Cholesterol's Interfacial Interactions with Galactosylceramides†

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ABSTRACT: Recently, the influence of acyl structure on galactosylceramide's (GalCer) interfacial phase behavior was studied [Ali, S., Smaby, J. M., & Brown, R. E. (1993) *Biochemistry 32*, 11696–11703]. Here, we show that acyl structure is a key parameter controlling GalCer's ability to interact with cholesterol. Different chain-pure GalCer species containing saturated (24:0, 18:0, or 10:0), or unsaturated (24:1$^{\Delta 15}$, 22:1$^{\Delta 13}$, or 18:2$^{\Delta 9,12}$) acyl chains were synthesized. After measurement of the force–area behavior of mixed cholesterol/GalCer films at 24 °C, the average molecular area and average compressibility were determined as a function of film composition. Cholesterol exerts only a slight condensing effect when the GalCer species are liquid-ordered [liquid-condensed], with maximum condensation occurring near 0.25 mole fraction. However, cholesterol exerts a marked condensing effect on liquid-disordered (liquid-expanded) GalCer species regardless of whether the acyl chain is saturated or unsaturated. Maximum condensation occurs at cholesterol mole fractions between 0.3 and 0.4. We also compared cholesterol's relative condensing effect on liquid-expanded GalCer versus sphingomyelin. Cholesterol's condensation of either bovine brain or egg sphingomyelin is 25–30% greater than that observed with different liquid-expanded GalCer species. Aside from average area behavior, we assessed cholesterol's interfacial interactions with the various sphingolipids by determining the average compressibility as a function of composition. The compressibility of condensed GalCer derivatives changes very little upon addition of cholesterol. In contrast, cholesterol causes dramatic changes when combined with liquid-expanded GalCer derivatives, which all have compressibilities 4–5-fold higher than bovine brain GalCer. The nature of the cholesterol-induced change in average compressibility for liquid-expanded GalCer derivatives depends on acyl structure and surface pressure.

Because cholesterol is a major lipid component of many cellular membranes, a great deal of effort has gone into understanding how this sterol interacts with other membrane lipids [e.g., Finegold (1993)]. To date, the majority of these efforts have focused on cholesterol's mixing behavior with glycerol-based phospholipids, but occasionally they have included sphingomyelin, a sphingoid-based phospholipid. The results from many, but not all, of the studies suggest that cholesterol has a greater "affinity" for sphingomyelin compared with other phospholipids. Less clear is whether other simple sphingolipids (e.g., monoglycosylceramides) display a similar high "affinity" for cholesterol and to what extent changes in ceramide and/or headgroup composition affect the interaction with cholesterol.

Investigating the interfacial interactions of cholesterol and monoglycosylceramides is important physiologically because of the unique structural properties conferred upon membranes (e.g., myelin and brush border membranes) by these lipids. Maintaining proper function requires not only that these lipids be present but also that their membrane concentration be optimized. In certain pathological conditions, membrane concentrations of galactosylceramides are deficient (e.g., Pelizaeius–Merzbacher's disease) or grossly exceed optimum levels (e.g., globoid cell leukodystrophy), and membrane function is impaired (Witter et al., 1980; Suzuki & Suzuki, 1989). Because the molecular basis for this impairment is not clear, investigations including high as well as low mole fractions of monoglycosylceramides should help to define the underlying physicochemical properties responsible for the fundamental defect in the diseased membranes.

A particularly informative approach for assessing lipid lateral interactions is to determine the molecular area of mixed-lipid monolayers as a function of surface pressure. Using this approach, previous investigations indicated that cholesterol has a relatively greater condensing effect on sphingomyelin than on phosphatidylcholine (Lund-Katz et al., 1988). The effect could not be adequately explained by differences in acyl chain composition. Here we have used the monolayer approach to assess condensation and compressibility in mixed films composed of cholesterol and galactosylceramides containing various homogeneous acyl chain compositions. An important advantage of studying lipid–lipid interactions in this way is that the range of molecular areas known to occur in membrane systems can be investigated systematically while avoiding mesophasic changes that often occur in bulk aqueous systems (e.g., bilayer vesicles, micelles) as lipid composition is varied. Moreover, with lipid monolayers, other complicating issues such as lipid compositional changes due to either transbilayer asymmetry or vesicle-to-vesicle variation are avoided.

While the monolayer approach has been useful for studying the mixing behavior of many phospholipids and neutral lipids [for a review, see Dörfler (1990)], little has been reported for glycosphingolipid mixing with cholesterol. Unlike previous investigations in which cholesterol mixing was studied with galactosylceramides bearing heterogeneous acyl chains (Johnston & Chapman, 1988; Slotte et al., 1993), this report focuses on cholesterol's ability to condense and lower the compressibility of different molecular species of galactosylcermide containing homogeneous acyl chains that bear either

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zero, one, or two double bonds in their acyl chains. The results clearly show that cholesterol exerts a large condensing effect on liquid-expanded, but not on liquid-condensed, GalCer.[1] Nonetheless, cholesterol's condensing effect on sphingomyelin is 25–30% larger than on liquid-expanded GalCer. A preliminary report of portions of this work has appeared elsewhere (Ali et al., 1992).

