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Interaction of ceramides with phosphatidylcholine, sphingomyelin and sphingomyelin/cholesterol bilayers

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Abstract

Ceramides (Cers) may exert their biological activity through changes in membrane structure and organization. To understand this mechanism, the effect of Cer on the biophysical properties of phosphatidylcholine, sphingomyelin (SM) and SM/cholesterol bilayers was determined using fluorescence probe techniques. The Cers were bovine brain Cer and synthetic Cers that contained a single acyl chain species. The phospholipids were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glyero-3-phosphocholine (DPPC) and bovine brain, egg yolk and bovine erythrocyte SM. The addition of Cer to POPC and DPPC bilayers that were in the liquid-crystalline phase resulted in a linear increase in acyl chain order and decrease in membrane polarity. The addition of Cer to DPPC and SM bilayers also resulted in a linear increase in the gel to liquid-crystalline phase transition temperature (T_M). The magnitude of the change was dependent upon Cer lipid composition and was much higher in SM bilayers than DPPC bilayers. The addition of 33 mol% cholesterol essentially eliminated the thermal transition of SM and SM/Cer bilayers. However, there is still a linear increase in acyl chain order induced by the addition of Cer. The results are interpreted as the formation of DPPC/Cer and SM/Cer lipid complexes. SM/Cer lipid complexes have higher T_Ms than the corresponding SM because the addition of Cer reduces the repulsion between the bulky headgroup and allows closer packing of the acyl chains. The biophysical properties of a SM/Cer-rich bilayer are dependent upon the amount of cholesterol present. In a cholesterol-poor membrane, a sphingomyelinase could catalyze the isothermal conversion of a liquid-crystalline SM bilayer to a gel phase SM/Cer complex at physiological temperature.

Keywords: Ceramide; Phosphatidylcholine; Sphingomyelin; Cholesterol; Fluorescence; Model membrane

Abbreviations: BB-Cer, bovine brain ceramide; BB-SM, bovine brain sphingomyelin; BE-SM, bovine erythrocyte sphingomyelin; Cer, ceramide; C24:1-Cer, *N*-nervonoyl-sphingosine; C18:1-Cer, *N*-oleoyl-sphingosine; C16:0-Cer, *N*-palmitoyl-sphingosine; C18:0-Cer, *N*-stearoyl-sphingosine; C16:0-SM, *N*-(palmitoyl)sphingosylphosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPH-Cer, *N*-3-(4-(6-phenyl)-1,3,5-hexatrienyl)phenylpropanoyl)sphingosine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPG, 1,2-dipalmitoyl-*sn*-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DRM, detergent-resistant membrane domains; EY-SM, egg yolk sphingomyelin; G.P., generalized polarization; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 1,2-POG, 1-palmitoyl-2-oleoyl-*sn*-glycerol; Prodan, 6-propionyl-2-dimethylaminonaphthalene; SM, sphingomyelin; T_M, midpoint temperature of the gel to liquid-crystalline phase transition

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1. Introduction

Sphingolipid and cholesterol-rich microdomains are involved in plasma membrane signal transduction, cholesterol efflux and intracellular lipid and protein trafficking [1,2]. These membrane microdomains can be isolated as detergent-resistant membrane domains (DRMs) for structural and functional studies [3,4]. Sphingolipid and cholesterol-rich microdomains are in a liquid-ordered phase where the sphingolipid molecules have restricted acyl chain motion but have high lateral mobility [1,2]. Sphingomyelin (SM) is the major sphingolipid in the plasma membrane and is the substrate for the enzyme catalyzed formation of the lipid second messenger, ceramide (Cer) [5]. The cellular concentration of Cer can be quite high in reaching levels of 1–10 mol% of the total phospholipid concentration and thus Cer can be a major lipid component in SM-rich microdomains [6]. One possible mechanism for the biological action of Cers is through changes in membrane structure and organization through the formation of Cer-rich microdomains [7–11]. However, there is little information on the lipid–lipid interactions between SM, Cer and cholesterol to understand their role in regulating the membrane physical properties of sphingolipid and cholesterol-rich microdomains.

When compared to physiological phosphatidylcholines, SMs are more saturated, are asymmetric due to the amide linkage of very long chain fatty acids and are more prone to intermolecular hydrogen bonding [1,2]. Unlike phosphatidylcholines, naturally occurring SMs demonstrate gel to liquid-crystalline phase transitions in the physiological temperature range [12]. The *N*-linked acyl chain composition can be important in determining the interaction of SMs with other phospholipids [13] and with cholesterol [14–16]. Also, *N*-linked acyl chain composition may be important in determining SM and Cer metabolism [17–18] and Cer function [19]. Cers, which have a single hydroxyl polar head group, are the most condensed sphingolipid [20,21] and demonstrate the highest thermal transition temperature [22–24]. In bilayer mixtures of Cer and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), laterally segregated Cer-enriched microdomains can be formed [8,24,25]. Cers can segregate into microdomains in lamellar phase phosphatidylethanolamine bilayers and also facilitate the lamellar hexagonal phase transition [9,10]. Cer-enriched microdomains may also be formed in liquid-crystalline phase phosphatidylcholine bilayers [11]. Diacylglycerols and Cers are membrane-associated lipid second messenger molecules that are formed by the enzymatic removal of the phosphorylcholine headgroup of phosphatidylcholine and SM. However, they can have very different effects on membrane physical properties due to differences in their fatty acyl chain composition. This has been demonstrated in model systems where phospholipase C catalyzed diacylglycerol formation-induced vesicle fusion and sphingomyelinase catalyzed Cer formation-induced vesicle leakage [26]. Thus, the interaction of Cers with other membrane components, e.g. SM, should be an important factor in determining membrane lateral organization and physical properties. Also, the effect of cholesterol on the interaction of Cer with SM may also be important in regulating signal transduction processes within sphingolipid and cholesterol–cholesterol microdomains.

We have used fluorescence probe techniques to measure the bilayer physical properties of mixtures of Cer, SM and phosphatidylcholine. We have also determined the effect of cholesterol on mixtures of bovine brain SM (BB-SM) and bovine brain Cer (BB-Cer). To vary the *N*-linked acyl chain, we have used the naturally occurring SMs, BB-SM (containing primarily stearic (C18:0) and nervonic (C24:$1^{\Delta 15}$) acids), bovine erythrocyte SM (containing primarily lignoceric (C24:0) acid) and chicken egg yolk SM (containing primarily palmitic (C16:0) acid). The fatty acid composition of BB-SM is similar to those found in cells, e.g. U937 monocytic cells [18], from which DRMs have been isolated [27]. For comparison, we also studied an unsaturated (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, POPC) and a saturated (DPPC) phosphatidylcholine (PC). BB-Cer and synthetic Cers with specific *N*-linked acyl chains were used. The interaction between SM and Cer is analogous to the interaction between saturated phosphatidylcholines and diacylglycerols [28,29]. These studies have indicated the formation of specific phosphatidylcholine/diacylglycerol complexes. Our results demonstrate that the interaction of Cer with SMs and phosphatidylcholines is different and that the

N-linked acyl chain can be a factor in these interactions. These molecular species differences may be due to differences in acyl chain interactions that allow the formation of tighter or weaker lipid complexes. We have also demonstrated that cholesterol and Cer have additive effects on the physical properties of SM bilayers. The addition of sufficient amounts of cholesterol to form a liquid-ordered phase may disrupt the formation of SM/Cer complexes.

2. Materials and methods

2.1. Materials

POPC, DPPC, BB-SM, BB-Cer, egg yolk sphingomyelin (EY-SM), and egg D-*erythro*-sphingosine were from Avanti Polar Lipids (Alabaster, AL, USA). The BB-Cer was derived from BB-SM and both have identical fatty acid compositions. Bovine erythrocyte sphingomyelin (BE-SM), *N*-nervonoyl-sphingosine (C24:1-Cer), *N*-oleoyl-sphingosine (C18:1-Cer), *N*-palmitoyl-sphingosine (C16:0-Cer) and *N*-stearoyl-sphingosine (C18:0-Cer) were from Sigma (St. Louis, MO, USA). 1,6-Diphenyl-1,3,5-hexatriene (DPH), 6-propionyl-2-dimethylaminonaphthalene (prodan), 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan) and 3-(4-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid were from Molecular Probes (Eugene, OR, USA). High purity cholesterol was from Calbiochem-Novabiochem (La Jolla, CA, USA). The synthesis of *N*-3-(4-(6-phenyl)-1,3,5-hexatrienyl)phenylpropanoyl)sphingosine (DPH-Cer) was performed by *N*-acylation of D-*erythro*-sphingosine with the *N*-hydroxysuccinimidyl ester of the fluorescent fatty acid [30].

2.2. Liposome preparation

The required amounts of phospholipids, Cers, cholesterol and fluorescent probe were mixed in chloroform. The organic solvent was evaporated under a stream of nitrogen and the sample dried under vacuum for at least 3 h in a lyophilizer. The dried lipids were dispersed in buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4) by vortexing. The lipids were warmed above their gel to liquid-crystalline phase transition temperatures to ensure complete hydration. The fluorescent probe to phospholipid molar ratio was 1–250 mol/mol, unless stated otherwise.

