Domain Formation in Models of the Renal Brush Border Membrane Outer Leaflet

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ABSTRACT The plasma membrane outer leaflet plays a key role in determining the existence of rafts and detergent-resistant membrane domains. Monolayers with lipid composition mimicking that of the outer leaflet of renal brush border membranes (BBM) have been deposited on mica and studied by atomic force microscopy. Sphingomyelin (SM) and palmitoyloleoyl phosphatidylcholine (POPC) mixtures, at molar ratios varying from 2:1 to 4:1, were phase-separated into liquid condensed (LC) SM-enriched phase and liquid expanded (LE) POPC-enriched phase. The LC phase accounted for 33 and 58% of the monolayers surface for 2:1 and 4:1 mixtures, respectively. Addition of 20–50 mol % cholesterol (Chl) to the SM/POPC (3:1) mixtures induced marked changes in the topology of monolayers. Whereas Chl promoted the connection between SM domains at 20 mol %, increasing Chl concentration progressively reduced the size of domains and the height differences between the phases. Lateral heterogeneity was, however, still present at 33 mol % Chl. The results indicate that the lipid composition of the outer leaflet is most likely responsible for the BBM thermotropic transition properties. They also strongly suggest that the common maneuver that consists of depleting membrane cholesterol to suppress rafts does not abolish the lateral heterogeneity of BBM membranes.

INTRODUCTION

Data accumulated over the last two decades indicate that the organization of biological membranes in in-plane microdomains (Jain and White, 1977; Karnovsky et al., 1982; Kinnunen, 1991; Tocanne et al., 1994; Jacobson et al., 1995) plays a key role in the expression and regulation of membrane functions (Simons and Ikonen, 1998; Brown and London, 1998). Recent studies show that, among the mechanisms involved in the formation and organization of microdomains and rafts (Kusumi and Sako, 1996; Simons and Ikonen, 1998), the physical state of membrane lipids is of primary importance (Schroeder et al., 1994; Ahmed et al., 1997). Thus, the resistance to low-temperature detergent extraction of numerous membrane proteins, including the glycophosphatidylinositol-anchored proteins (GPI), is associated to a liquid ordered or gel state of membrane lipids. Formation of the liquid ordered phase (L_o) depends on the presence of cholesterol (Ipsen et al., 1987; Sankaram and Thompson, 1990) and numerous experiments on cultured cells confirm that membrane microdomains vanish upon cholesterol depletion of the plasma membrane (Klein et al., 1995; Pike and Miller, 1998; Pralle et al., 2000; Röper et al., 2000). Besides cholesterol, the presence of glycosphingolipids, and more generally sphingolipids, appears to be the determinant for the existence of plasma membrane microdomains (Simons and Ikonen, 1998; Brown and London, 1998). Natural sphingolipids have the peculiarity of undergoing very broad gel to liquid crystal phase transitions that can extend from below 20°C to 55°C, and thus include the physiological temperature (reviewed in Barenholz, 1984). Furthermore, in natural phospholipid-cholesterol mixtures, cholesterol preferentially interacts with sphingolipids rather than with the other phospholipid species (Demel et al., 1977; Barenholz, 1984; Sankaram and Thompson, 1990; Slotte, 1999). In biological membranes, sphingolipids are essentially localized on the outer leaflet of the plasma membrane (Op den Kamp, 1981). This strongly suggests that this membrane leaflet plays a crucial role in the existence of microdomains.

The lipid composition of the apical membrane of renal and intestinal epithelial cells (brush border membranes, BBM) is, in that respect, of particular interest. In BBM, sphingolipids account for >30% of total lipids, a value significantly higher than that usually found in other plasma membranes (Carmel et al., 1985; Schachter, 1985; Le Grimellec et al., 1988; 1992; Van Meer and Simons, 1988). Taking into account the asymmetrical distribution of sphingolipids in membranes, this indicates that the outer leaflet of BBM must be predominantly constituted of sphingolipids, a fact established for renal BBM isolated from the proximal tubule. The phospholipid composition of their outer leaflet is ~75% sphingomyelin (SM) and 25% zwitterionic phospholipids, essentially phosphatidylcholine (PC) (Vénien and Le Grimellec, 1988). In contrast to intestinal BBM, the renal BBM show a very low content in glycosphingolipids (Spiegel et al., 1988).

In this paper we have studied the existence, size, shape, and in-plane distribution of domains in lipid mixtures made of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), bo-

Received for publication 29 November 2000 and in final form 28 March 2001.