MATERIALS AND METHODS

Cholesterol was obtained from Nu-Chek Prep (Elysian, MN). Purity was greater than 99% based on thin-layer chromatographic (TLC) analysis [petroleum ether/diethyl ether/acetic acid (7:3:1)]. A stock solution for film balance studies was made by dissolving cholesterol in $CHCl_3/CH_3OH$/petroleum ether (5:1:4) and was stored under argon at −70 °C when not is use. Various *N*-acylated GalSph species were synthesized and characterized as described previously (Ali et al., 1991, 1993, 1994). Monolayer studies were performed using a Langmuir-type automated film balance (Brockman et al., 1980, 1984; Ali et al., 1991). Similar results were obtained when either $CHCl_3/CH_3OH$/petroleum ether (5:1:4) or hexane/2-propanol/H_2O (7:3:0.25) was used as spreading solvent.

Analysis of Isotherms. Mixing behavior of two-component lipid monolayers was analyzed by mean molecular area–composition diagrams (Goodrich, 1957), by mean compressibility–composition diagrams, and, when appropriate, by examination of the surface pressure–composition behavior (Crisp, 1949; Cadenhead et al., 1976; Smaby & Brockman, 1992).

The mean molecular area ($\bar{A}_\pi$) of the two components 1 and 2 in a mixture at a given surface pressure (π) was calculated using (Goodrich, 1957)

$$\bar{A}_\pi = X_1(A_1)_\pi + (1 - X_1)(A_2)_\pi \quad (1)$$

where X_1 is the mole fraction of component 1. $(A_1)_\pi$ and $(A_2)_\pi$ are the mean molecular areas of two pure components 1 and 2 at identical surface pressures. The nature of lipid mixing is reflected by deviations from simple additivity for $\bar{A}_\pi$. A negative deviation from additivity indicates condensation and implies intermolecular accommodation and/or dehydration interactions between the lipids in the mixed films (e.g., Cadenhead & Müller-Landau, 1980).

The relative condensation was compared for different lipid mixtures by calculating their percent monolayer condensation at a constant surface pressure:

$$\% \text{ condensation} = [(\bar{A}_{\text{ideal}} - \bar{A}_{\text{obs}})/\bar{A}_{\text{ideal}}]100 \quad (2)$$

where $\bar{A}_{\text{ideal}}$ represents the additive ideal mean molecular area and $\bar{A}_{\text{obs}}$ represents the experimentally observed mean molecular area.

Earlier studies have shown the value of compressibility data for providing insights into cholesterol's interfacial interactions with phosphatidylcholine [e.g., Albrecht et al. (1981) and Hirshfeld and Seul (1990)]. In the present study, monolayer compressibility for pure lipid species at a given surface pressure (π) was calculated from π–A data using

$$k_\pi = (-1/A_\pi)(dA/d\pi)_\pi \quad (3)$$

where A_π is the area per molecule (angstroms squared) at the indicated surface pressures and π is the corresponding surface pressure in millinewtons per meter. Mean compressibilities ($\bar{k}_\pi$) for mixtures were calculated analogously to mean molecular areas except that the relative contribution of each lipid's compressibility apportions according to molecular area as well as by mole fraction (see Appendix). Thus, at a given constant surface pressure (π)

$$\bar{k}_\pi = -1/\bar{A}_\pi[(k_1a_1)_\pi X_1 + (k_2a_2)_\pi X_2] \quad (4)$$

and $\bar{k}_\pi$ is additive with respect to the product $(k_ia_i)_\pi$ rather than just $(k_i)_\pi$ for either ideal miscibility or immiscibility. Deviations from additivity in the experimental values indicate interactions between the components in the mixed monolayers.

Monolayer phase transitions between liquid-expanded and liquid-condensed phases or between monolayer and bulk phases were identified using a combination of second and third derivatives of π with respect to A as previously described (Ali et al. 1991, 1993).

RESULTS

In previous investigations involving cholesterol/GalCer mixed monolayers, only naturally derived GalCer subfractions were studied (Johnston & Chapman, 1988; Slotte et al., 1993). The results showed that, at low surface pressures, cholesterol has a much greater condensing effect on a bovine brain GalCer subfraction containing no hydroxy acyl chains (NFA-GalCer) compared to a GalCer subfraction containing only hydroxy acyl chains (HFA-GalCer). At high surface pressures, almost no condensation was observed with either GalCer subfraction. Because of naturally derived GalCer's heterogeneity [e.g., Johnson and Brown (1992)], it was not possible to determine what role acyl chain length and saturation play in modulating GalCer's interaction with cholesterol. To address this issue, we synthesized a number of GalCer derivatives which contained different homogeneous acyl chains. The acyl residues included lignocerate (24:0), stearate (18:0), nervonate ($24{:}1^{\Delta 15}$), decanoate (10:0), linoleate ($18{:}2^{\Delta 9,12}$), and docosenoate ($22{:}1^{\Delta 13}$). The surface pressure versus molecular area (π–A) and surface potential versus molecular area (ΔV–A) behavior of these GalCer derivatives have been reported previously [see Ali et al. (1991, 1993, 1994) and references therein].

Here, we investigated the effect of cholesterol on the force-area isotherms of GalCer species containing the different homogeneous acyl groups described above (Figures 1 and 2). For comparison with earlier studies (Johnston & Chapman, 1988; Slotte et al., 1993), we also examined mixed monolayers containing cholesterol and either bovine brain GalCer or its subfraction containing nonhydroxylated acyl chains (NFA-GalCer). To facilitate viewing, data were plotted as the apparent molecular area of GalCer versus surface pressure. The apparent molecular area of GalCer was calculated by dividing the total, measured, surface area of the cholesterol/GalCer mixed monolayers by the number of GalCer molecules in the surface. The isotherms in Figures 1 and 2 clearly show that, at equivalent mole fractions, cholesterol's relative expansion of GalCer's apparent molecular area was directly dependent on GalCer's acyl composition. In fact, little expansion occurred in the apparent molecular area of GalCer derivatives displaying liquid-expanded behavior. This behavior indicated a large condensation effect of cholesterol on these GalCer derivatives.