2.3. Fluorescence measurements

DPH and DPH-Cer were used to measure phospholipid acyl chain motion [31,32]. The fluorescence polarization measurements were performed as previously described [31,32] where the excitation wavelength was 350 nm and the emission was monitored with a Corning 3-144 cutoff filter. The fluorescence properties of prodan and laurdan were used to measure the microenvironment of the phospholipid bilayers [33–35]. Prodan appears to be a better probe for the bilayer surface than laurdan which occupies a deeper region of the bilayer. The fluorescence spectra of prodan and laurdan were recorded and the measured fluorescence intensity at 420 and at 480 nm were used to calculate the generalized polarization values (G.P.) [34,35]. The excitation wavelength was 350 nm.

3. Results

3.1. Fluorescence polarization of DPH and DPH-Cer

The fluorescence polarization of DPH and DPH-Cer was used to determine the effect of the addition of BB-Cer on the acyl chain motion and phase properties of POPC, DPPC, BB-SM, EY-SM and BE-SM bilayers. The polarization measurements of DPH in liquid-crystalline phase POPC bilayers increased only slightly upon the incorporation of BB-Cer and demonstrated essentially no change with temperature (FigA. 1). At 25°C, a linear correlation was found for the change in fluorescence polarization with the mol% Cer in the bilayer when the BB-Cer concentration was varied up to 30 mol% (Fig. 1A, insert). For gel phase DPPC bilayers, there was a small increase in the polarization values with the addition of BB-Cer. There was a dramatic decrease in the fluorescence polarization values at the gel to liquid phase transition temperature ($T_M = 42$°C) of DPPC. The addition of BB-Cer shifted the temperature at which there was a 50% change in the fluorescence polarization values (midpoint temperature) to slightly higher temperatures. Above T_M in the liquid-crystalline

phase, the polarization values in DPPC bilayers was similar to that found in POPC (Fig. 1B). BB-SM, EY-SM and BE-SM have different acyl chain compositions and different thermal properties [36,37].

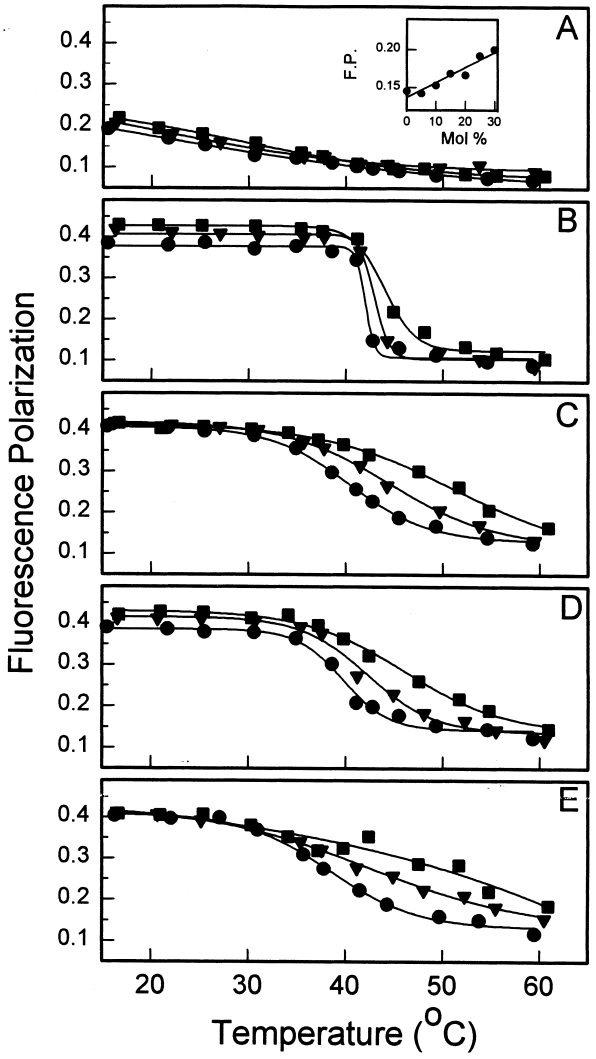

Fig. 1. The effect of BB-Cer on the fluorescence polarization of DPH in POPC (A), DPPC (B), BB-SM (C), EY-SM (D) and BE-SM (E) bilayers was measured as a function of temperature. The measurements are for bilayers that contain 0 (●), 5 (▼) and 10 (■) mol% BB-Cer. The insert in (A) demonstrates the fluorescence polarization (F.P.) as a function of the mol% BB-Cer in POPC bilayers when measured at 25°C. The data for DPPC, BB-SM, EY-SM, and BE-SM were fitted with a sigmoidal regression analysis (solid line) to obtain a value for the midpoint temperature (T_M) for a 50% change in the fluorescence polarization values. The equation used was $p(T)=p_0+a/(1+\exp(-(T-T_M))/b)$, where $p(T)$ was the measured polarization value at a given temperature (T) and p_0, a, b and T_M were constants. The phospholipid concentration was 0.1 mg/ml and the probe/phospholipid molar ratio was 1/250.

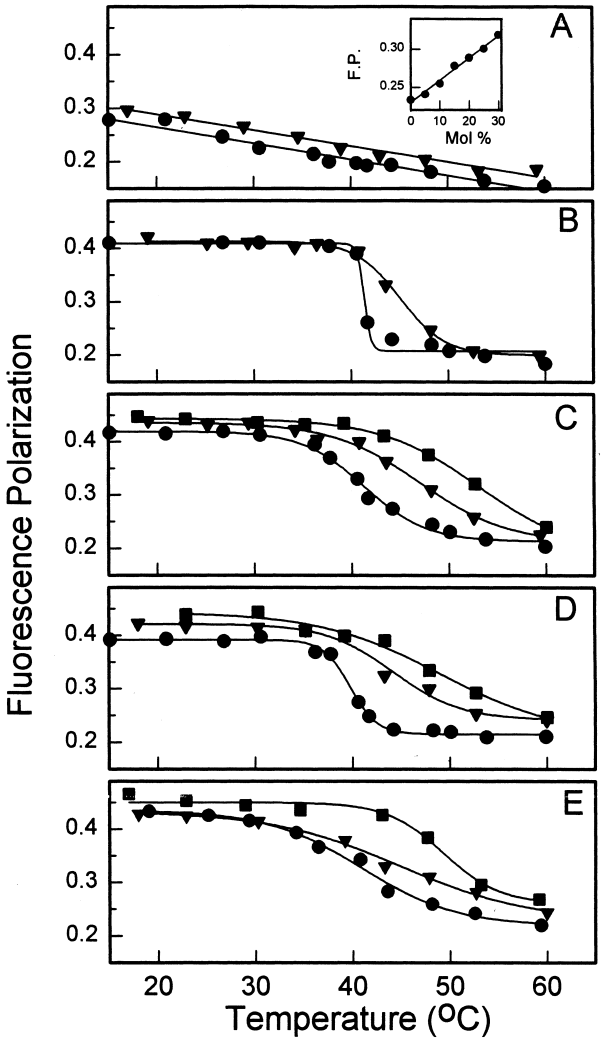

Fig. 2. The effect of BB-Cer on the fluorescence polarization of DPH-Cer in POPC (A), DPPC (B), BB-SM (C), EY-SM (D) and BE-SM (E) bilayers was measured as a function of temperature. The concentration of DPH-Cer was 1 mol% and BB-Cer was added to give a final concentration of Cer of 1, 5 and 10 mol%. For (A), the samples consist of POPC/1 mol% Cer (●) and POPC/10 mol% Cer (▼). In (B), the samples were DPPC/1 mol% Cer (●) and DPPC/10 mol% Cer (▼). For (B), (C) and (D), the samples consist of BB-SM, EY-SM and BE-SM, respectively, at 1 (●), 5 (▼) and 10 (■) mol% Cer. The insert in (A) demonstrates the fluorescence polarization (F.P.) as a function of the mol% Cer (0.25 mol% DPH-Cer and the remainder BB-Cer) in POPC bilayers when measured at 25°C. The data for DPPC, BB-SM, EY-SM and BE-SM were analyzed as in Fig. 1 to obtain a value for T_M where the solid line demonstrates the regression fit to the data. The phospholipid concentration was 0.1 mg/ml.