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0006-3495/01/07/547/09 $2.00

vine brain SM (SM), and cholesterol (Chl) in proportions mimicking the composition of the outer membrane leaflet of renal BBM. To define the properties of the membrane outer leaflet independently of the possible influence of the inner leaflet, the experiments were done on lipid monolayers. Langmuir-Blodgett (LB) films provide good models for such studies on membrane hemi-leaflets (Brockman, 1999). Lipid domain topology was determined by atomic force microscopy (AFM). Because of its capacity to image structures with a resolution that extends from the molecular to the microscopic level (Radmacher et al., 1992; Shao et al., 1996; Engel et al., 1997), the AFM is a useful tool for probing the mesoscopic lateral organization of lipid mixtures (Zazadzinski et al., 1994; ten Grotenhuis et al., 1996; Dufrêne et al., 1997; Vié et al., 1998).

The results obtained strongly suggest that in BBM outer leaflet the phospholipids are most likely under liquid condensed (LC)-liquid expanded (LE) phase separation conditions. They also indicate that cholesterol concentrations up to 33 mol % are not sufficient to suppress the lateral heterogeneity of the monolayer. These data provide a better understanding of the existence of thermotropic transitions of physiological relevance in epithelial brush borders (Brasitus et al., 1980; Le Grimellec et al., 1982, 1983; Levi et al., 1993). Finally, they raise questions about the effect of cholesterol depletion on rafts.

MATERIALS AND METHODS

1-Hexadecanoyl-2-[*cis*-9-octadecanoyl]-*sn*-glycero-3-phosphocholine, bovine brain sphingomyelin, and cholesterol were purchased from Sigma-Aldrich (Saint Quentin, France). Lipids were dissolved in a chloroform/methanol solution (2:1 v/v) at concentrations of 1 mM. Phospholipids and cholesterol concentrations in solutions were determined as previously described (Vénien and Le Grimellec, 1988). The water used as subphase was purified by passage through a Milli-RO, then a Milli-Q, purification system (Millipore, Molsheim, France) to yield a product with a resistivity of 18.2 MΩ/cm.

Monolayers of pure or mixed lipids were prepared at the air-water interface (Vié et al., 1998; Van Mau et al., 1999) in a Nima Langmuir-Blodgett trough 611 MC (Nima Technology Ltd., Coventry, UK) at room temperature (22 ± 2°C). According to the lipid mixture, 6–10 nmol were spread on the surface. After evaporation of the solvent (10 min), they were compressed with a speed not exceeding 5 $Å^2$/molecule/min. Monolayers were deposited at a constant surface pressure of 30 mN/m, after a 5-min relaxation time, by raising vertically (5 mm/min) freshly cleaved mica through the air-water interface. This value of the surface pressure is considered as a good approximation of the situation found in biological membranes (Demel et al., 1975; Albrecht et al., 1978).

AFM imaging of LB films was performed in contact mode using a Nanoscope IIIa atomic force microscope (Digital Instruments, Santa Barbara, CA) under ambient conditions, using a 14 × 14 μm scanner (Vié et al., 1998; Van Mau et al., 1999). Height images, which report on the topology of the hydrophobic face of the monolayer, were acquired in constant-force mode using silicon nitride tips on integral cantilevers with a nominal spring constant of 0.06 N/m. Typically, the estimated imaging forces were below 0.5 nN. Scan rates varied from 1 to 2.5 Hz. Images were obtained from at least three different samples prepared on different days with at least five macroscopically separated areas on each sample. The surface area of domains was determined using the bearing analysis routine included in the Nanoscope IIIa software.

RESULTS

Imaging of pure phospholipid species

AFM of pure POPC, which remains in the LE or fluid phase up to the point of monolayer collapse (Grönberg and Slotte, 1990; Ali et al., 1991; Worthman et al., 1997), provided homogeneous and flat aspect images even using a vertical scale of 3 nm (Fig. 1, *A* and *C*), with corrugations smaller than 1 Å (Fig. 1 *B*), as previously reported for DOPC monolayers transferred at a similar surface pressure (Vié et al., 1998; Van Mau et al., 1999). The standard deviation of the height fluctuations (RMS) was 0.2 Å. However, SM gave images typical of phase separation (Fig. 1, *D* and *F*), the LC phase protruding from the LE phase by 1.0 ± 0.1 nm (Fig. 1 *E*). This value compared with the height difference between LE and LC phases in dioleoyl-dipalmitoyl PC, dipalmitoyl phosphatidylcholine/dioleoylphosphatidyl serine, and dioleoyl-dipalmitoyl PE mixtures as determined by AFM (Vié et al., 1998; Dufrêne et al., 1997; Reviakine et al., 2000). LC SM-branched domains occupied the largest part (76 ± 2%) of the sample surface.

