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LIPID PHASES IN RENAL BRUSH BORDER MEMBRANES REVEALED BY LAURDAN FLUORESCENCE*

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Abstract—Steady-state fluorescence properties of 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) have been used to determine the coexistence of separate lipid-phase domains in apical brush border membrane vesicles isolated from the rat renal cortex. The temperature dependence of generalized polarization has been utilized to quantitate the lipid phases. Finally, the effect of cholesterol enrichment on these parameters has been studied. The results indicate the coexistence of lipid-phase domains in brush border membranes based on the values of the generalized polarization and assuming that the membrane state can be described by coexistence of gel and liquid crystalline state. At 37°C, approximately 19–32% of the brush border membrane lipids are in the gel phase, and cholesterol enrichment causes a further concentration-dependent increase in the gel phase of brush border membrane lipids.

INTRODUCTION

In several transporting epithelial cells, including the renal proximal tubule, the plasma membrane exhibits a high degree of morphological, structural and functional polarity. The functional polarity of epithelial plasma membranes is of critical importance for vectorial transport of solutes and water. In kidney proximal tubular cells, the apical membrane, also commonly known as the brush border membrane (BBM),[‡] which borders the lumen, and the basolateral membrane, which faces the interstitium, also markedly differ from each other in their lipid composition. Compared to the basolateral membrane, the BBM has markedly higher cholesterol (Chol) to phospholipid, sphingomyelin to phosphatidylcholine (PC) and saturated to unsaturated fatty acid mol ratios.^{1–3} In addition, there are differences in glycolipid composition.⁴ These lipid compositional differences between the basolateral membrane and the BBM result in a lower fluidity of BBM isolated from the kidney of several species.^{1,5–8} Interestingly, the BBM also exhibits asymmetry of distribution of phospholipids into its two leaflets. Sphingomyelin accounts for 75% of the amount of phospholipids present on the external membrane leaflet, which faces the lumen. The inner leaflet, which faces the cytoplasm, is richer in phosphatidylethanolamine, phosphatidylserine, PC and phosphatidylinositides.⁹

The large compositional heterogeneity of BBM lipids and their asymmetrical distribution in the two leaflets suggest that in BBM there may be the presence of lipid domains that may differ in their individual lipid composition as well as their phase state. In BBM from human kidney cortex electron spin resonance experiments with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) indicate two distinct thermotropic transitions, at 20°C and 40°C.⁵ In BBM from rabbit kidney cortex steady-state fluorescence anisotropy measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) similarly indicate two distinct thermotropic transitions, at 22°C and 47°C.⁸ In BBM from rabbit kidney cortex fluorescence lifetime characteristics of *trans*- and *cis*-parinaric acid suggest the presence of coexisting solid-phase and fluid-phase lipid domains. In fact, at the physiologic temperature (37°C), approximately 15% of the BBM lipids are seemingly in the solid phase.⁸ Similarly, in rat intestinal BBM, fluorescence anisotropy of DPH and differential scanning calorimetry (DSC) measurements also indicate broad thermotropic transitions that occur near or at the physiologic temperature.^{10,11}

The purpose of the present study is to utilize the sensitivity of the excitation and emission spectra of 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) to the properties of the lipid environment^{12,13} to demonstrate and quantitate lipid phases in BBM isolated from the renal cortex. In addition, because alterations in BBM Chol content modulate BBM alkaline phosphatase^{2,3,14,15} and phosphate transport activity,^{2,15,16} we also determine the effect of BBM Chol enrichment on BBM lipid-phase characteristics.

MATERIALS AND METHODS

Brush border membrane isolation. All studies were performed in male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN). Brush border membranes from the renal superficial cortex were isolated by differential centrifugation and magnesium precipitation as previously described from our laboratory.^{3,15–17} Protein was determined by the method of Lowry using crystalline bovine serum albumin as standard.¹⁸ Enzyme activity measurements including alkaline phosphatase, maltase, leucine aminopeptidase, gamma-glutamyl transferase (BBM-specific) and Na,K-ATPase (basolateral membrane-specific) were measured by kinetic spectrophotometric techniques

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‡Abbreviations: BBM, brush border membrane; Chol, cholesterol; CHS, cholesteryl hemisuccinate; DPPC, dipalmitoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; GP, generalized polarization; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; PC, phosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TLC, thin layer chromatography.

Figure 1. Excitation spectra of Laurdan in BBM: (—) at 13°C, (·····) at 25°C, (---) at 37°C, (-·-·-) at 49°C, (- - - -) at 61°C. Emission at 440 nm. Spectra are normalized at 340 nm.

