231
16. Le Grimezellec, C., Friedlander, G., el Yandouzi, E. H., Zlatkine, P., and Giocondi, M. C. (1992) Kidney Int. 42, 825–836
17. Venien, C., Aubry, M., Crine, P., and Le Grimezellec, C. (1988) Anal. Biochem. 174, 325–330
18. Milhiet, P. E., Doeneck, C., Giocondi, M. C., Van Mau, N., Heitz, F., and Le Grimezellec, C. (2001) Biophys. J. 81, 547–555
19. Dufrêne, Y. F., Burger, W. R., Green, J. D., and Lee, G. U. (1997) Langmuir 13, 4779–4784
20. Shao, Z., and Zhang, Y. (1996) Ultramicroscopy 66, 141–152
21. Vie, V., Van Mau, N., Lesniewska, E., Goudonnet, J. P., Heitz, F., and Le Grimezellec, C. (1998) Langmuir 14, 4574–4583
22. Yuan, C. B., and Johnsen, L. J. (2001) Biophys. J. 81, 1059–1069
23. Giocondi, M. C., Vie, V., Lesniewska, E., Milhiet, P. E., Zinke-Almang, M., and Le Grimezellec, C. (2001) Langmuir 17, 1653–1659
24. Broekman, H. (1999) Curr. Opin. Struct. Biol. 9, 438–443
25. Carmel, G., Rodrigue, F., Carriere, S., and Le Grimezellec, C. (1985) Biochim. Biophys. Acta 818, 149–157
26. Untracht, S. H., and Shipley, G. (1977) J. Biol. Chem. 252, 4449–4457
27. Koyanova, R., and Caffrey, M. (1995) Biochim. Biophys. Acta 1255, 213–236
28. Koyanova, R., and Caffrey, M. (1998) Biochim. Biophys. Acta 1376, 91–145
29. El Yandouzi, E. H., and Le Grimezellec, C. (1993) Biochimica et Biophysica Acta 115, 2977–2982
30. Revaikeine, I., Bergsma Schutter, W., Mazeres Dubut, C., Govorukhina, N., and Brisson, A. (2000) J. Struct. Biol. 131, 234–239
31. Hollars, C. W., and Dunn, R. C. (1998) Biophys. J. 75, 342–353
32. Dietrich, C., Bagatolli, L. A., Velovysk, Z. N., Zim, N. L., Levi, M., Jacobson, K., and Gratton, E. (2001) Biophys. J. 80, 1417–1428
33. Maulik, P. R., and Shipley, G. G. (1996) Biochemistry 35, 8025–8034
34. Johnson, S. J., Bayerl, T. M., McDermott, D. C., Adam, G. W., Rennie, A. R., Thomas, R. K., and Sackmann, E. (1991) Biophys. J. 59, 289–294
35. Maulik, P. R., and Shipley, G. G. (1996) Biochemistry 35, 289–294
36. Angell, K., and Bloom, M. (1992) Biophys. J. 61, 1176–1183
37. Hernandez-Borre, J., and Keough, K. M. (1993) Biophys. J. 1186, 277–282
38. Worthman, L. A., Nag, K., Davis, P. J., and Keough, K. M. (2001) Biophys. J. 79, 2569–2580
39. Rinia, H. A., Snel, M. M. E., Van der Eerden, J., and de Kruijff, B. (2001) FEBS Lett. 501, 92–96
40. Simons, K., and van Meer, G. (1988) Biochim. Biophys. Acta 987, 619–620
41. Hansen, G. H., Immerdal, L., Thorsen, E., Niels Christiansen, L. L., Nyström, B. T., Demant, E. J. F., and Danielsen, E. M. (2001) J. Biol. Chem. 276, 32338–32344