Mean Molecular Area versus Composition in Cholesterol/GalCer Films. To compare quantitatively the magnitude of cholesterol's condensing effect on the different GalCer derivatives, we determined the additivity of the mean molecular areas as a function of composition (Goodrich, 1957). Figures

[1] Abbreviations: GalCer, galactosylceramide; π, surface pressure; LE, liquid-expanded; LC, liquid-condensed; *N*-acylGalSph, *N*-acylgalactosylsphingosine.

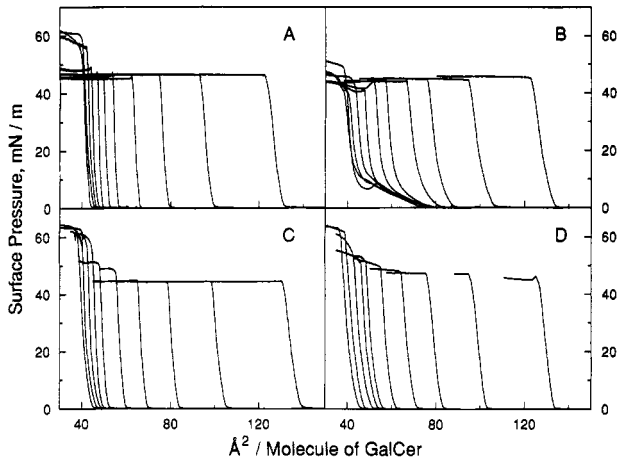

FIGURE 1: Surface pressure vs apparent molecular area of GalCer. Isotherms were recorded at 24 °C. The subphase consisted of 10 mM potassium phosphate (pH 6.6) containing 100 mM NaCl and 0.02% (w/v) NaN_3. Panel A shows bovine brain galactosylceramide with increasing mole fractions of cholesterol (from left to right, X_c = 0. 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, and 0.7). Panel B shows non-hydroxy fatty acyl- (NFA-) galactosylceramide with increasing mole fractions of cholesterol (same X_c as in panel A). Panel C shows *N*-24:0-GalSph with increasing mole fractions of cholesterol (from left to right, X_c = 0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7). Panel D shows *N*-18:0-GalSph with increasing mole fractions of cholesterol (same X_c as in panel C).

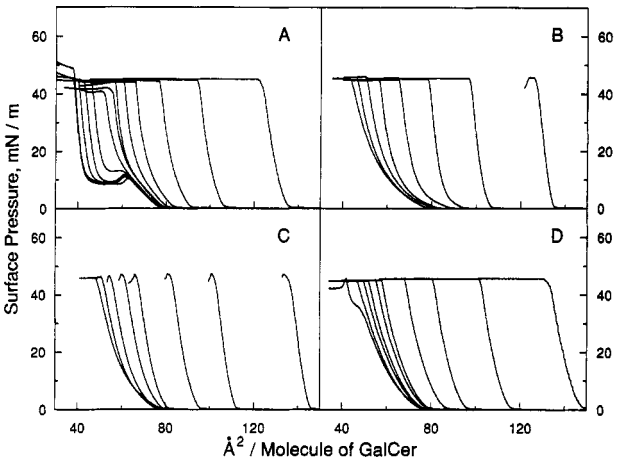

FIGURE 2: Surface pressure vs apparent molecular area of GalCer. Conditions were the same as for Figure 1. Panel A shows *N*-24:$1^{\Delta 15}$-GalSph with increasing mole fractions of cholesterol (from left to right, X_c = 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.276, 0.3, 0.35, 0.4, 0.5, 0.6, and 0.7). Panel B shows *N*-10:0-GalSph with increasing mole fractions of cholesterol (from left to right, X_c = 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7). Panel C shows *N*-$18:2^{\Delta 9,12}$-GalSph with increasing mole fractions of cholesterol (same X_c as in panel B). Panel D shows *N*-22:$1^{\Delta 13}$-GalSph with increasing mole fractions of cholesterol (from left to right, X_c = 0, 0.11, 0.165, 0.22, 0.275, 0.326, 0.43, 0.53, 0.63, and 0.726).

3 and 4 show the results at three different surface pressures (5, 15, and 30 mN/m). Solid lines represent ideal additivity (see Materials and Methods). It was clear that negative deviations from additivity occurred for all GalCer species. However, the magnitude of cholesterol's condensing effect was strongly modulated by GalCer's acyl structure. First, we considered cholesterol's effects on bovine brain GalCer (Figure 3A). Cholesterol had only a slight condensing effect on bovine brain GalCer. The condensation was relatively insensitive to surface pressure and reached a maximum (approximately 7.6% difference from additivity) near X_c = 0.2 (Figure 3A; Table 1). As shown in Figure 3C,D, cholesterol had very similar effects on both *N*-24:0-GalSph and *N*-18:0-GalSph except that the magnitude of the condensation ranged from about