Figure 2. Emission spectra of Laurdan in BBM: (—) at 13°C, (·····) at 25°C, (---) at 37°C, (-·-·-) at 49°C, (- - - -) at 61°C. Excitation at 340 nm. Spectra are normalized at 430 nm.

on homogenate and BBM fractions to determine the specific activity and enrichment factor (specific activity in BBM fraction/specific activity in homogenate) of each BBM preparation.^{3,15-17} Typically the BBM were enriched at least 12-fold for alkaline phosphatase, maltase, leucine aminopeptidase and gamma-glutamyl transferase and less than 1.3-fold for Na,K-ATPase.

Modulation of BBM Chol content. Modulation of BBM Chol content was achieved by incorporation of cholesteryl hemisuccinate (CHS) (Steraloids Inc., Pawling, NY), hydrophilic Chol ester, as previously described.¹⁶ Briefly, freshly isolated BBM were suspended in 3.5% polyvinylpyrrolidone, 1.0% bovine serum albumin, 0.5% glucose in phosphate-buffered saline, pH 7.5. Ethanol or CHS freshly dissolved in ethanol was then added, and the BBM were incubated in a shaking water bath at 37°C for 60 min. In additional experiments to determine for nonspecific sticking rather than specific incorporation of the lipid to the BBM, Chol freshly dissolved in ethanol was also added. BBM were then washed three times in an ice-cold buffer consisting of 300 mM mannitol, 16 mM Hepes, 10 mM Tris, pH 7.5 and then aliquoted for simultaneous measurements of (1) lipid composition, and (2) fluorescence measurements.

Brush border membrane lipid composition measurements. Total lipids were extracted by the method of Bligh and Dyer.¹⁹ Coprostanol (Supelco Inc., Bellefonte, PA) was added as an internal standard for Chol determination. To determine the BBM total Chol content, *i.e.* free Chol plus cholesteryl ester, an aliquot of the BBM sample was subjected to alkaline hydrolysis in a reaction mixture consisting of 0.3 mL of 33% KOH and 3.0 mL of 88% ethanol, at 70°C for 30 min. Cholesterol content was determined by gas chromatography as previously described from our laboratory.^{3,15-17} BBM Chol content was also determined by measuring free Chol and cholesteryl ester content separately. Free and esterified Chol were separated by one-dimensional thin layer chromatography (TLC: silica gel; Eastman Kodak, Rochester, NY) with a solvent system that consisted of heptane/diethylether/glacial acetic acid (85:15:2, vol/vol). The amount of Chol in the Chol and cholesteryl ester spots was then measured as above. Individual phospholipid polar head group species were separated by two-dimensional TLC (silica gel 60; E. Merck, Darmstadt, West Germany) as previously described from our laboratory.^{3,15-17} Phospholipid content in the total lipid and individual phospholipid extract was determined by measuring the phosphorus content by the method of Ames and Dubin.²⁰

Brush border membrane fluorescence measurements. Brush border membrane samples corresponding to 0.3 mg BBM protein were resuspended in 150 mM NaCl, mM Hepes, 10 mM Tris, pH 7.40, previously deoxygenated by vigorous nitrogen bubbling for 1 h. One microliter of Laurdan (Molecular Probes, Eugene, OR) was then added from a 1 mM stock solution, resulting in a probe/lipid ratio of 1:360. Steady-state excitation and emission spectra were acquired on a spectrofluorometer (model 4800C, SLM Instruments, Urbana, IL) equipped with a xenon-arc lamp, using 4 nm bandwidth. Temperature was controlled by a water circulating bath (Neslab Instruments, Portsmouth, NH), and the actual temperature was measured in the sample cuvette.

RESULTS

Laurdan excitation and emission spectra

Fluorescence excitation spectra for Laurdan in BBM as a function of temperature are shown in Fig. 1. In BBM at 13°C the excitation spectrum is characterized by two maxima, at 357 nm (blue band) and 393 nm (red band). Increasing the temperature to 61°C causes a slight spectral shift and a gradual decrease in the fluorescence intensity at both excitation maxima. The decrease in the red band, however, is greater than the decrease in the blue band, resulting in a marked decrease in the 410 nm/340 nm fluorescence intensity ratio at emission wavelengths of either 440 nm or 490 nm.

Fluorescence emission spectra for Laurdan in BBM as a function of temperature are shown in Fig. 2. In BBM at 13°C the emission spectrum is characterized by a maximum at 430 nm (blue band). Increasing the temperature to 61°C causes the appearance of a second emission maximum at 488 nm (red band). This results in a marked increase in the 490 nm/440 nm fluorescence intensity ratio at either excitation wavelength of 340 nm or 410 nm.