SM/POPC mixtures

In mammals, the amount of sphingomyelin in renal BBM can vary between ~30 and 45% of total phospholipids as a function of the species, the localization of the tubule in the renal cortex, the diet, and aging (Carmel et al., 1985; Levi et al., 1989; Levi, 1990; Le Grimellec et al., 1992). Because SM accounts for ~75% of the phospholipids present in renal BBM outer leaflet (Vénien and Le Grimellec, 1988), we investigated the topology of mixtures with molar SM/POPC ratios varying from 2:1 to 4:1. In the 2:1 monolayers, LC domains were still present but much more dispersed than in pure SM. As compared to 76% in pure SM, they occupied only 33 ± 3% of the sample surface (Fig. 2 *A*) and their average area was 1.06 ± 0.1 × 10^4 nm^2 (n = 257), with values ranging from 2.0 × 10^2 to 9.0 × 10^4 nm^2. Thus, in the absence of Chl, a clear LE-LC phase separation took place even with the lowest SM concentration compatible with the composition of renal BBM outer leaflet. As expected, increasing the SM concentration of the monolayer resulted in an increase in the proportion of the LC phase, which accounted for 58 ± 4% of the sample surface in 4:1 SM/POPC mixtures (Fig. 2 *B*). For this SM concentration, the shape of domains became very irregular, resembling that obtained with pure SM but with little branching (mean area: 2.89 ± 0.5 × 10^4 nm^2, n = 115). The topology of the 3:1 SM/POPC monolayers was intermediate between those of the 2:1 and 4:1 mixtures (Fig. 3 *A*).

SM/POPC/Chl monolayers

In purified renal BBM, the cholesterol/phospholipid molar ratio (C/P) was found to vary from ~0.5 to 0.9 (reviewed in Le