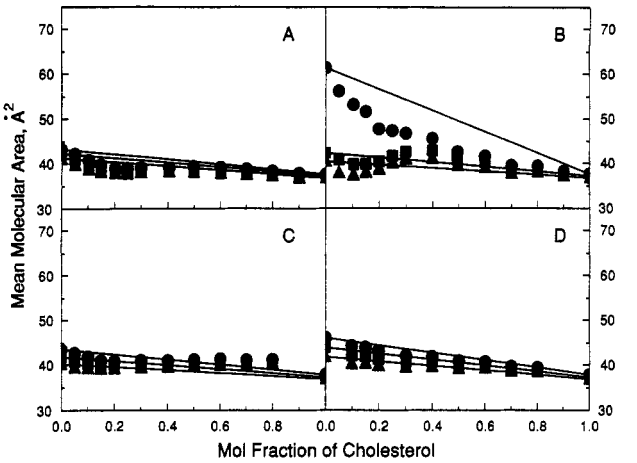

FIGURE 3: Mean molecular area vs composition. In each panel (●) 5 mN/m; (■) 15 mN/m; (▲) 30 mN/m. Ideal additivity of mean molecular area is represented by the solid line. (A) Bovine brain GalCer; (B) NFA-GalCer; (C) *N*-24:0-GalSph; (D) *N*-18:0-GalSph.

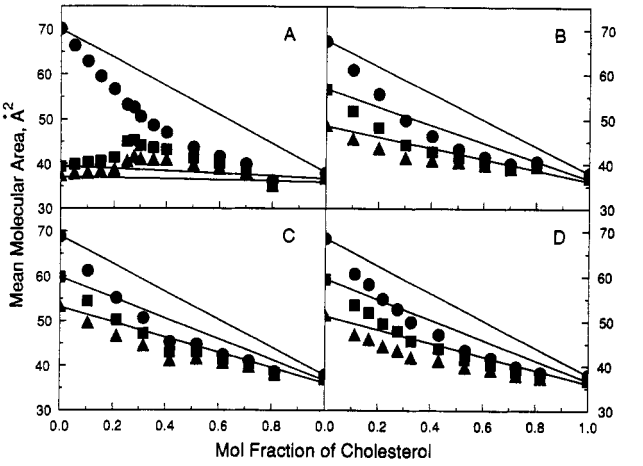

FIGURE 4: Mean molecular area vs composition. In each panel (●) 5 mN/m; (■) 15 mN/m; (▲) 30 mN/m. Ideal additivity of mean molecular area is represented by the solid line. (A) *N*-24:$1^{\Delta 15}$-GalSph; (B) *N*-10:0-GalSph; (C) *N*-$18:2^{\Delta 9,12}$-GalSph; (D) *N*-22:$1^{\Delta 13}$-GalSph.

3.5% change from additivity, at low pressures and X_c = 0.2, to zero (or slightly positive values) at X_c greater than 0.5.

In contrast, cholesterol had dramatic condensing effects on *N*-$18:2^{\Delta 9,12}$-GalSph and *N*-10:0-GalSph. For these liquid-expanded derivatives, condensation was evident at all mole fractions with maximum deviations from additivity (approximately 15–17.5% at 5 mN/m) occurring between X_c = 0.4 and 0.5 (Figure 4B,C; Table 1). The relative condensation at low surface pressure (5 mN/m) was about 2–3-fold greater than that observed at high surface pressure (30 mN/m) (Table 1). Cholesterol had similar condensing effects on *N*-22:$1^{\Delta 13}$-GalSph, a derivative with a liquid-expanded-to-condensed phase transition near 35 mN/m at 24 °C (Ali et al., 1993, 1994) and displayed liquid-expanded characteristics at 5, 15, and 30 mN/m (Figure 4D). Thus, the relative degree of condensation by cholesterol was strongly influenced by the physical character of the GalCer films.

In agreement with this observation was the fact that cholesterol's condensing effect on *N*-24:$1^{\Delta 15}$-GalSph and on NFA-GalCer changed substantially with the phase state of the films (Figures 4A and 3B, respectively). At low pressures (5 mN/m) where the GalCer films were liquid-expanded, dramatic condensation was induced by cholesterol with maximum relative values [e.g., 19% and 14%, respectively, at 5 mN/m] between X_c = 0.4 and 0.6 (Table 1). However, once the surface pressure was raised sufficiently high as to

Table 1: Cholesterol's Condensation of Galactosylceramides and Sphingomyelins in Mixed Monolayers