Generalized polarization of Laurdan

The fluorescence intensity of Laurdan is quantitated by the generalized polarization (GP), which is defined as

$$GP = \frac{I_B - I_R}{I_B + I_R} \quad (1)$$

where I_B and I_R are the emission intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum, respectively.

In Fig. 3 the GP for Laurdan in BBM is plotted as a function of temperature. Increasing temperature causes a decrease in the GP; however, in contrast to phospholipid vesicles,^{12,13} the slope of the change in GP as a function of temperature is very broad with possible deflection points at approximately 20°C and 60°C. It should be noted that the temperature-dependent decrease in GP is reversible upon cooling when the BBM are warmed up to 61°C. At higher temperatures, especially 73°C and higher, the GP values are not reversible upon cooling. Thus, the temperature-dependent decrease in GP at temperatures above 73°C may indicate protein de-

Figure 3. Generalized polarization of Laurdan in BBM as a function of temperature. (a): Excitation at 340 nm. (b): Excitation at 410 nm. Emission at 440 nm and 490 nm.

naturation and hence may reflect the contribution of protein-lipid interactions to GP of Laurdan in BBM.

The addition rule of GP¹³ can be used to quantitate the lipid phases at physiologic temperature (37°C) for BBM. The addition rule can be expressed as

$$GP(37^\circ C) = \frac{xGP_G S_G + (1-x)GP_L S_L}{xS_G + (1-x)S_L} \quad (2)$$

where GP_G and GP_L and the GP of the pure gel and pure liquid crystalline phases, respectively. S_G is the sum of the intensities I_B + I_R for the gel phase, and S_L is the same but for the liquid crystalline phase. X is the relative fraction of gel phase.

Assuming that at 0°C the BBM are in the gel phase and at 80°C the BBM are in the liquid crystalline phase, for BBM at 37°C we calculate that at an excitation wavelength of 340 nm, 32% of the BBM lipids, and at an excitation wavelength of 410 nm, 19% of the BBM lipids are in the gel or gel-like phase.

For BBM the possible coexistence of lipid phases at a physiological temperature of 37°C is also suggested by the dependence of the Laurdan emission spectra on the excitation wavelength.²¹ In Fig. 4 we see that the ratio of the two emission spectra, the emission obtained at an excitation of 340 nm divided by the emission obtained at an excitation of 410 nm, is highly dependent on the excitation wavelength.

Figure 4. Ratio spectra between Laurdan steady-state emission spectra obtained using excitation at 340 nm and 410 nm. BBM (—) at 13°C, (·····) at 25°C, (---) at 37°C, (-·-·-) at 49°C, (- - - -) at 61°C.

Laurdan excitation and emission spectra in Chol-enriched BBM

Fluorescence excitation spectra for Laurdan in control and Chol-enriched BBM at 37°C are shown in Fig. 5. Cholesterol enrichment causes a gradual increase in the fluorescence intensity at both excitation maxima. The increase in the red band however is greater than the increase in the blue band. Cholesterol enrichment also has a significant effect on the emission spectra, causing a gradual decrease in the fluorescence intensity at the red band (Fig. 6). The effects of Chol enrichment on the Laurdan excitation and emission spectra are strikingly similar to the effects of decreasing temperature (Figs. 1 and 2). In fact, the effect of a 48 mol% enrichment in BBM Chol, resulting in an increase in Chol content from 46.2 to 55.9 mol%, is similar to the effect of decreasing BBM temperature from 37°C to 25°C.

Generalized polarization of Laurdan in control and Chol-enriched BBM

In Fig. 7 the GP for Laurdan in control and Chol-enriched BBM are plotted as a function of temperature. At tempera-

Figure 5. Excitation spectra of Laurdan in BBM at 37°C. (—) Control (46.2 mol% Chol); (·····) +12% cholesterol (49.0 mol% Chol); (---) +24% cholesterol (51.5 mol% Chol); (-·-·-) +48% cholesterol (55.9 mol% Chol). Emission at 440 nm. Spectra are normalized at 340 nm.

Figure 6. Emission spectra of Laurdan in BBM at 37°C. (—) Control (46.2 mol% Chol); (·····) +12% cholesterol (49.0 mol% Chol); (---) +24% cholesterol (51.5 mol% Chol); (-·-·-) +48% cholesterol (55.9 mol% Chol). Excitation at 340 nm. Spectra are normalized at 340 nm.

tures of 25°C and above, Chol enrichment causes dose-dependent marked increases in GP. Cholesterol enrichment also causes a marked broadening of the slope of the change in GP as a function of increasing temperature.