BB-SM has a broad thermal transition in the temperature range of 30–40°C, BE-SM has a broad transition between 20 and 35°C, and EY-SM demonstrates a sharp transition at 39°C. The addition of BB-Cer to BB-SM (Fig. 1C) and BE-SM (Fig. 1E) did not increase the polarization values in the gel state, whereas for EY-SM (Fig. 1D), there was a slight increase. However, above the T_M of the neat SMs, the addition of BB-Cer increased the polarization values in BB-SM, BE-SM and EY-SM bilayers. When the fluorescence polarization versus temperature curves for each of the SMs were analyzed by a sigmoidal regression analysis, the data demonstrated that the addition of BB-Cer increased the temperature where a 50% change in the polarization values occurs. This indicated that the addition of BB-Cer

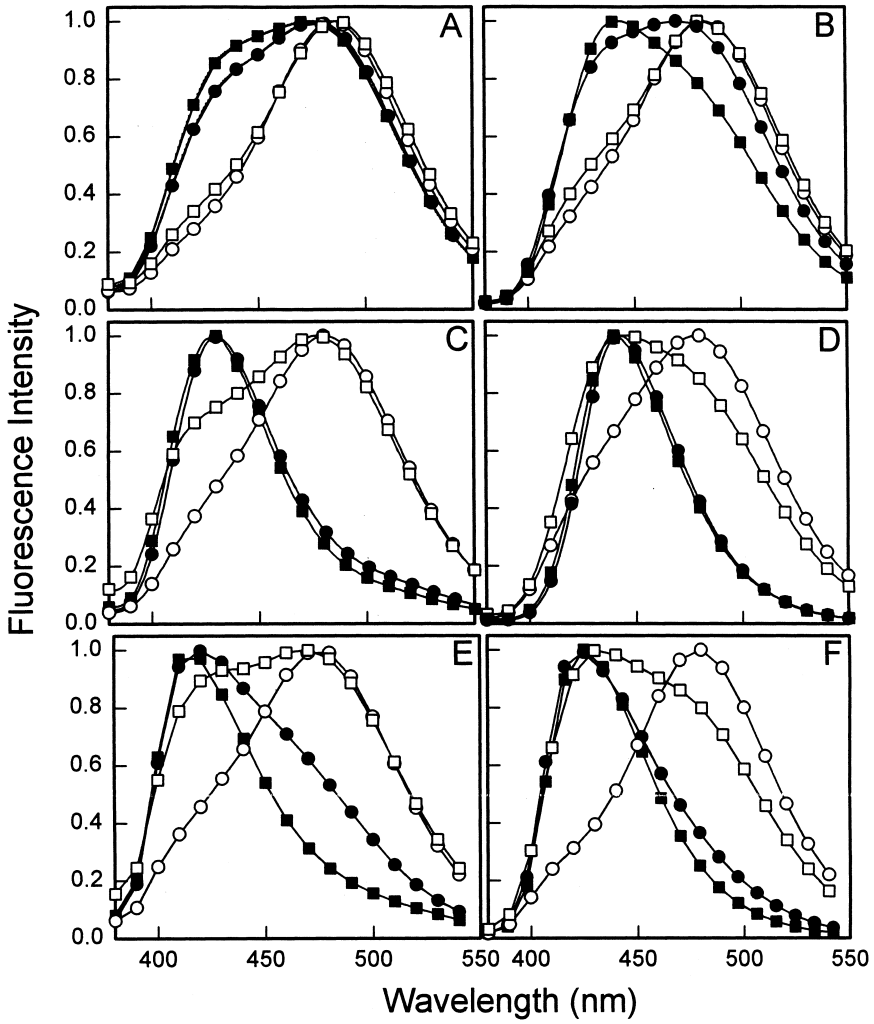

Fig. 3. The fluorescence spectra of prodan and laurdan were measured in POPC, DPPC and BB-SM bilayers containing BB-Cer. The spectra are for POPC (A and B), DPPC (C and D) and BB-SM (E and F) bilayers containing prodan (A, C and E) and laurdan (B, D and F). The data are for the phospholipid bilayer alone (●, ○) and containing 25 mol% BB-Cer (■, □). The spectra were recorded at 25°C (●, ■) and 55°C (○, □). The phospholipid concentration for samples containing laurdan were 0.3 mg/ml at a probe/phospholipid molar ratio of 1/250 and for prodan the phospholipid concentration was 1.0 mg/ml at a probe/phospholipid molar ratio of 1/833 such that the amount of fluorescent probe was kept constant for the prodan and laurdan samples. For fluid phospholipid bilayers, the fluorescence wavelength maxima varied from 477 to 486 nm for prodan and from 470 to 473 nm for laurdan. In gel phase DPPC bilayers, the fluorescence wavelength maxima were 430 nm for prodan and varied from 440 to 443 nm for laurdan. In gel phase BB-SM bilayers, the fluorescence wavelength maxima varied from 415 to 419 nm for prodan and from 423 to 425 nm for laurdan. In the gel phase, the spectra for both probes were blue shifted in BB-SM bilayers when compared to DPPC bilayers.

shifted the gel to liquid-crystalline phase transition to higher temperatures. The temperature for the onset of the thermal transitions was not altered, however, the temperature for the completion of the thermal transition increased with the amount of BB-Cer added [31].

For POPC bilayers, there was a slight increase in the polarization values of DPH-Cer with the addition of BB-Cer (Fig. 2A). At 25°C, a linear correlation was found for the change in fluorescence polarization with the mol% Cer in the bilayer when the BB-Cer concentration was varied up to 30 mol% (Fig. 2A, insert). For DPH-Cer, there was no change in the polarization values on the addition of BB-Cer to DPPC bilayers (Fig. 2B) in the gel phase. The addition of Cer did shift the temperature for a 50% change in fluorescence polarization to higher temperatures. For BB-SM (Fig. 2C), EY-SM (Fig. 2D) and BE-SM (Fig. 2E), increasing the BB-Cer concentration increased the DPH-Cer polarization values in gel phase bilayers. These increases may be due to changing the physical properties of the bilayer, however, because of the DPH-Cer concentration (1 mol%) used, there may also be some effect in changing the lateral distribution of the fluorescent probe [38]. Similar to the DPH measurements, the addition of BB-Cer shifted the midpoint temperature for a 50% change in the fluorescence polarization values to higher temperatures. These results demonstrate that in SM bilayers the addition of BB-Cer shifted the gel to liquid-crystalline phase transition to higher temperatures.

3.2. Fluorescence spectra of prodan and laurdan

The environmentally sensitive fluorescence spectroscopic properties of prodan and laurdan were used to investigate Cer dependent changes in the properties of phospholipid bilayers. For prodan and laurdan in liquid-crystalline POPC bilayers (Fig. 3A,B), there was a blue shifted shoulder in the fluorescence spectra upon increasing the amount of BB-Cer from 0 to 25 mol% at both 25 and 55°C. An increase in the intensity of the blue part of the emission spectra can indicate a slight decrease in interfacial polarity. For gel phase DPPC bilayers (Fig. 3C,D), there was essentially no effect of the addition of BB-Cer on the fluorescence emission spectra of prodan or laurdan. For gel phase BB-SM bilayers (Fig. 3E,F), the addition of BB-Cer decreased the width of the spectra for both prodan and laurdan. For gel phase DPPC bilayers, the emission maxima for prodan and laurdan were 430 and 440 nm, respectively, and for BB-SM bilayers were 415 and 423 nm, respectively. The blue shift in the emission indicates a more non-polar and rigid environment for the probes in the BB-SM bilayer compared to a DPPC bilayer. These results are consistent with SMs being more tightly packed. At 55°C, the fluorescence emission spectra of prodan and laurdan in DPPC and BB-SM bilayers were similar to that found in liquid-crystalline POPC bilayers. The addition of BB-Cer to both DPPC and BB-SM bilayers resulted in a blue shift in the spectra for both prodan and laurdan. These results demonstrated a more hydrophobic and rigid microenvironment for the probes.

The fluorescence spectra of prodan and laurdan were analyzed to determine G.P. values. The addition of BB-Cer to POPC bilayers (Fig. 4A,D) slightly increased the G.P. values for prodan and laurdan. The G.P. values essentially did not change with changes in temperature. At 25°C for both fluorescent probes, a linear correlation was found for the change in G.P. values with the mol% Cer in the bilayer when the BB-Cer concentration was varied up to 25 mol%

Table 1
Effect of the molecular species of Cer on the thermal transition temperature of DPPC, BB-SM, EY-SM and BE-SM bilayers

Cer	Phospholipid bilayer	$T_{M(o)}$ (°C)	ΔT_M/mol% Cer (°C/mol%)
BB-Cer	DPPC	42.3	0.32
	BB-SM[1]	38.8	0.69
	EY-SM	40.4	0.51
	BE-SM	36.8	1.15
C18:1-Cer	DPPC	43.2	0.04
	BB-SM	36.5	0.34
C24:1-Cer	DPPC	43.0	0.13
	BB-SM	36.7	0.71
C16:0-Cer	DPPC	42.9	0.24
	BB-SM	35.9	0.82
C18:0-Cer	DPPC	43.2	0.33
	BB-SM	35.9	0.81

[1]When the data for BB-SM were analyzed separately as the data for DPH and the data for laurdan (Fig. 5B), the results for $T_{M(o)}$ were 40.1 and 36.0°C and for ΔT_M/mol% Cer were 0.77 and 0.87°C/mol% Cer, respectively.