sphingolipid (SL) monolayer	surface pressure (mN/m)	sphingolipid (SL) molecular area: pure SL monolayer (Å²/mol)	mixed SL/cholesterol monolayers (Å²/mol of SL)[a] 4:1[c]	mixed SL/cholesterol monolayers (Å²/mol of SL)[a] 1:1[c]	condensation of SL packing (Å²/mol of SL) 4:1	condensation of SL packing (Å²/mol of SL) 1:1	% condensation of SL[b] 4:1	% condensation of SL[b] 1:1
bovine brain GalCer	5	43.3	39.3	41.1	4.0	2.2	7.6	2.7
	15	42.3	38.7	40.3	3.6	2.0	7.0	2.5
	30	41.3	38.2	39.5	3.1	1.8	6.2	2.3
NFA-GalCer	5	61.4	50.3	47.6	11.1	13.8	15.6	13.9
	15	42.4	41.3	43.7	1.1	NC[d]	2.3	NC
	30	40.5	39.3	41.5	1.2	NC	2.5	NC
N-24:0-GalSph	5	43.6	41.8	44.9	1.8	NC	3.5	NC
	15	41.8	40.7	43.8	1.1	NC	2.1	NC
	30	40.3	39.9	42.8	0.4	NC	0.9	NC
N-18:0-GalSph	5	46.2	44.7	44.4	1.5	1.8	2.7	2.1
	15	44.0	42.5	43.0	1.5	1.0	2.8	1.3
	30	41.9	40.8	41.6	1.1	0.3	2.2	0.4
N-24:1$^{\Delta 15}$-GalSph	5	70.1	61.3	49.6	8.8	20.5	11.0	19.0
	15	39.4	42.4	45.3	NC	NC	NC	NC
	30	37.3	38.9	42.2	NC	NC	NC	NC
N-10:0-GalSph	5	67.3	60.1	49.0	7.2	18.3	9.4	17.5
	15	56.5	50.8	45.7	5.7	10.8	8.7	11.5
	30	48.5	45.0	43.5	3.5	5.0	6.0	5.8
N-18:2$^{\Delta 9,12}$-GalSph	5	68.8	59.8	52.4	9.0	16.4	11.4	15.0
	15	59.7	53.7	49.2	6.0	10.5	8.6	10.5
	30	53.3	49.0	46.3	4.3	7.0	6.7	7.6
N-22:1$^{\Delta 13}$-GalSph	5	68.3	59.8	50.1	8.5	18.2	10.7	16.5
	15	59.3	52.9	46.2	6.4	13.1	9.1	12.9
	30	51.2	46.3	42.4	4.9	8.8	8.0	9.5
bovine brain sphingomyelin	5	74.3	61.7	45.1	12.6	29.2	15.0	26.1
	15	62.9	52.2	43.2	10.7	19.7	14.8	19.7
	30	49.7	44.8	41.5	4.9	8.2	8.3	9.4
egg sphingomyelin	5	70.9	57.3	43.2	13.6	27.7	16.9	25.5
	15	60.8	49.4	41.9	11.4	18.9	16.2	19.3
	30	48.4	43.3	40.7	5.1	7.7	8.9	9.0

[a] Areas occupied by cholesterol molecules are 37.6, 37.2, and 36.8 Å² at surface pressures of 5, 15, and 30 mN/m, respectively. [b] Calculation described under Materials and Methods. [c] Molar mixing ratio of sphingolipid:cholesterol, except for *N*-22:1$^{\Delta 13}$-GalSph, where data were calculated at cholesterol mole fractions of 0.22 and 0.53. [d] NC, no condensation.

induce condensed behavior to *N*-24:1$^{\Delta 15}$-GalSph and NFA-GalCer, the relative condensing effect dropped to almost nil. In fact, positive deviations from additivity were observed for X_c between 0.2 and 0.6.

Comparison of Cholesterol's Condensation of Sphingomyelin and GalCer. We also compared cholesterol's condensing effect on GalCer with that on sphingomyelin (SM). In earlier investigations, Phillips and co-workers reported that cholesterol's condensing effect on SM was larger than that on PC (Lund-Katz et al., 1988). Under our experimental conditions, we observed nearly identical condensation values for cholesterol on either bovine brain or egg SM. Table 1 shows values obtained at two mixing ratios of cholesterol/SM and cholesterol/GalCer. Note that significant condensation occurred only if sphingolipid films are liquid-expanded. More importantly, it was clear that cholesterol's condensing effect on SM is about 25–30% greater than its effect on GalCer. This was true despite various manipulations to GalCer's acyl chain involving hydrocarbon length and unsaturation.

Mean Compressibility versus Composition of Cholesterol/GalCer Films. To evaluate the effect that cholesterol had on the interfacial cohesive properties of GalCer and the extent to which GalCer's acyl structure modulated these interactions, we determined the compressibility as a function of composition for the different GalCer derivatives. Figures 5 and 6 show the results at three different surface pressures (5, 15, and 30 mN/m). Solid lines represent ideal compressibility and were calculated by apportioning values of pure cholesterol and GalCer as described in the Appendix. As shown in Figure 5A, addition of cholesterol had very little effect on the already very low compressibility of bovine brain GalCer films. This

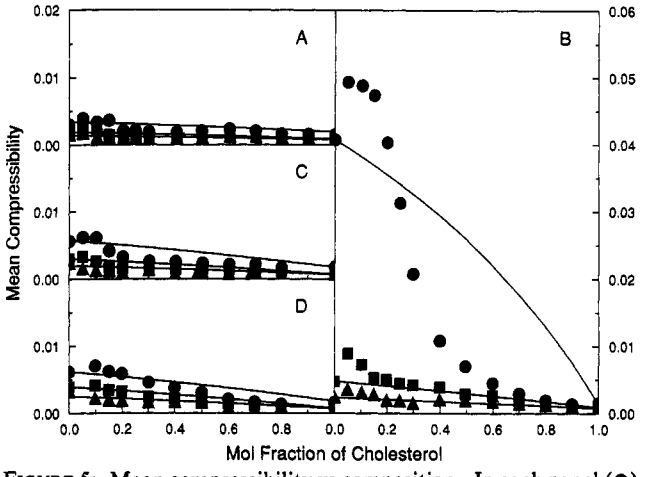

FIGURE 5: Mean compressibility vs composition. In each panel (●) 5 mN/m; (■) 15 mN/m; (▲) 30 mN/m. Ideal additivity of mean compressibility is represented by the solid line. (A) Bovine brain GalCer; (B) NFA-GalCer; (C) *N*-24:0-GalSph; (D) *N*-18:0-GalSph.

behavior could be attributed, in large part, to the high levels of long, saturated acyl chains because the compressibilities of *N*-24:0-GalSph and *N*-18:0-GalSph were also quite low and were only slightly affected by increasing amounts of cholesterol regardless of surface pressure (Figure 5, panels C and D, respectively).