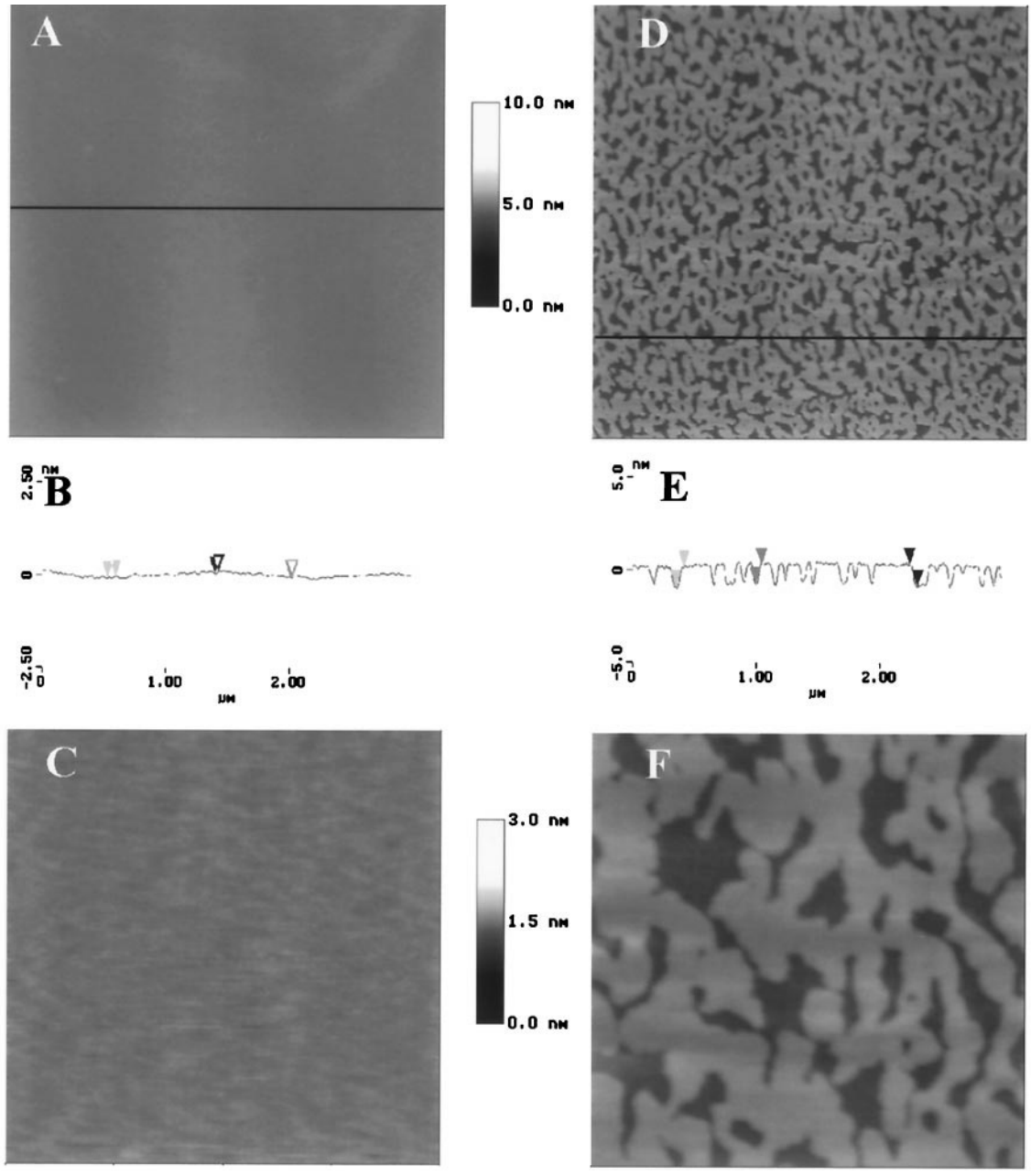

FIGURE 1 AFM images of POPC and SM monolayers. Monolayers were transferred at a surface pressure of 30 mN/m. (*A*) Representative 3 × 3 μm scan of a POPC monolayer. The horizontal black line marks the location of the section analysis shown in (*B*). Vertical distances between arrows were 0.3, 0.7, and 0.3 Å from the left to the right, respectively. (*C*) 1 × 1 μm scan of a POPC monolayer. Note the difference in vertical *z* color scale between the 3 μm (z = 10 nm) and the 1 μm (z = 3 nm) scans. *D–F* are the corresponding figures for pure SM. The vertical distances in *E* were, from the left to the right, 1.1, 1.0, and 1.2 nm.

Grimellec et al., 1992). Although two different Chl pools have been identified, the Chl distribution between the outer and inner BBM leaflets remains unknown (El Yandouzi and Le Grimellec, 1992). Taking this uncertainty into account, Chl has been added to the SM/POPC (3:1) mixture at 20, 25, 33, and 50 mol %, corresponding to C/P values of 0.25, 0.33, 0.5, and 1, respectively. The results obtained are presented in Fig. 3 for low magnification images and in Fig. 4 for images at a higher resolution. Addition of 20 mol % Chl to the phospholipid mixture markedly modified the topology of the monolayer (Figs. 3 *B* and 4 *B*). Light domains became connected, with an increase in their relative surface from 48 ± 4% to 68 ± 5% upon Chl addition. They surrounded a discontinuous darker matrix, located at a relative depth of 1.0 ± 0.1 nm, i.e., a value comparable to that obtained for SM/POPC (3:1) in absence of Chl (1.1 ± 0.1 nm). Raising the Chl molar fraction to 25%

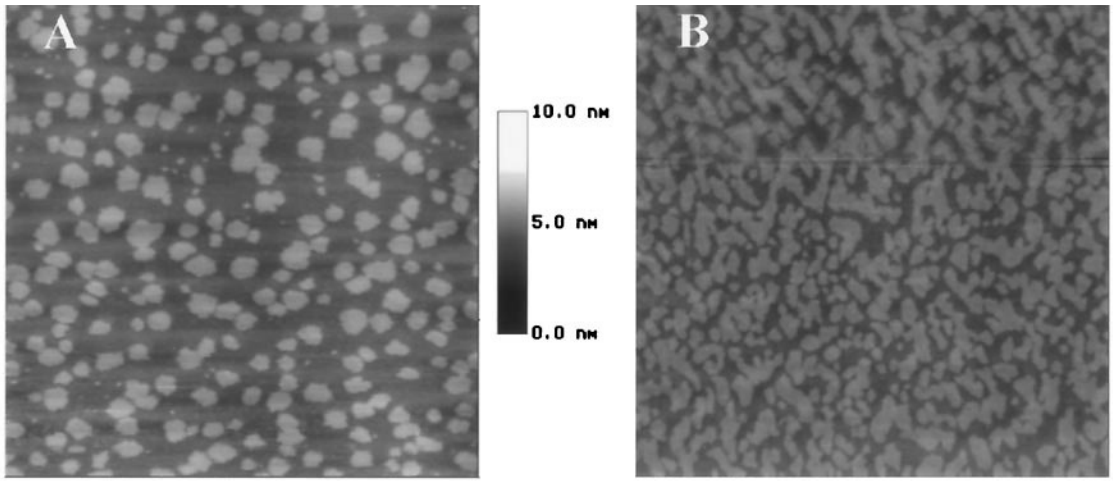

FIGURE 2 AFM images of SM/POPC (2:1, *A*) and (4:1, *B*) monolayers. Scan size: 3 × 3 μm.

resulted in a decrease in the total area (61 ± 5%) and width of light domains, and in their partial disconnection (Figs. 3 *C* and 4 *C*). This was accompanied by a significant reduction of the height difference between the darker and lighter domains (0.7 ± 0.1 nm). At 33 mol % Chl the monolayer surface was still heterogeneous, appearing mostly granular (Fig. 3 *D*). Images at a higher magnification (Fig. 4, *D* and *F*) showed the presence of small light domains (20–70 nm in diameter) close to each others and often connected, protruding by 0.4 ± 0.1 nm from darker domains of a comparable size. Finally, although difficult to estimate from the largest scans (Fig. 3 *E*), the roughness of samples containing 50 mol % Chl remained more pronounced (Fig. 4, *E* and *G*) than that of pure POPC (Fig. 4 *H*), with an RMS of the height fluctuation of 0.4 Å. However, the limited contrast of images did not allow us to ascertain, solely from topological grounds, the presence, or not, of heterogeneity at the surface of the samples.