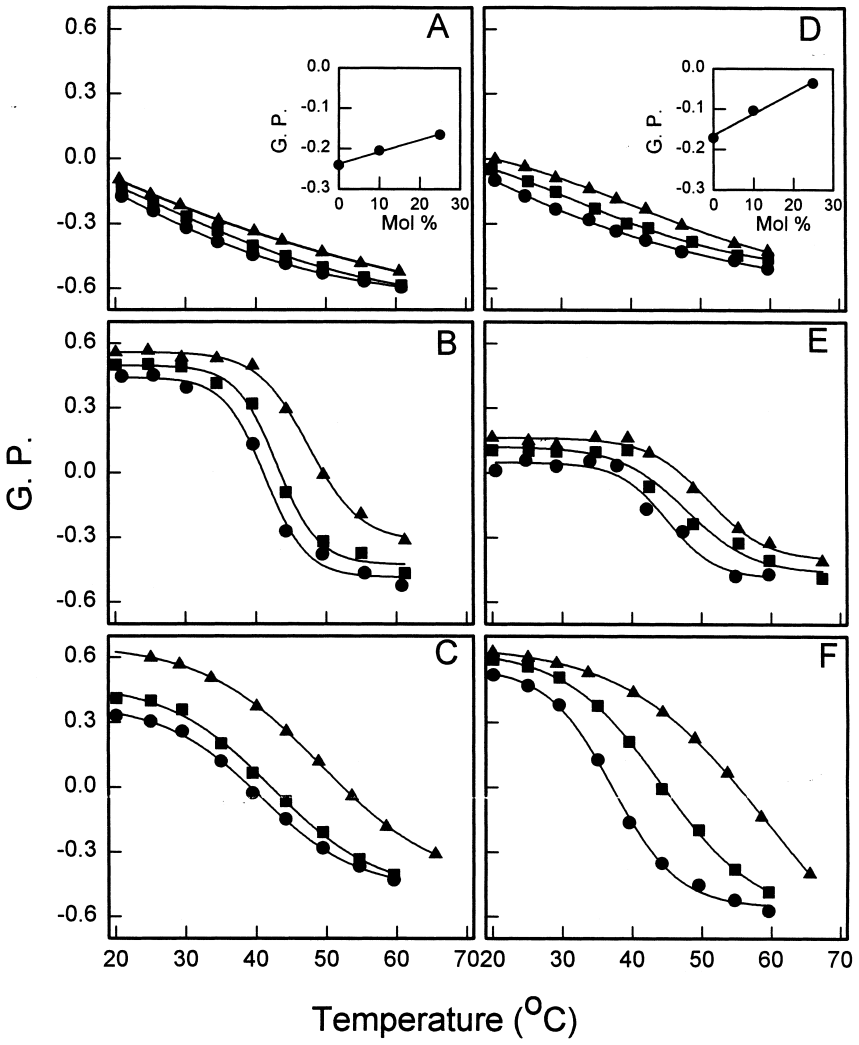

Fig. 4. The fluorescence spectra of prodan and laurdan were analyzed to determine G.P. values as a function of temperature. The measurements are for POPC, DPPC and BB-SM bilayers containing 0 (●), 10 (■) and 25 (▲) mol% BB-Cer. The data are for POPC (A and D), DPPC (B and E) and BB-SM (C and F) containing prodan (A, B and C) and laurdan (D, E and F). The inserts in (A) and (D) demonstrate the G.P. measurements as a function of the mol% BB-Cer in POPC bilayers when measured at 25°C. The data for DPPC and BB-SM were analyzed as in Fig. 1 to obtain a value for T_M that is the temperature where there was a 50% change in the G.P. values. The solid line demonstrates the regression fit to the data.

(Fig. 4A,D, inserts). At all temperatures, the addition of BB-Cer increased the G.P. values for prodan and laurdan in DPPC (Fig. 4B,E) and BB-SM (Fig. 4C,F) bilayers. Also for both DPPC and BB-SM bilayers, the midpoint temperature for a 50% change in the G.P. values was shifted to higher temperatures with the addition of BB-Cer.

3.3. Effect of Cer concentration on T_M

The effect of the BB-Cer concentration on the midpoint temperature, T_M, was analyzed for DPPC (Fig. 5A), BB-SM (Fig. 5B), EY-SM, and BE-SM (Fig. 5C). The T_M values were calculated from the sigmoidal regression analysis of the temperature versus fluorescent measurement data for DPH, DPH-Cer, prodan and laurdan. There was a linear correlation between an increase in the mol% Cer and an increase in T_M for all four phospholipid bilayers. The $T_{M(o)}$ values (Table 1) that were determined by our fluorescent measurements were similar to the temperatures for the gel to liquid-crystalline phase transitions

determined by differential scanning calorimeter. When comparing the data for BB-SM bilayers, an analysis of the DPH fluorescent polarization data gave higher values for T_M and $T_{M(o)}$ than the analysis of the laurdan data (Fig. 5B and Table 1). However, we used all of the data for our linear analysis. The values for the slopes, ΔT_M/mol% Cer, were 0.32, 0.69, 0.51 and 1.15°C/mol% Cer for DPPC, BB-SM, EY-SM and BE-SM bilayers, respectively. The results demonstrate that the increase in the gel to liquid-phase transition with the addition of BB-Cer was more pronounced in SM bilayers than in DPPC bilayers. Cers with specific *N*-linked acyl chains, C18:1-Cer, C24:1-Cer, C16:0-Cer and C18:0-Cer, were investigated for their effect on the T_M of DPPC and BB-SM bilayers (Fig. 5D and Table 1). C18:1-Cer, which would be a minor naturally occurring Cer species, was the least effective in raising T_M in both DPPC and BB-SM bilayers. In BB-SM bilayers, C24:1-Cer, C16:0-Cer and C18:0-Cer demonstrated similar results. In DPPC bilayers, the saturated C16:0-Cer and C18:0-Cer were more effective in raising T_M than C24:1-Cer. It should be noted that SMs containing oleic (C18:$1^{\Delta 9}$) and nervonic (C24:$1^{\Delta 15}$) acid have different physical properties due to the difference in the location of the double bond in bilayer [15]. The results demonstrate that for different molecular species of Cer the increase in the gel to liquid-phase transition was higher in BB-SM bilayers (ΔT_M/mol% Cer values varied from 0.34 to 0.82°C/mol%) than in DPPC bilayers (ΔT_M/mol% Cer varied from 0.04 to 0.33°C/mol%).

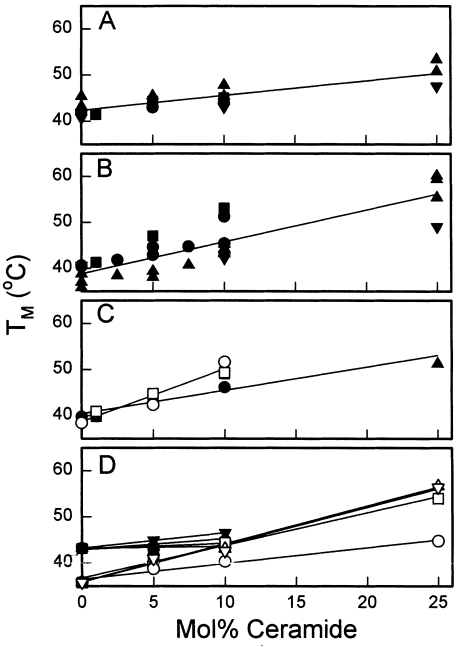

Fig. 5. The effect of different molecular species of Cer on the midpoint temperature (T_M) for the thermal transitions of DPPC, BB-SM, EY-SM and BE-SM was determined. The results are for BB-Cer in DPPC bilayers (A), BB-Cer in BB-SM bilayers (B) and BB-Cer in EY-SM (●, ■) and in BE-SM (○, □) bilayers (C). The values for T_M were derived from analysis of the fluorescent measurement versus temperature curves for DPH (●, ○), for DPH-Cer (■, □), for laurdan (▲) and for prodan (▼). The data were analyzed by linear regression using the equation: $T_{M(Cer)} = T_{M(o)} + M \times$(mol% Cer), where $T_{M(Cer)}$ was the measured value for T_M at a certain concentration of Cer (mol% Cer), M was the slope, and $T_{M(o)}$ was the characteristic transition temperature of the specific phospholipid in the absence of added Cer. The results of this analysis are in Table 1. In (D), the effect of different Cer molecular species on T_M was measured in DPPC (●, ■, ▲, ▼) and BB-SM (○, □, △, ▽) bilayers. The molecular species of Cer were C18:1-Cer (●, ○), C24:1-Cer (■, □), C16:0-Cer (▲, △) and C18:0-Cer (▼, ▽). The results of the linear regression analysis are in Table 1.