Using the relationship of GP to the GP of the pure gel and pure liquid crystalline phases (Eq. 2), and assuming that at 0°C the BBM are in the gel phase and at 61°C the BBM are in the liquid crystalline phase, we estimate that at 37°C Chol enrichment causes dose-dependent marked increases in the gel phase of BBM (Table 1). The dependency of the GP and the calculated % gel phase on the excitation wavelength is of interest and suggests that in BBM Laurdan molecules sur-

Figure 7. Generalized polarization of Laurdan in BBM as a function of temperature. (—) Control (46.2 mol% Chol); (·····) +12% cholesterol (49.0 mol% Chol); (---) +24% cholesterol (51.5 mol% Chol); (-·-·-) +48% cholesterol (55.9 mol% Chol). (a): Excitation at 340 nm. (b): Excitation at 410 nm. Emission at 440 nm and 490 nm.

Table 1. Percent gel phase in control and cholesterol (Chol)-enriched brush border membranes (BBM)

	Excitation 340 nm	Excitation 410 nm
BBM (control)	32.4 ± 1.4	19.0 ± 1.1
BBM (+12% Chol)	42.2 ± 0.9	26.2 ± 0.6
BBM (+24% Chol)	49.9 ± 1.4	31.9 ± 1.0
BBM (+48% Chol)	57.7 ± 1.8	41.7 ± 1.3

Percent gel phase for BBM at 37°C were calculated from the Laurdan emission intensities at 440 nm and 490 nm at 0°C, 37°C and 60°C as described in the Materials and Methods (Eq. 2).

rounded by lipids in the gel phase are preferentially excited in the blue part of the spectrum, whereas Laurdan molecules surrounded by lipids in the liquid crystalline phase are preferentially excited in the red part of the excitation spectrum.

The possible coexistence of lipid phases at 37°C is further suggested by the dependence of the Laurdan emission spectra on the excitation wavelength. In Fig. 8 we see that the ratio of the two emission spectra, the emission obtained at excitation of 340 nm divided by the emission obtained at excitation of 410 nm, is highly dependent on the excitation wavelength.

DISCUSSION

Previous studies in BBM isolated from human and rabbit kidney revealed the existence of a broad thermotropic transition extending from approximately 20–22°C to 42–47°C.^{6,8} These studies therefore suggested that at the physiological temperature of 37°C in BBM there may be coexistence of lipid phases in the gel and liquid crystalline phases. In the present study we utilized the highly phase-sensitive fluorescence probe Laurdan,^{12,13} and we found evidence for the coexistence of lipid phases in rat BBM at 37°C. The emission spectra of Laurdan in BBM demonstrated that the emission

Figure 8. Ratio spectra between Laurdan steady-state emission spectra in BBM at 37°C obtained using excitation at 340 nm and 410 nm. (—) Control (46.2 mol% Chol); (·····) +12% cholesterol (49.0 mol% Chol); (---) +24% cholesterol (51.5 mol% Chol); (-·-·-) +48% cholesterol (55.9 mol% Chol).

spectral shift from 440 nm to 490 nm, which occurs when phospholipid vesicles undergo phase transition from the gel to the liquid crystalline phase,^{12,13} does not occur until the BBM are at 49–61°C. Furthermore, using the addition rule of GP of Laurdan (Eq. 2), we estimated that at 37°C 19–32% of the BBM lipids are in the gel or gel-like phase. These results are in agreement with the parinaric acid fluorescence lifetime data in rabbit BBM, which estimated that at 37°C at least 10–15% of the BBM lipids are in the gel phase.⁸ The dependence of the ratio of the two emission spectra, the emission obtained at an excitation of 340 nm divided by the emission obtained at an excitation of 410 nm, on the excitation wavelength (Fig. 4) further suggests the coexistence of more than one phase.²¹

In a recent study of Chol/dipalmitoylphosphatidylcholine (DPPC) mixtures, ²H nuclear magnetic resonance and DSC measurements have identified the presence of (1) three distinct phases: the liquid crystalline phase, the gel phase and a high Chol concentration phase, which is termed a liquid-ordered phase, and (2) three regions of two-phase coexistence.²² At the present time we do not know whether these three phases also exist in renal BBM, and whether Laurdan fluorescence can distinguish between the gel phase or the liquid-ordered phase. Detailed studies of Laurdan fluorescence in Chol/DPPC or Chol/BBM phospholipid mixtures containing various amounts of Chol and as a function of temperature need to be performed. For the time being we use the term gel phase-like to indicate the possible existence of the classical gel phase as well as the liquid-ordered phase.