DISCUSSION

The present experiments indicate that in monolayers made of a phospholipid mixture that mimics that of the outer membrane leaflet of renal proximal cells, large domains are formed due to an LC/LE phase separation. Addition of cholesterol markedly affects the domain organization in a concentration-dependent manner. Domains are clearly observed, on a topological basis, up to 33 mol % cholesterol. These data strongly suggest that, at least for renal epithelial cells, the disappearance of rafts following cholesterol depletion is not necessarily due to the loss of the lateral heterogeneity of membrane lipids.

Existence of domains in pure SM and SM/POPC mixtures

The present observation of a phase separation in pure SM samples agrees with the fact that the LE-LC transition for SM is quite broad, with onset occurring near 12 mN/m and ending above 30 mN/m (Smaby et al., 1996). This result is also in accordance with those obtained by differential scanning calorimetry on SM liposomes where the gel to liquid crystal transition extends from below 20°C to above 45°C (Barenholz, 1984). A comparable thermotropic behavior, occurring in the same temperature range, was reported for the sphingomyelin extracted from renal BBM (Carmel et al., 1985), justifying the use of the bovine brain SM in the present study.

Our data show that in Langmuir-Blodgett monolayers, SM-enriched domains phase-separate from POPC for SM/POPC molar ratios varying from 2:1 to 4:1. They agree with the differential scanning calorimetry data of Untracht and Shipley (1977) on multilamellar SM/egg yolk PC (EPC)/water who reported that, at room temperature, a phase separation occurs for SM/EPC ratios higher than 1:3. For 2:1 and 3:1 molar ratios, the gel-liquid crystal phase separation extends over an ~20°C temperature range, which includes the physiological temperature. One has to recall that the fatty acid composition of EPC (42% of C 16:0 and 52% C 18:1, Untracht and Shipley, 1977) is close to that of POPC. More recently, Ahmed et al. (1997) also observed that, in liposomes made of SM/1-palmitoyl-2-(12-doxyl) stearoyl-phosphatidylcholine (12SLPC) mixtures, phase separation occurs at room temperature when the SM concentration exceeds 30 mol %.

Effects of cholesterol on the topology of SM/POPC (3:1) monolayers

These first results about the existence in LB monolayers of a mesoscopic scale phase separation in ternary phosphatidylcholine/sphingolipid/cholesterol mixtures show that introducing 20 mol % Chl in 3:1 SM/POPC monolayers induced an increase in the area occupied by light domains that became connected. This suggests that cholesterol preferen-

tially interacts with the LC SM-enriched domains. Preferential interaction of cholesterol with sphingomyelin in phosphatidylcholine-sphingomyelin mixtures made from lipids extracted from biomembranes most likely reflects both the better hydrophobic matching between Chl and the longer saturated acyl chains of SM and the possible formation of hydrogen bonds between the cholesterol hydroxyl group and the amide function in sphingomyelins (Brown, 1998; Ramstedt and Slotte, 1999). The absence of significant change in the height difference between the matrix and the protruding domains supports this interpretation. The light domains visualized by AFM are thus likely to be constituted of a mixture of L_o cholesterol-enriched SM domains (Sankaram and Thompson, 1990) coexisting with LC SM-enriched domains. The observation that, at some concentrations, Chl enhances the lateral segregation of the SM/POPC mixture is in agreement with the results obtained on phase-separated phosphatidylcholine and phosphatidylcholine/sphingomyelin mixtures (Silvius et al., 1996; Ahmed et al., 1997).

Increasing the Chl concentration to 25 mol %, which results in a decrease of the size of light domains and their disconnection, is associated with a decrease in the image contrast corresponding to a reduction in the height difference with the matrix. This suggests that for this concentration, Chl now also interacts with the POPC-enriched phase, thereby increasing its thickness. Such an increase in the LE thickness would favor the mixing of the SM species having the shortest acyl chains with the LE phase resulting in a decrease in the size of the light domains. This phenomenon will be accentuated when increasing the Chl concentration to 33 mol %, giving samples where the monolayer is still heterogeneous, and consists of a "network" composed of small (20–70 nm) branched domains emerging from the matrix by ~0.4 nm. Our data showing that a cholesterol concentration higher than 33 mol % is required to suppress the phase separation in membranes made from 3:1 SM/POPC mixtures are not surprising if one considers that Chl concentrations ≥50 mol % can be required to suppress the gel to liquid crystal transition of sphingomyelins (Maulik and Shipley, 1996) and that, up to 30 mol % cholesterol, POPC/Chl monolayers remain in the LE phase at all surface pressures (Worthman et al., 1997). Finally, although the roughness of the 50 mol % Chl samples was significantly higher than that of pure POPC monolayers, the limited contrast of images did not allow us to ascertain the persistence of a phase separation for this Chl concentration.