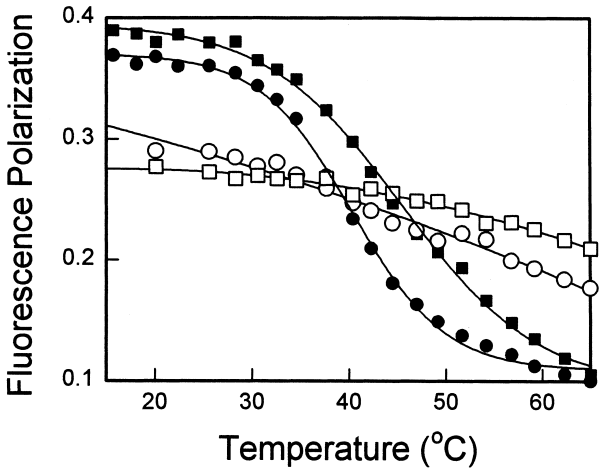

Fig. 6. The fluorescence polarization of DPH was used to measure the effect of 33 mol% cholesterol on the physical properties of BB-SM bilayers and BB-SM bilayers containing 10 mol% BB-Cer. The data are for BB-SM/BB-Cer/cholesterol (100/0/0 mol/mol) (●), BB-SM/BB-Cer/cholesterol (90/10/0 mol/mol) (■), BB-SM/BB-Cer/cholesterol (67/0/33 mol/mol) (○) and BB-SM/BB-Cer/cholesterol (57/10/33 mol/mol) (□) bilayers.

3.4. *Fluorescence polarization of DPH in BB-SM/BB-Cer/cholesterol bilayers*

The effect of cholesterol on BB-SM bilayers and

BB-SM bilayers containing BB-Cer was determined by DPH fluorescence polarization measurements. As previously demonstrated, the fluorescence polarization versus temperature curve for BB-SM/BB-Cer (90/10 mol/mol) bilayers was shifted to higher temperatures when compared to BB-SM bilayers (Fig. 6). With the addition of 33 mol% cholesterol, the magnitude of the changes in the fluorescence polarization values with temperature were dramatically reduced and demonstrated that cholesterol eliminated the gel to liquid-crystalline phase transition [16]. However, the fluorescence polarization versus temperature curves for BB-SM/BB-Cer/cholesterol (67/0/33 mol/mol) and BB-SM/BB-Cer/cholesterol (57/10/33 mol/mol) bilayers were different. Around physiological temperature, the BB-SM, BB-SM/BB-Cer/cholesterol (67/0/33 mol/mol) and BB-SM/BB-Cer/cholesterol (57/10/33 mol/mol) bilayers have similar DPH polarization values. Under these conditions, the BB-SM bilayers should consist of a mixture of lipids in both the gel and liquid-crystalline phases, whereas the BB-SM/BB-Cer/cholesterol (67/0/33 mol/mol) and BB-SM/BB-Cer/cholesterol (57/10/33 mol/mol) bilayers should be in a liquid-ordered phase [39,40].

Experiments were performed where the BB-Cer concentration (from 0 to 10 mol%) was varied in BB-SM bilayers and BB-SM bilayers containing 33 mol% cholesterol and where the cholesterol concentration (from 0 to 33 mol%) was varied in BB-SM bilayers and BB-SM bilayers containing 10 mol% BB-Cer. DPH polarization measurements were made at temperatures below ($T = 20$°C), around ($T = 37$°C), and above ($T = 60$°C) the gel to liquid-crystalline phase transition temperature of BB-SM. At 20°C, in comparing BB-SM bilayers and BB-SM bilayers containing 33 mol% cholesterol, the bilayers with cholesterol had lower DPH polarization values (Fig. 7A). However, increasing the BB-Cer concentration had little effect on the DPH polarization measurements. In BB-SM bilayers and BB-SM bilayers containing 10 mol%, there was a linear decrease in the fluorescence polarization values with increasing cholesterol concentration (Fig. 7B). These results were consistent with cholesterol decreasing acyl chain order in gel phase phospholipid bilayers (Fig. 7B) and Cer having little effect. At 60°C, BB-SM bilayers with 33 mol% cholesterol had higher polarization values than BB-SM bilayers (Fig. 7E). However, in both lipid mixtures, the polarization values increased with increasing amounts of Cer. Also, in BB-SM and BB-SM bilayers containing 10 mol% BB-Cer, there was a linear increase in fluorescence polarization values with cholesterol concentration (Fig. 7F) where the values were higher in the bilayers containing BB-Cer. At 60°C, Cer and cholesterol additively increased acyl chain order in liquid-crystalline bilayers. At 37°C, the addition of BB-Cer resulted in an increase in the DPH polarization values in BB-SM bilayers that was not observed in BB-SM bilayers containing 33 mol% cholesterol (Fig. 7C). Also, cholesterol linearly decreased the fluorescence polarization values in BB-SM bilayers containing 10 mol% BB-Cer but not in BB-SM bilayers (Fig. 7D). At 37°C, Cer and cholesterol have opposite effects. Cer increases T_M and thus increases the amount of gel phase phospholipid that increases the fluorescence polarization values and cholesterol decreases the amount of gel phase phospholipid, which decreases the fluorescence polarization values.

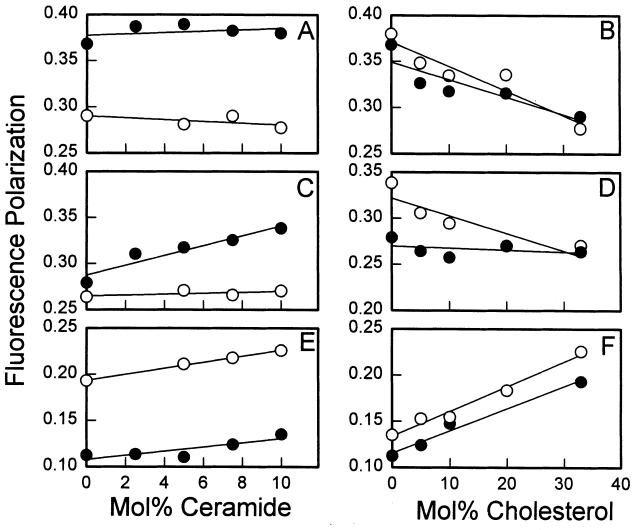

Fig. 7. The fluorescence polarization of DPH was measured in BB-SM/BB-Cer/cholesterol bilayers as a function of changes in mol% Cer (A, C and E) and mol% cholesterol (B, D and F). The concentration of Cer was varied in BB-SM bilayers (●) and in BB-SM bilayers containing 33 mol% cholesterol (○). The concentration of cholesterol was varied in BB-SM bilayers (●) and in BB-SM bilayers containing 10 mol% BB-Cer (○). The measurements were made at 20°C (A and B), 37°C (C and D) and 60°C (E and F).

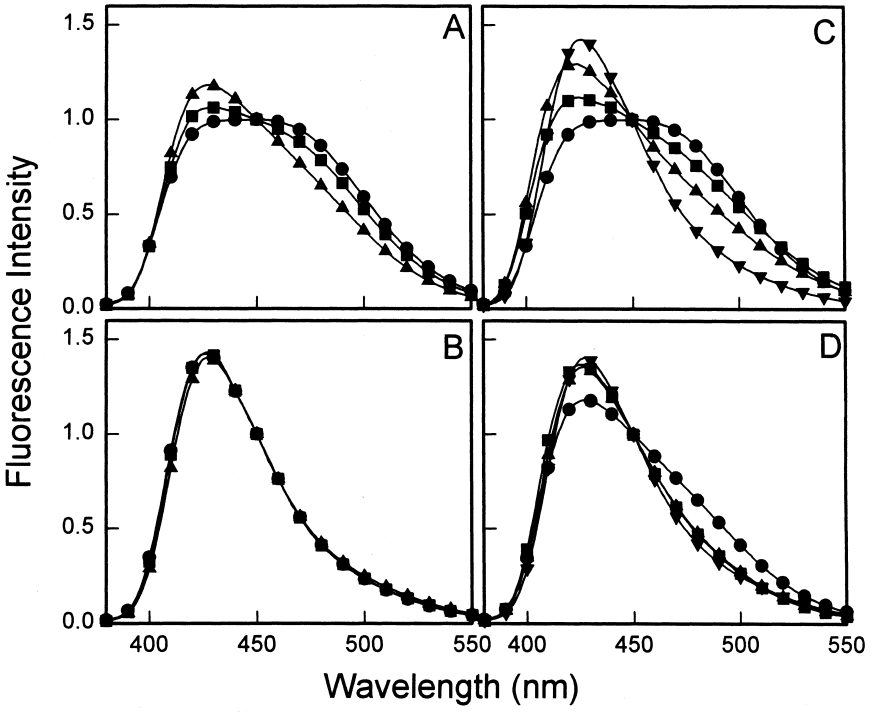

Fig. 8. The fluorescence spectra of laurdan were measured in BB-SM bilayers where the Cer and cholesterol concentrations were systematically varied. The spectra were recorded at 37°C. In (A), the spectra are for BB-SM bilayers containing 0 (●), 5 (■) and 10 (▲) mol% BB-Cer. In (B), the spectra are for BB-SM bilayers with 33 mol% cholesterol and 0 (●), 5 (■) and 10 (▲) mol% BB-Cer. In (C), the spectra are for BB-SM bilayers containing 0 (●), 5(■), 10 (▲) and 33 (▼) mol% cholesterol. In (D), the spectra are for BB-SM bilayers containing 10 mol% BB-Cer and 0 (●), 5(■), 10 (▲) and 33 (▼) mol% cholesterol. For all of the spectra, the fluorescence intensity was normalized to a value of 1 at 450 nm.