In sharp contrast, cholesterol caused dramatic changes in the film compressibility of liquid-expanded GalCer derivatives. In the absence of cholesterol, GalCer derivatives such as *N*-18:2$^{\Delta 9,12}$-GalSph (Figure 6C), *N*-10:0-GalSph (Figure 6B), and *N*-22:1$^{\Delta 13}$-GalSph (Figure 6D) all had mean compressibilities

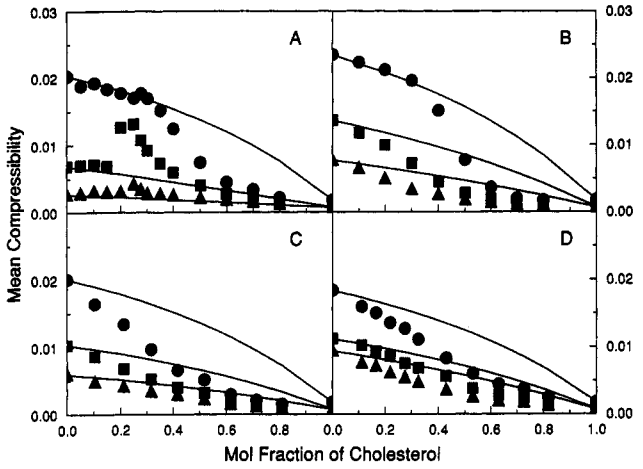

FIGURE 6: Mean compressibility vs composition. In each panel (●) 5 mN/m; (■) 15 mN/m; (▲) 30 mN/m. Ideal additivity of mean molecular area is represented by the solid line. (A) *N*-24:1$^{\Delta 15}$-GalSph; (B) *N*-10:0-GalSph; (C) *N*-18:2$^{\Delta 9,12}$-GalSph; (D) *N*-22:1$^{\Delta 13}$-GalSph.

about 4–5-fold higher than bovine brain GalCer. The addition of cholesterol decreased the mean compressibilities of the films particularly at X_c between 0 and 0.5. For *N*-18:2$^{\Delta 9,12}$-GalSph/cholesterol as well as for *N*-22:1$^{\Delta 13}$-GalSph/cholesterol films, the extent of the decrease exceeded the calculated ideal values and resulted in negative deviations from additivity (Figure 6C,D). In contrast, the mean compressibility of *N*-10:0-GalSph/cholesterol films, at low but not high pressures, showed slightly positive deviations from additivity between 0 and 40 mol % cholesterol and then went sharply negative at higher cholesterol mole fractions (Figure 6B). Cholesterol mole fractions between 0.4 and 0.6 produced maximum negative deviations in mean compressibility for all liquid-expanded GalCer species.

The mean compressibility for *N*-24:1$^{\Delta 15}$-GalSph/cholesterol and for NFA-GalCer/cholesterol mixtures were quite sensitive to surface pressure as well as composition. For instance, positive deviations from additivity were observed for *N*-24:1$^{\Delta 15}$-GalSph at X_c between 0.10 and 0.40 when the surface pressure was 5 or 15 mN/m (Figure 6A). However, at higher cholesterol compositions (X_c = 0.4–1.0), the compressibilities did show negative deviations from additivity but only when the films were liquid-expanded (e.g., 5 mN/m). Similar positive deviations from additivity were also observed for NFA-GalCer/cholesterol at X_c between 0.05 and 0.30 when the surface pressure was 5 or 15 mN/m, respectively (Figure 5B). In general, this behavior probably reflected the contribution of the two-dimensional phase transition in these GalCer derivatives which tended to oppose the negative deviation attributable to condensation. Regardless, at higher surface pressures (e.g., 30 mN/m), the effect of increasing cholesterol on the compressibilities in these mixtures was dampened considerably.

DISCUSSION

Cholesterol's Interaction with GalCer. Our results show clearly that acyl composition has a major impact on the way that different GalCer species interact with cholesterol. This finding is evident from the compressibility and condensation data calculated from the surface pressure versus molecular area isotherms measured at 24 °C.

One factor affecting cholesterol's ability to alter the lateral compressibility of GalCer films is monolayer phase state. If the films are already ordered and condensed by virtue of their acyl composition, cholesterol promotes very little additional condensation. However, if the GalCer films are liquid-expanded, cholesterol produces dramatic decreases in compressibility. In fact, compressibility values observed at 50 mol % cholesterol and 30 mN/m for liquid-expanded GalCer derivatives are quite similar to values for condensed GalCer species lacking cholesterol. This is consistent with the known "solidifying effect" of cholesterol on liquid crystalline phospholipids in bilayers [e.g., see Finegold (1993)].

Monolayer phase state and the structure of GalCer's acyl residues also have dramatic effects on cholesterol's ability to condense GalCer. First, consider the phase state of GalCer monolayers. Under conditions where the films are liquid-expanded, cholesterol interacts strongly with GalCer, as is indicated by the large condensing effect at X_c between 0.2 and 0.6. This interaction occurs regardless of whether the acyl chains are saturated (*N*-10:0-GalSph), monounsaturated (*N*-24:1$^{\Delta 15}$-GalSph and *N*-22:1$^{\Delta 13}$-GalSph), diunsaturated (*N*-18:2$^{\Delta 9,12}$-GalSph), or a saturated/monounsaturated mixture (NFA-GalCer). In contrast, it is clear that cholesterol has little or no condensing effect on GalCer monolayers which already display condensed film behavior. This is true for bovine brain GalCer, *N*-24:0-GalSph, and *N*-18:0-GalSph as well as for *N*-24:1$^{\Delta 15}$-GalSph and NFA-GalCer at high surface pressures. The importance of the monolayer phase state in determining the extent of interaction between cholesterol and glycerol-based phospholipids has been noted previously [e.g., Chapman et al. (1969)]. Our results explain why Slotte et al. (1993) failed to observe any interaction between cholesterol and GalCer. These investigators reported no condensing effect of 50 mole % cholesterol on bovine brain GalCer as well as its subfractions containing either hydroxylated and nonhydroxylated acyl chains at 20 mN/m.