Supported monolayers as compared to free monolayers and bilayers

The present data illustrate the complementarity between AFM and more classical optical microscopy approaches of phase separation phenomena in membranes. Fluorescence microscopy has been used to observe lipid-lipid interactions and two-dimensional phase transition of phosphatidylcholine/cholesterol monolayers at an air-water interface (Slotte, 1995; Worthman et al., 1997). These studies have provided evidence for the existence of laterally segregated sterol-rich domains coexisting with phospholipid-rich domains at certain compositions and lateral surface pressures. However, due to the properties of the fluorescent probes used and to the resolution of the optical microscope, fluorescence images at surface pressures around 30 mN/m, which approximate the conditions of biological membranes, generally bring limited information. The AFM, by giving access to the mesoscopic scale, demonstrates the existence of lateral segregation in SM and SM/POPC/Chl samples that would have appeared homogeneous using optical microscopy. However, AFM experiments are performed on supported monolayers and an important issue concerns the effect of film transfer and of the underlying substratum on the film organization. Indeed, it was shown that the transfer properties of monolayers strongly depend on the surface pressure of the monolayer (Egusa et al., 1990) and that this phenomenon is very sensitive at low surface pressures. However, when the transfer takes place at high surface pressures, as done in this work, this effect is minimized (Rana et al., 1994) and the lateral organization and the lipid-packing density of the supported film are comparable to those of the film at the air-solution interface (Silvestro and Axelsen, 1998; Schief et al., 2000). It is noteworthy that the 30 mN/m surface pressure chosen for the present experiments is considered to result in a physical state of the phospholipids close to that found in biological membrane bilayers (Demel et al., 1975; Albrecht et al., 1978; Marsh, 1996). Lipid monolayers constitute a valuable tool for the study of lipid-lipid and lipid-protein interactions in membranes (Brockman, 1999). To what extent the superimposition of two monolayers affects their organization remains, however, poorly known. For instance, each monolayer seems to behave independently when forming bilayers from phase-separated dipalmitoylphosphatidylcholine films using the LB technique (Hollars and Dunn, 1998), whereas coupling between leaflets can occur in supported bilayers formed by vesicle fusion (Giocondi et al., 2001). Coupling between the two leaflets is also observed in giant liposomes (Korlach et al., 1999; Bagatolli and Gratton, 2000).

Implications for the lateral organization of epithelial cell brush border membranes

The outer plasma membrane leaflet is assumed to play a key role in the formation of detergent-resistant membrane domains (DRMs) and rafts, two categories of microdomains enriched in sphingolipids and cholesterol. The present data suggest that, in absence of cholesterol, the lipids constituting BBM outer leaflet spontaneously phase-separate and form domains. They also show that a cholesterol concentration higher than 33 mol % is required to eventually suppress the membrane lateral heterogeneity.

Regarding the glycerophospholipid/sphingolipid/cholesterol ratio, the lipid composition of renal BBM (0.9:0.7:1) compares with that of intestinal BBM (1:1:1) (Carmel et al.,