3.5. *Laurdan fluorescence measurements in BB-SM/BB-Cer/cholesterol bilayers*

Fig. 8 demonstrates laurdan fluorescence spectra measured at 37°C for BB-SM bilayers containing different amounts of Cer and cholesterol. The addition of BB-Cer to BB-SM bilayers resulted in a concentration dependent increase in the fluorescence intensity at 425 nm. (Fig. 8A). Similarly, the addition of cholesterol to BB-SM bilayers increased the fluorescence intensity at 425 nm. (Fig. 8C) where the maximal change occurred at 33 mol% cholesterol. In BB-SM bilayers containing 33 mol% cholesterol, the addition of BB-Cer did not further increase the fluorescence intensity at 425 nm (Fig. 8B). In BB-SM bilayers containing 10 mol% BB-Cer, the addition of only 5 mol% cholesterol resulted in the maximal fluorescence intensity at 425 nm. At 37°C, both Cer and cholesterol induced a blue shift in the fluorescence spectra by increasing the fluorescence intensity at 425 nm. The microenvironment of laurdan was affected by Cer that induced the formation of gel phase phospholipid and by cholesterol that decreased bilayer hydration and polarity [33,34]. Unlike the DPH polarization measurements, the fluorescence spectra of laurdan were different in BB-SM, BB-SM/BB-Cer/cholesterol (67/0/33 mol/mol) and BB-SM/BB-Cer/cholesterol (57/10/33 mol/mol) bilayers at 37°C.

The G.P. values for laurdan were measured in BB-SM bilayers as a function of Cer and cholesterol concentration (Fig. 9). In BB-SM bilayers, the addition of BB-Cer shifted the temperature dependence of the G.P. values to higher temperatures (Fig. 9A). The addition of cholesterol to BB-SM bilayers increased the G.P. values at all temperatures (Fig. 9C). At 33 mol% cholesterol, there was no longer a large change in G.P. values as a function of temperature. Also, there was essentially no change in the G.P. values when BB-Cer was added to BB-SM bilayers containing 33 mol% cholesterol (Fig. 9B). In BB-SM bilayers containing 10 mol% BB-Cer, there

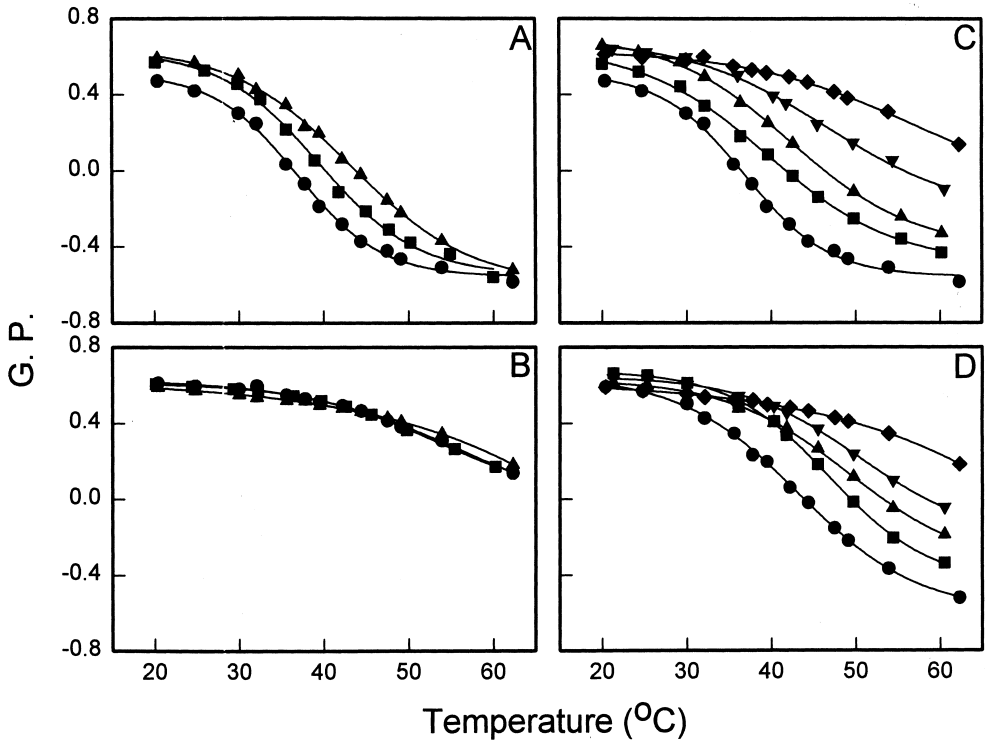

Fig. 9. The fluorescence spectra of laurdan were analyzed to determine G.P. values in BB-SM bilayers were both the cholesterol and BB-Cer concentrations were systematically varied. In (A), the concentration of BB-Cer was varied in BB-SM bilayers containing no cholesterol. The concentrations of BB-Cer were 0 (●), 5 (■) and 10 (▲) mol%. In (B), the concentration of BB-Cer was varied in BB-SM bilayers containing 33 mol% cholesterol. The concentrations of BB-Cer were 0 (●), 5 (■) and 10 (▲) mol%. In (C), G.P. values were determined for laurdan in BB-SM bilayers where the cholesterol concentrations were 0 (●), 5(■), 10 (▲), 20 (▼) and 33 (♦) mol%. In (D), G.P. values were determined for laurdan in BB-SM bilayers containing 10 mol% BB-Cer where the cholesterol concentrations were 0 (●), 5(■), 10 (▲), 20 (▼) and 33 (♦) mol%.

were concentration dependent changes in G.P. values with the addition of cholesterol (Fig. 9D). The data was further analyzed in Fig. 10 where G.P. values were determined at 20, 37 and 60°C and plotted on an expanded scale. At 20°C, there appears to be a maximal G.P. value (G.P. = ~0.6) for BB-SM bilayers containing 33 mol% cholesterol (Fig. 10A) or 10 mol% BB-Cer (Fig. 10B). In BB-SM bilayers, increasing the Cer concentration (Fig. 10A) or the cholesterol concentration (Fig. 10B) increased the G.P. value to this maximal level. At 60°C, there was a small increase in G.P. values with increasing Cer in BB-SM and BB-SM bilayers containing 33 mol% cholesterol (Fig. 10E). However, there was a much larger increase demonstrated by increasing the cholesterol concentration (Fig. 10F). The effect of BB-Cer and cholesterol to each increase the G.P. values appeared to be additive. At 37°C, there was a maximal value for G.P. (G.P. = ~0.5) in BB-SM bilayers containing 33 mol% cholesterol (Fig. 10C) that did not change with the addition of Cer. The addition of Cer to BB-SM bilayers had a greater effect on increasing the G.P. values at 37°C (Fig. 10C) than at 60°C (Fig. 10E). At 37°C, the addition of cholesterol increased the G.P. values to the maximal value in both BB-SM and BB-SM bilayers containing 10 mol% BB-Cer (Fig. 10D). The results clearly demonstrate that in the temperature region of the phase transition of BB-SM, the concentration of both Cer and cholesterol affect the membrane microenvironment measured by laurdan fluorescence.

4. Discussion

As a model system for sphingolipid and cholesterol-rich microdomains, we have studied the interaction between naturally occurring SMs and Cers and

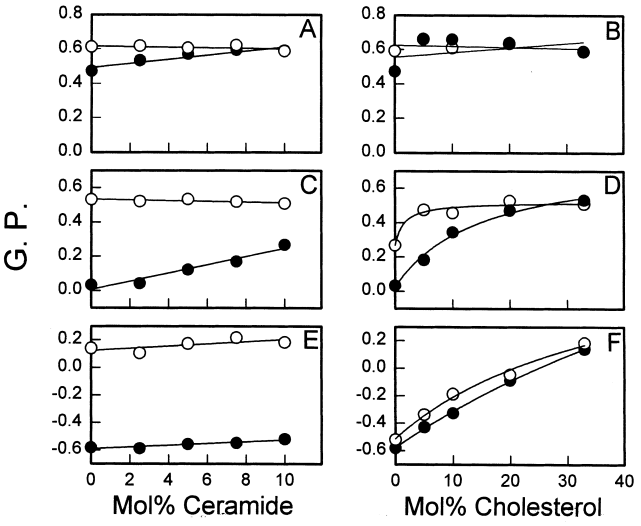

Fig. 10. The G.P. values for laurdan were measured in BB-SM/BB-Cer/cholesterol bilayers as a function of changes in mol% Cer (A, C and E) and mol% cholesterol (B, D and F). The concentration of Cer was varied in BB-SM bilayers (●) and in BB-SM bilayers containing 33 mol% cholesterol (○). The concentration of cholesterol was varied in BB-SM bilayers (●) and in BB-SM bilayers containing 10 mol% BB-Cer (○). The measurements were made at 20°C (A and B), 37°C (C and D) and 60°C (E and F).

the effect of cholesterol on the interaction between BB-SM and BB-Cer. For comparison, the interaction of BB-Cer with the POPC and DPPC was also studied. Physiological SMs and Cers have mixed *N*-linked fatty acyl chains and they can be asymmetric because a fatty acyl chain greater than 20 carbon atoms is much longer than the sphingosine methylene chain. In cells, the characterizations of the acyl chain composition of SMs and Cers have suggested that there is a selective metabolism of different molecular species of sphingolipids in the synthesis or metabolism of SM [18,41,42]. In this regard, C16:0-Cer, C24:0-Cer and C24:1-Cer have been the major molecular species of Cer identified in basal and stimulated cells. The bulk of the cellular SM and cholesterol is located in plasma membrane microdomains that can be isolated as DRMs [3,43]. In specific cases, the stimulated formation of Cer has been observed in these sphingolipid and cholesterol-rich microdomains [7]. One mechanism for the biological activity of Cers may be through changes in membrane structure and organization by the formation of Cer-rich microdomains [7].