Another important consideration with respect to cholesterol's interaction with GalCer is the position of the double bond(s) in the acyl chain. The large condensing effect that cholesterol exerts on liquid-expanded *N*-24:1$^{\Delta 15}$-GalSph, *N*-22:1$^{\Delta 13}$-GalSph, and *N*-18:2$^{\Delta 9,12}$-GalSph is consistent with cholesterol being anchored to the polar interface by its hydroxyl group, probably in the vicinity of the carbonyl group of GalCer's amide linkage, with its steroid ring penetrating the hydrocarbon domain of the GalCer species. The positions of the *cis* double bonds and their associated Δtg kinks (Huang, 1977; Davies et al., 1990) are distal enough from the interface so that the steroid ring can align closely with the linear portion of the acyl chain to promote strong van der Waals attractive interactions. Indeed, the large condensation also observed with *N*-10:0-GalSph supports this vision of optimal cholesterol interaction and is consistent with previous ideas concerning acyl chain length and unsaturation proposed for PC/cholesterol interactions in monolayers (Demel et al., 1972; Gosh et al., 1973). Not surprisingly, cholesterol's interactions with phospholipids in bilayer assemblies are also known to be quite sensitive to double-bond position (Ayanoglu et al., 1986, 1988, 1990; Davis & Keough, 1984a,b; Keough et al., 1989; Kariel et al., 1991).

Other classical indicators of mixing in two-component monolayers, such as collapse pressure versus composition, have quite limited value in elucidating cholesterol mixing with most of the GalCer derivatives studied here. For *N*-24:0-GalSph, *N*-18:0-GalSph, and bovine brain GalCer, the highly ordered nature of these films raises the possibility of kinetic limitations on mixing with cholesterol, especially at surface pressures near collapse [e.g., Cadenhead et al. (1976)]. This being the case, further experiments are needed to verify the apparent macroscopic miscibility, albeit highly nonideal, suggested by the decline in collapse pressure as cholesterol increases from 0 to 0.6 mole fraction (Figure 1). At higher cholesterol mole

fractions, the collapse pressures are quite similar to the value for pure cholesterol and suggest immisicibility. On the other hand, with several of the GalCer derivatives displaying liquid-expanded behavior (e.g., *N*-18:2$^{\Delta 9,12}$-GalSph and *N*-10:0-GalSph), the relatively small differences in their collapse pressures and that of cholesterol make unequivocal determination of mixing behavior unreliable. This also is the case for 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) mixing with liquid-expanded GalCer derivatives (Ali et al., 1991). Corroborating experimental evidence using fluorescence microscopy would be most helpful in assessing mixing behavior in these systems [e.g., McConnell (1991) and Hirshfeld and Seul (1990)].

The presence of a liquid-expanded-to-condensed phase transition provides an avenue for gaining limited insight into the interfacial mixing of *N*-24:1$^{\Delta 15}$-GalSph and NFA-GalCer with cholesterol. However, the metastable nature of this transition complicates interpretation in that it is very difficult to determine the width and slope of the transition as well as cholesterol's effects on these parameters. Using the transition onset pressure as an indicator [e.g., Ali et al. (1991)], cholesterol does appear to have limited miscibility with the liquid-expanded phases of *N*-24:1$^{\Delta 15}$-GalSph and NFA-GalCer. However, between X_c = 0.2 and 0.25, *N*-24:1$^{\Delta 15}$-GalSph's 2-D transition suddenly disappears completely. NFA-GalCer's 2-D transition disappears between X_c = 0.15 and 0.2.

A similar disappearance of dipalmitoylphosphatidylcholine's (DPPC) liquid-expanded-to-liquid condensed monolayer transition has been reported at X_c between 0.2 and 0.3 (Cadenhead & Müller-Landau, 1979; Müller-Landau & Cadenhead, 1979; Albrecht et al., 1981). In a series of more recent reports, McConnell and co-workers used both force–area and fluorescence microscopic approaches to determine the mixing of cholesterol with DMPC and DPPC [for review, see McConnell (1991)]. These investigators showed that two immisicible liquid phases can coexist in a single mixed monolayer and identified a critical composition at 30 mol % cholesterol above which the two liquid phases, one enriched in cholesterol and the other enriched in DPPC, merge into a single phase of uniform composition on increasing lateral surface pressure. On the basis of the force–area isotherms, the cholesterol/*N*-24:1$^{\Delta 15}$-GalSph mixed monolayers show some interesting similarities to the cholesterol/DPPC case. However, further experiments will be needed to establish the extent of similarity in these systems.

Cholesterol's Interaction with Sphingomyelin Compared to GalCer. A variety of studies have suggested that cholesterol interacts more favorably with sphingomyelin than with membrane phospholipids such as phosphatidylcholine [for review, see Schroeder et al. (1991)]. The results of this study show that, despite being sphingoid rather than glycerol-based, GalCer is not condensed by cholesterol to the same extent as sphingomyelin. The reason that cholesterol condenses SM more than GalCer is because, at equivalent surface pressures, the liquid-expanded SM is larger than the GalCer (in the absence of cholesterol) and because the apparent molecular area of SM is smaller than that of GalCer in the presence of cholesterol (Table 1). At 5 mN/m, bovine brain and egg SM molecular areas are 74 and 71 Å^2, respectively, whereas GalCer molecular areas range from 61 to 70 Å^2 depending on acyl composition. In contrast, at 0.5 mole fraction cholesterol, the apparent molecular areas of SM are smaller (approximately 41–45 Å^2) than those of the "condensable" GalCer derivatives (approximately 48–52 Å^2). The net result is a larger condensation for SM than for GalCer.