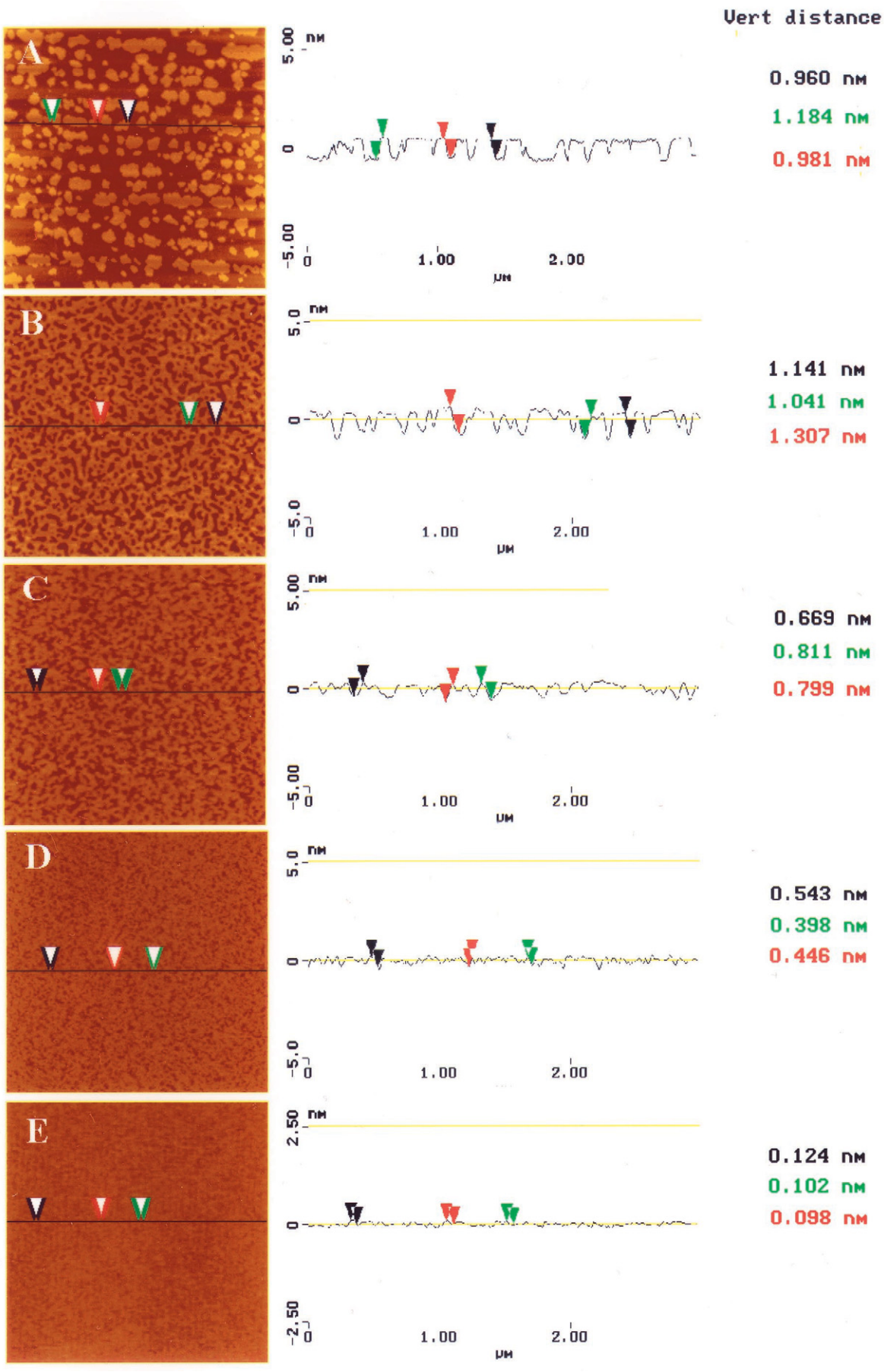
Vert distance
A
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FIGURE 3