4.1. Cer in POPC bilayer

POPC bilayers have a T_M of −7°C and are in the liquid-crystalline state at physiological temperatures [44]. Fluorescence polarization measurements demonstrate that the addition of up to 25 mol% BB-Cer to POPC bilayers induced a small increase in acyl chain order. Laurdan and prodan fluorescence measurements indicate that BB-Cer also induces a small decrease in the polarity of the bilayer. BB-Cer has a small headgroup and predominantly saturated acyl chains (~60% are stearic acid). Surface monolayer studies demonstrate it has a small mean molecular surface area [20,21,24]. Thus, it could be considered a very compact molecule. The addition of BB-Cer to liquid-expanded DPPC or BB-SM monolayers results in decreased mean molecular surface areas. This is probably due to the presence of BB-Cer with its small polar headgroup inducing closer packing of the acyl chains by reducing the repulsion between the phospholipid headgroups [44]. Similarly, the decreases in fluidity and polarity are due to an increase in the packing density of the acyl chains of the bilayer caused by the addition of the very compact Cer molecule.

Unlike other studies, we did not observe any discontinuities in our fluorescence measurements with changes in BB-Cer concentration in POPC bilayers that would indicate the formation of Cer-enriched microdomains or specific phospholipid–Cer complexes [8,11]. The addition of another naturally occurring Cer to liquid-crystalline DMPC bilayers demonstrated a change in the physical properties of the bilayer at Cer concentrations greater than 10 mol%. This was interpreted to be the formation of Cer-enriched microdomains [8]. This Cer had ~47% of the *N*-linked fatty acids as hydroxyl fatty acids and ~80% of the *N*-linked fatty acids had an acyl chain length greater than 20 carbon atoms. The formation of the Cer-enriched microdomains in DMPC bilayers was due to the hydrophobic mismatch between the relatively short myristoyl chains and the longer *N*-linked acyl chains [8]. In other sphingolipid/phospholipid mixtures, microdomain formation has also been attributed to the differences in fatty acyl chain length and unsaturation between the Cer and diacylglycerol moieties [45–47]. Additionally, the Cer backbone of sphingolipids has a free hydroxyl and

an amide group that act as hydrogen bond donors and hydrogen bond acceptors whereas for glycerol based phospholipids the esterified carbonyl groups linked to the glycerol backbone can only act as hydrogen bond acceptors [47]. Thus, the formation of a network of intermolecular hydrogen bonds involving sphingolipid molecules can also help facilitate the formation of Cer-rich microdomains [47]. The lack of formation of Cer-rich microdomains in POPC bilayers may be due to POPC and BB-Cer having similar length fatty acyl chains. In another study, Cer-enriched microdomains were found for C16:0-Cer in POPC bilayers and by the action of a sphingomyelinase on POPC/*N*-(palmitoyl)sphingosylphosphocholine (C16:0-SM) bilayer mixtures [11]. C16:0-SM and C16:0-Cer are symmetric molecules where the *N*-linked acyl chain is of nearly equal length to the sphingosine methylene chain. However, the order–disorder acyl chain melting temperature of C16:0-Cer is much higher ($T_M = 90°C$) [22] than that of C16:0-SM ($T_M = 41°C$) [13,15,16]. The increased tendency of C16:0-Cer to form a gel phase should help support lateral phase separation. In POPC bilayers, the very homogeneous and symmetric C16:0-Cer molecules may form microdomains, whereas the much more heterogeneous and asymmetric BB-Cer molecules do not. In liquid-crystalline PC bilayers, the formation of Cer-rich microdomains appears to be dependent upon the molecular structure of the Cer and the phospholipid.

4.2. Cer in DPPC bilayers

In liquid-crystalline phase DPPC bilayers, we found that the effects of BB-Cer on the physical properties of the bilayer were similar to those found for POPC. BB-Cer induced a small increase in acyl chain order as demonstrated by increases in DPH and DPH-Cer fluorescence polarization measurements and also induces a small decrease in the polarity of the bilayer as demonstrated by laurdan and prodan fluorescence measurements. In gel phase DPPC bilayers, the addition of BB-Cer slightly increased the acyl chain order and had only a small effect on the polarity of the bilayer. This is probably due to the formation of a separate gel phase DPPC/BB-Cer complex that has slightly different biophysical properties than a pure DPPC phase [24]. The addition of BB-Cer increased the transition temperature, T_M, of DPPC bilayers as determined by DPH, DPH-Cer, prodan and laurdan fluorescent measurements (Fig. 5A). The increase in T_M was linear with the amount of Cer in the bilayer. These results have also been demonstrated by differential scanning calorimetry [24]. The magnitude of the increase in T_M was dependent upon the Cer *N*-acyl chain composition (Fig. 5D and Table 1). C18:1-Cer which is symmetric with an unsaturated fatty acyl chain demonstrated the smallest effect on changing T_M, C16:0-Cer and C18:0-Cer which are symmetric with saturated fatty acyl chains demonstrated the greatest effect on increasing T_M, and C24:1-Cer which is asymmetric with a long unsaturated fatty acyl chain had a moderate effect.

The interaction of DPPC and BB-Cer [24] and interaction between phosphatidylcholines and diacylglycerols have been studied by differential scanning calorimetry [38,29,44]. These systems have been analyzed as having gel phase immiscibility where there is a pure phosphatidylcholine phase and a phase that contains a complex between phosphatidylcholine/Cer or phosphatidylcholine/diacylglyerol. For DPPC/BB-Cer mixtures, the lipid complex contained 8–12 mol% BB-Cer [24]. In several phosphatidylcholine/diacylglyerol mixtures, the lipid complexes contained 40–50 mol% diacylglyerol [38,29,44]. Lipid complexes have higher melting temperatures than the pure phosphatidylcholine because the addition of diacylglycerols or Cers, which have small headgroups, reduces the repulsion between the bulky phosphocholine headgroups and allows closer packing of the hydrocarbon chains. This increases the van der Waal's cohesive forces between the hydrocarbon chains and leads to an increase in the bilayer stability and an increase in the gel to liquid-crystalline phase transition temperatures [44]. Our fluorescent measurements do not distinguish between the presence of different lipid domains. The increase in the T_M of the lipid mixtures would be due to the weighted average of the thermal properties of the pure DPPC phase and a Cer-rich phase. The order–disorder transitions of BB-Cer, C16:0-Cer and C18:0-Cer are above the T_M of DPPC such that a more ordered, higher melting Cer-rich domain would be formed. C18:1-Cer, which should have a low order–disorder thermal transition temperature, would have little ef-

fect on the measured T_M. Whereas, C16:0-Cer, which has a higher thermal transition temperature, should have a much greater effect.

4.3. Cer in SM bilayers

BB-SM, EY-SM and BE-SM have different *N*-linked acyl chain compositions and different gel to liquid-crystalline phase transitions. For each of the SM bilayers, BB-Cer slightly increased acyl chain order in the gel phase. This was most clearly demonstrated for BB-SM (Figs. 6 and 7A). The fluorescence spectra of both prodan and laurdan are blue shifted in gel phase BB-SM bilayers when compared to DPPC bilayers. This indicated a more nonpolar or a more rigid microenvironment for these probes in gel phase BB-SM bilayers and is consistent with SM bilayers being more tightly packed than phosphatidylcholine bilayers. The addition of BB-Cer increases the G.P. values for prodan and laurdan in the gel phase that indicates reduced dipolar relaxation and is consistent with BB-Cer increasing acyl chain order. For each of the SM bilayers, there was a dramatic effect of BB-Cer on the fluorescence polarization values of DPH and on the prodan and laurdan G.P. values in the temperature region of the gel to liquid-crystalline phase transition. The addition of BB-Cer shifted the T_M values to higher temperatures where the ΔT_M/mol% Cer values (Fig. 5 and Table 1) are different for each of the SM bilayers. BE-SM, which is an asymmetric sphingolipid containing primarily lignoceric acid, was affected the most by the addition of BB-Cer. The ΔT_M/mol% Cer values for BB-Cer are higher in the SM bilayers than the DPPC bilayer (Fig. 5 and Table 1). In BB-SM bilayers, C24: 1-Cer, C16:0-Cer and C18:0-Cer had ΔT_M/mol% Cer values which were similar to BB-Cer, however, the addition of C18:1-Cer produced a smaller effect. The ΔT_M/mol% Cer values for all of the Cers that contained a single fatty acid were much higher for BB-SM bilayers than for the DPPC bilayers (Fig. 5 and Table 1).