Interestingly, our results indicate that differences in SM and GalCer acyl composition are not sufficient to explain the differences in condensation with cholesterol. Rather, differences in the headgroup structure also must play a role. For instance, a comparison of the force–area isotherms of GalCer and SM containing only palmitate acyl chains reveals condensed behavior for the GalCer derivative (Ali et al., 1993, 1994) but both liquid-expanded and -condensed behavior with a two-dimensional phase transition (21 mN/m) for the SM derivative (Lund-Katz et al., 1988). Because the ceramide structure of each derivative is identical, GalCer's headgroup must promote stronger intermolecular interactions. Indeed, an analogous situation exists for phosphatidylcholines compared to phosphatidylethanolamines (Chapman et al., 1969).

Two factors which could contribute to headgroup structural differences in GalCer and SM are hydrated bulk volume and average orientation. First, consider that X-ray studies of PC and PE monolayers do reveal differences in the headgroup region attributable to hydrated bulk volume (Vaknin et al., 1991; Helm et al., 1991). While similar structural data for GalCer and SM monolayers have not been reported yet, many investigators have noted similar hydration levels for PE and glycolipids bearing one sugar residue [e.g., Sen and Hui (1988)] as well as for SM and PC, which possess identical phosphocholine headgroups [e.g., Calhoun and Shipley (1979) and McIntosh et al. (1992)]. In fact, SM has a larger hydration shell than GalCer (Ruocco & Shipley, 1983; Sen & Hui, 1988).

Aside from the headgroup hydration, it appears likely that the headgroups of GalCer and SM assume different average orientations in the chain-disordered (liquid-expanded) state. GalCer's sugar headgroup, on average, extends normal to the interface in fully hydrated liquid-crystalline bilayers (Skarjune & Oldfield, 1982; Jarrell et al., 1992), whereas phosphocholine's orientation is more parallel (within 30°) to the plane of the membrane (Bruzik, 1988; Buldt et al., 1978; Seelig et al., 1987). The incorporation of cholesterol, with its planar steroid ring, reduces *trans–gauche* isomerization about the carbon–carbon bonds in the liquid-disordered acyl chains of GalCer and SM [e.g., Davies et al. (1990)]. As a result, condensation occurs, in large part, because of the tremendous gain in van der Waals attractive forces between molecules. A consequence of cholesterol being positioned near the interface with its extremely low hydration capability [e.g., Sen and Hui (1988)], could be reorientation of the headgroups of GalCer or SM in a way that prevents GalCer from achieving the same small molecular areas as SM (see Table 1). Indeed, subtle chemical alterations of GalCer's headgroup (Nyholm et al., 1990) and changes to SM's isomeric configuration (Bruzik, 1988) are known to promote drastic conformational changes to their headgroups. Whether similar changes are also a consequence of molecular condensation by cholesterol will require additional experiments. Thus, to summarize, we believe that GalCers pack more tightly (occupy smaller areas) in liquid-expanded films than SMs do and that they achieve somewhat larger areas upon mixing with equimolar cholesterol. The end result is a diminished condensing effect for liquid-disordered GalCers than for SMs.

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APPENDIX

When the mean compressibility is used to evaluate interactions between components in mixed monolayers, ideal

behavior is calculated and compared to the experimentally observed values at various lipid mixing ratios. Of central importance in calculating either ideal miscibility or immiscibility is whether the compressibility of each pure component should be apportioned simply by mole fraction prior to summation [e.g., see Bonosi et al. (1992)]. To test this hypothesis, consider the following: For a single lipid species (i) at a specified surface pressure (π)

$$k_i = (-1/a_i)\,(da_i/d\pi) \qquad \text{(A1)}$$

For a binary lipid mixture that is either ideally miscible or immiscible, the average compressibility $(\bar{k})_\pi$ at a specified surface pressure (π) is

$$\bar{k}_\pi = -1/\bar{A}_\pi\,(d\bar{A}/d\pi)_\pi \qquad \text{(A2)}$$

where $\bar{A}_\pi = X_1(a_1)_\pi + X_2(a_2)_\pi$. Substituting for $\bar{A}_\pi$ in eq A2 yields

$$\bar{k}_\pi = \left(\frac{-1}{a_1X_1 + a_2X_2}\right)_\pi [X_1(da_1/d\pi)_\pi + X_2(da_2/d\pi)_\pi]$$

However, according to eq A1, $(da_1/d\pi)_\pi = (-k_1a_1)_\pi$ and $(da_2/d\pi)_\pi = (-k_2a_2)_\pi$. Therefore

$$\bar{k}_\pi = \left(\frac{-1}{a_1X_1 + a_2X_2}\right)_\pi [X_1(-k_1a_1)_\pi + X_2(-k_2a_2)_\pi]$$

which simplifies to

$$\bar{k}_\pi = -1/\bar{A}_\pi[(k_1a_1)_\pi X_1 + (k_2a_2)_\pi X_2]$$

and shows that, for either ideal miscibility or immiscibility, $\bar{k}_\pi$ is additive with respect to the product $(k_ia_i)_\pi$ rather than just $(k_i)_\pi$ at a specified surface pressure.

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