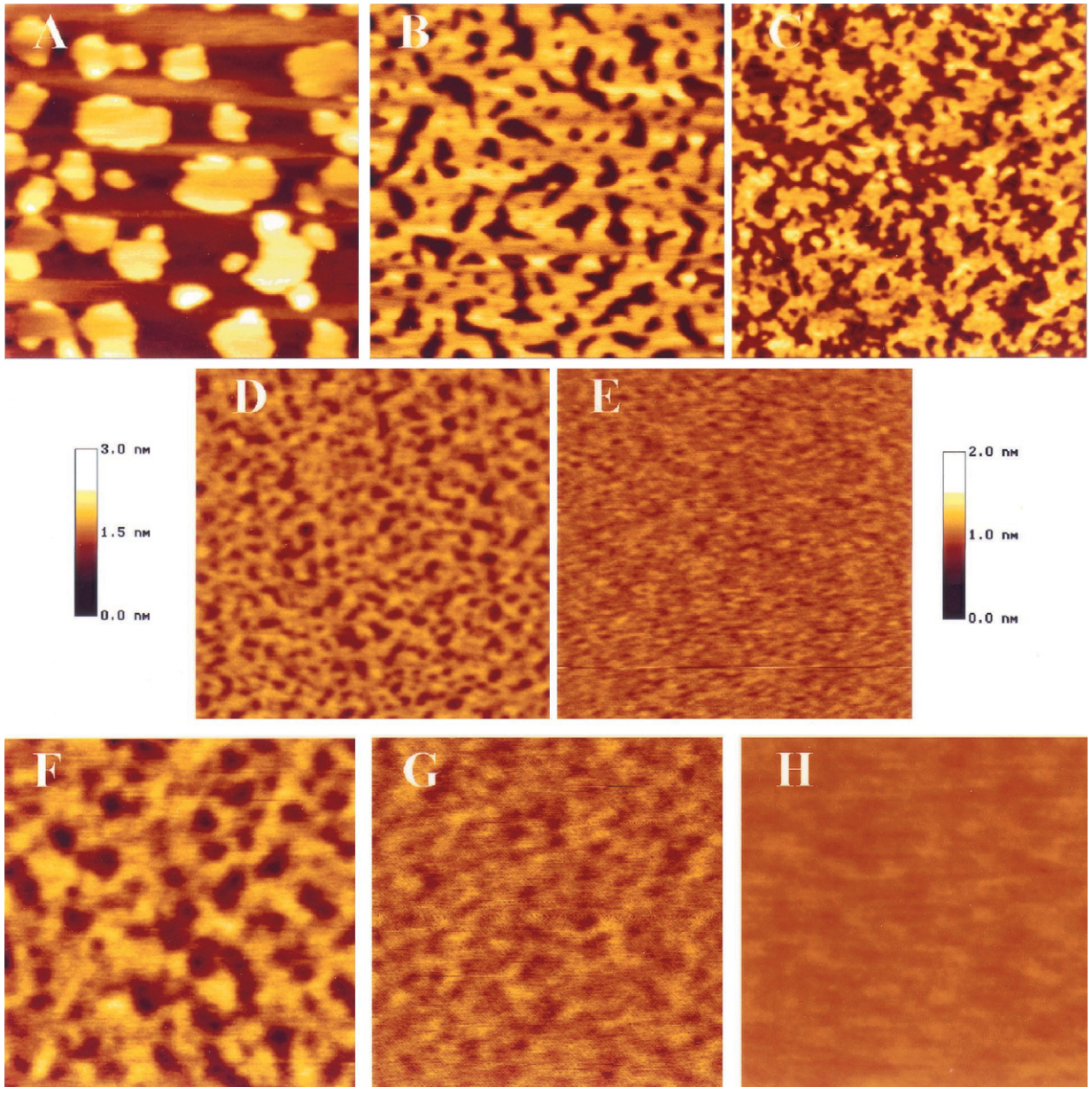

FIGURE 4 High-magnification images showing the effect of increasing cholesterol concentrations on the topology of SM/POPC (3:1) monolayers. (*A–E*) 1 × 1 μm scans, *z* scale (on left) = 3 nm. Cholesterol concentration in (*A*) 0 mol %; (*B*) 20 mol %; (*C*) 25 mol %; (*D*) 33 mol %; (*E*) 50 mol %. (*F–H*) 500 × 500 nm scans with a vertical scale of 2 nm (right scale). *F* and *G* correspond to monolayers with 33 and 50 mol % cholesterol, respectively. *H* is pure POPC monolayer, given for comparison purposes.

1985; Le Grimellec et al., 1988, 1992; Van Meer and Simons, 1988). The main difference between renal and intestinal cells resides in the replacement of the renal cells' sphingomyelin by glycosphingolipids in intestinal cells. The present monolayer studies provide new elements for understanding the presence, both in renal and intestinal BBM, of the broad thermotropic transitions between ~20° and 42°C, detected by DSC, ESR, and fluorescence spectroscopy (Brasitus et al., 1980; Le Grimellec et al., 1982, 1983; Levi et al., 1993). These thermotropic transitions are accompanied by

FIGURE 3 Low magnification images of the effects of cholesterol on the topology of SM/POPC (3:1) monolayers. The first column corresponds to 3 × 3 μm AFM height images of monolayers with the following cholesterol concentrations: (*A*) 0 mol %; (*B*) 20 mol %; (*C*) 25 mol %; (*D*) 33 mol %; (*E*) 50 mol %. The second column gives the virtual section profiles corresponding to the black lines drawn on the images. Vertical distance between the green, red, and black arrows of section analysis curves is given in the third column.

"breaks" in the Arrhenius plots of various transporters and enzymes (Schachter, 1985; Le Grimellec et al., 1992) toward their lower temperature end. Thermotropic transitions occur in BBM despite a high cholesterol content, between ~33 and 50 mol %. However, cholesterol oxidase experiments indicate that about one-third of the BBM cholesterol interacts poorly with other membrane lipids (El Yandouzi and Le Grimellec, 1993), which decreases the effective cholesterol concentration to a level compatible with the observation of the phase separation in monolayers (<33 mol %). In addition, the marked functional changes associated with temperature-dependent modifications of the BBM physical state might also be linked to different lipid-protein interactions between the two cholesterol-dependent liquid ordered phases, $L_{o\alpha}$ and $L_{o\beta}$, described by McMullen and McElhaney (1995).

Finally, the glycerophospholipid/sphingolipid/cholesterol ratios found in renal and intestinal BBM compare with the 1:1:1 ratio determined in the Triton X-100 detergent-resistant membrane fraction (DRM) isolated from MDCK cells by Brown and Rose (1992). Accordingly, the largest part of the apical membrane of renal and intestinal epithelial cells is expected to be resistant to Triton X-100 extraction. In numerous experiments on cultured living cells, the presence of rafts is indirectly characterized by their disappearance upon depletion of the membrane cholesterol using, for instance, β cyclodextrin (Klein et al., 1995; Pike and Miller, 1998; Röper et al., 2000; Pralle et al., 2000; Simons and Toomre, 2000). Our data indicate that, if one only considers the lipid constituents, cholesterol depletion in renal cells should maintain the membrane in a phase-separated state with the presence of gel phase (or $L_{o\beta}$?) enriched sphingomyelin domains, rather than dissipating membrane domains. Accordingly, in these cells, rafts might constitute an ensemble of subdomains recruiting certain categories of sphingolipids that dilute within the total sphingomyelin pool upon cholesterol depletion.

This work was supported by grants from La Fondation pour la Recherche Médicale, l'Association pour la Recherche sur le Cancer, la Région Languedoc-Roussillon, l'Université Montpellier I, and the CNRS-GDR 790.

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