In using the addition rule of GP of Laurdan to determine the % gel phase lipids at 37°C (Eq. 2), we assume that at 0°C the BBM lipids are in the gel phase and at 61°C the BBM lipids are in the liquid crystalline phase. Although these assumptions are probably accurate for phospholipid vesicle mixtures, as the GP of Laurdan as a function of temperature reaches an asymptotic value as the phospholipids are cooled to 0°C or warmed to 60°C,^{12,13} they may not be quite accurate for BBM because the GP of Laurdan does not reach a steady value at either 0°C or 80°C (Fig. 3). Within the experimental limitations, however, we make these assumptions to provide an approximate value for the % gel phase-like lipids in BBM at 37°C.

The BBM has a very complex and heterogeneous lipid composition. Each 1 mg of BBM protein has, on the average, 420 nmol Chol, 490 nmol total phospholipid, 184 nmol sphingomyelin, 104 nmol PC, 124 nmol phosphatidylethanolamine, 68 nmol phosphatidylserine and 10 nmol phosphatidylinositol.^{2,3} In addition, the BBM has a high saturated-to-unsaturated fatty acid ratio.^{1,17} Finally, the BBM contains a number of neutral glycolipids and gangliosides.⁴ The relatively high amount of Chol and, in addition, the presence of sphingomyelin, saturated fatty acids, glycolipids and gangliosides may explain the presence of gel phase-like lipids in BBM at 37°C. Differential scanning calorimetry studies in synthetic Chol-sphingomyelin and glucosylceramide-DPPC mixtures have indeed shown the gel-to-liquid crystalline phase transition to occur above 40°C.^{23–26}

The relatively high molar content of Chol is probably the major cause for the presence of the gel phase lipids in BBM, as well as for the absence of a distinct sharp phase transition of BBM lipids. Differential scanning calorimetry experiments

in synthetic Chol-sphingomyelin and Chol-DPPC mixtures have shown that above 25 mol% Chol there is absence of phase transition.^{23,24,27} In agreement with these studies, we have found that further enrichment of BBM with Chol causes a further concentration-dependent broadening or decrease in the slope of GP of Laurdan as a function of temperature. In addition, further enrichment with Chol also causes a further concentration-dependent increase in the % gel phase lipids of the BBM.

Protein-lipid interactions may also play a role in the coexistence of lipid phases in BBM. A previous study showed that in renal BBM the fluorescence polarization of DPH is lower in BBM total lipid extracts than in intact BBM.² In the present study we found that the temperature-dependent decrease in GP of Laurdan is completely reversible up to 61°C, but that there is an irreversible component beyond 73°C, which may be caused by protein denaturation.

Another important phenomenon that may exist in BBM is the presence of hydrocarbon chain interdigitation. The transbilayer distribution of phospholipids is highly asymmetrical in BBM, as sphingomyelin accounts for 75% of the phospholipids present in the external leaflet, whereas phosphatidylethanolamine, phosphatidylserine, PC and phosphatidylinositol comprise the majority of phospholipids present in the inner layer of the membrane.⁹ The sphingomyelin from BBM is a mixture of many molecular species with various fatty acyl chain moieties. Palmitic (16:0, 20%), stearic (18:0, 32%), arachidonic (20:4, 25%) and lignoceric (24:0, 15%) acids constitute the major fatty acyl groups.²⁸ The sphingosine moiety of sphingomyelin contributes one hydrocarbon chain of 15 carbons to the molecule. Consequently, the *N*-acyl chain contains more methylene units than does the other hydrocarbon chain contributed by sphingosine. Most of the molecular species of sphingomyelin thus exhibit a marked hydrocarbon chain-length asymmetry. In synthetic sphingomyelin bilayers with a lingoceroyl acyl group (C[24]:sphingomyelin), and in chain-length asymmetric saturated PC such as C(18):C(12) PC, studies with DSC and Raman spectroscopy have provided evidence for the formation of a mixed interdigitated chain packing, which is more ordered than the usual noninterdigitated bilayer.^{29–31} Although the presence of lipid interdigitation in BBM has not been proven, its presence would have profound effects on the biophysical state of the membrane.

In summary, Laurdan fluorescence data indicate the presence of coexisting lipid phases in rat renal BBM, which is further accentuated by Chol enrichment. The coexisting lipid phases may play an important role in the modulation of renal tubular physiology, including alterations in alkaline phosphate^{2,3,14,15} and phosphate transport activity.^{2,15,16}

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