The BB-SM and BB-Cer system is analogous to those of DPPC/1,2-dipalmitoyl-*sn*-glycerol (DPG), DMPC/1,2-dimyristoyl-*sn*-glycerol and POPC/1-palmitoyl-2-oleoyl-*sn*-glycerol (POG) where specific phosphatidylcholine/diacylglycerol gel phase lipid complexes have been demonstrated [38,29,44]. The phase diagrams for these lipid mixtures indicate that in the temperature region above the T_M of the pure phospholipid, there is a coexistence of diacylglycerol-poor domains which are in the liquid-crystalline phase and diacylglycerol-rich lipid complexes or domains which are in the gel phase. Our results can also be explained in terms of the formation of BB-SM/BB-Cer lipid complexes that have higher melting temperatures than BB-SM. The formation of small DPPC/BB-Cer complexes may be due to differences in the acyl chain length between DPPC and BB-Cer and the tendency of Cers to form hydrogen bond networks. However, DPPC/DPG complexes are 55/45 mixtures and have very cooperative behavior due to the homogeneity of the acyl chain lengths. We propose that the formation of SM and Cer complexes probably are very similar to the phosphatidylcholine/diacylglycerol complexes due to the ability to form hydrogen bond networks and also due to better mixing of the acyl chains. All of the Cers elevate the transition temperature of SM/Cer bilayer mixtures greater than DPPC/Cer mixtures. The more condensed BB-Cer and C16:0-Cer, which have high order–disorder thermal transitions, should form stronger complexes with SMs than the more expanded C18:1-Cer and thus have a greater effect in raising the temperatures of the lipid complexes [12]. Thus, the formation of sphingolipid/Cer lipid complexes should be dependent upon acyl chain length and unsaturation of both lipids. The formation of lipid complexes between saturated diacylglycerols and phosphatidylcholines and between POG and POPC (60/40 mol/mol) dramatically affects the thermal properties of the lipids. For example, the T_M for POPC and POG are −7 and 12°C, respectively, whereas the T_M of a POPC/POG complex (60/40 mol/mol) is 27°C [44]. Thus, at a temperature between the T_M of POPC and of the POPC/POG complex, a phospholipase C could convert a liquid-crystalline phase POPC bilayer to a bilayer containing a fluid POPC/POG-poor phase and a gel phase consisting of POPC/POG complexes or to a bilayer that is completely in the gel phase. This would depend upon the extent of POPC hydrolysis. However, naturally occurring phosphatidylcholines and diacylglycerols have thermal phase transitions well below physiological temperature and thus the formation of phosphatidylcholine/diacylglyerol complexes may not occur

in cells. Physiological temperature is within the temperature range of the gel to liquid-crystalline phase transition of naturally occurring SMs and below that of naturally occurring Cers. Thus, at 37°C, the enzymatic formation of Cer by a sphingomyelinase could dramatically alter the physical properties of a SM-rich membrane microdomain by isothermally converting a fluid liquid-crystalline phase SM bilayer to a gel phase SM/Cer bilayer. In model membranes, differences in the effect of phospholipase C and sphingomyelinase in inducing vesicle fusion versus vesicle leakage could be due to the differences in the formation and properties of specific lipid complexes [26]

4.4. Cholesterol in BB-SM/BB-Cer bilayers

The biophysical properties of BB-SM bilayers were dependent upon the concentration of both cholesterol (from 0 to 33 mol%) and BB-Cer (0 to 10 mol%). As expected, the addition of cholesterol decreased the acyl chain order in BB-SM and BB-SM/BB-Cer bilayers that were in the gel phase and increased acyl chain order when they were in the liquid-crystalline phase (Fig. 6). At a concentration of cholesterol (33 mol%) that would be found in a plasma membrane sphingolipid–cholesterol microdomain, the gel to liquid-crystalline phase transition was essentially eliminated in BB-SM and BB-SM/BB-Cer bilayers. However, the fluorescence polarization versus temperature profiles are not identical and demonstrate that the presence of Cer has an effect on acyl chain motion. This is clearly demonstrated in liquid-crystalline phase bilayers (Fig. 7E,F), where the effect of Cer and cholesterol on DPH polarization measurements was additive. The presence of BB-Cer in BB-SM bilayers caused tighter packing of the acyl chains to decrease acyl chain motion and cholesterol has it's known effect of restricting acyl chain motion. Also, gel phase BB-SM bilayers demonstrated that both BB-Cer and cholesterol separately affected acyl chain order (Fig. 7A,B). In the temperature region ($T = 37$°C) of the broad phase transition of BB-SM, Cer and cholesterol can have opposite effects on acyl chain motion. Cer decreases acyl chain motion by increasing the amount of gel phase lipid by formation of SM/Cer complexes (Fig. 7C) and cholesterol increases acyl chain motion by decreasing the amount of gel phase lipid (Fig. 7D). However, the presence of 33 mol% cholesterol in BB-SM bilayers effectively eliminates any changes in acyl chain order induced by the presence of BB-Cer.

At 37°C, the addition of BB-Cer to BB-SM bilayers results in a blue shift in the fluorescence spectra of laurdan. This is due to changes in the phase properties of the bilayer (Fig. 8). Cholesterol is known to cause a blue shift in the fluorescence spectra of laurdan that may be due to a decreased amount of hydration of phospholipid bilayers [34]. Laurdan fluorescence properties in BB-SM bilayers are determined by both cholesterol and BB-Cer. This is demonstrated in the G.P. versus temperature curves (Fig. 9) where the amount of cholesterol and/or Cer in BB-SM bilayers determined the measured G.P. values at all temperatures studied. At 37°C (Fig. 10), small changes in the amount of either cholesterol or Cer can change the G.P. values. However, in 33 mol% cholesterol a maximal change in the G.P. values was observed and the presence of BB-Cer had no additional effect. In a liquid-ordered bilayer, there was no change in bilayer polarity by the addition of Cer. Both the laurdan and DPH fluorescence data indicate that the presence of 33 mol% cholesterol eliminate the formation of gel phase SM/Cer complexes. Thus, at 37°C, the enzymatic formation of Cer would not dramatically alter the biophysical properties of a SM-rich membrane containing 33 mol% cholesterol.

4.5. Physiological relevance

An increase in cellular levels of Cer has been indicated to be an important event is signaling processes related to apoptosis, cell cycle arrest and cell senescence [48,49]. Elevation of Cer levels can occur by de novo synthesis where Cer synthase is an important regulatory enzyme or by the action of acidic or neutral sphingomyelinases on distinct metabolic pools of SM [5,50]. Cer synthase is located in mitochondrial and endoplasmic reticular membranes [7]. These membranes have little SM but are rich in phospholipids with phase properties similar to POPC. However, our results indicate that BB-Cer is miscible in POPC bilayers and suggest that newly de novo synthesized Cers probably would not form a Cer-rich microdomain. Membrane-associated signal-

ing proteins can have two membrane-targeting motifs where each binding site has low affinity for a specific class of lipid, however, occupancy of both sites generates high affinity binding [51]. Analogous to protein kinase C, a Cer-activated protein could contain a specific Cer binding site and a phospholipid-binding site. In a fluid bilayer, a Cer molecule being miscible with the surrounding phospholipid matrix, e.g. phosphatidylserine, could facilitate a Cer-activated protein in binding of multiple lipid ligands.

The cellular location of SM microdomains involved in signal transduction has not been clarified. Several sphingomyelinases have been identified and their cellular locations include lysosomes and endosomes, endoplasmic reticulum, plasma membrane and cytosol [52–55]. The action of sphingomyelinases on SM can generate a high local concentration of Cer and could induce the formation of SM/Cer-rich microdomains. The physical properties of a SM/Cer-rich microdomain would dependent upon the amount of cholesterol present. In a cholesterol-poor membrane, a gel phase SM/Cer complex would be formed which would dramatically alter the biophysical properties of the membrane and thus catalyze other membrane-associated events. For example, membrane defects formed by the coexistence of liquid-crystalline and gel phases are known to increase bilayer permeability and facilitate membrane protein association such as phospholipase A_2 activation [25,56]. In the analogous system, phosphatidylcholine/diacylglycerol complexes have been suggested to be important in regulating protein kinase C activity [57,58]. Some studies have indicated that agonist stimulated Cer formation occurs in the sphingolipid and cholesterol-rich microdomains which are isolated as DRMs [7]. The formation of Cer in these microdomains would not dramatically alter the biophysical properties of the bilayer. However, in this liquid-ordered phase, the Cer molecules would have high lateral mobility and would be completely miscible with the other lipids. As argued above, this could facilitate the interaction with membrane-associated proteins that have multiple lipid binding sites. The formation of Cer in these microdomains would facilitate cross talk between different signaling pathways that are associated with these microdomains [7].

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