ANNUAL REVIEWS

Annual Review of Biophysics

The Physical Properties of Ceramides in Membranes

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Annu. Rev. Biophys. 2018. 47:633–54

First published as a Review in Advance on April 4, 2018

The *Annual Review of Biophysics* is online at biophys.annualreviews.org

https://doi.org/10.1146/annurev-biophys-070317-033309

Keywords

sphingolipids, sphingosine, ceramides, membrane domains, lipid phases, fatty acids

Abstract

Ceramides are sphingolipids containing a sphingosine or a related base, to which a fatty acid is linked through an amide bond. When incorporated into a lipid bilayer, ceramides exhibit a number of properties not shared by almost any other membrane lipid: Ceramides (*a*) are extremely hydrophobic and thus cannot exist in suspension in aqueous media; (*b*) increase the molecular order (rigidity) of phospholipids in membranes; (*c*) give rise to lateral phase separation and domain formation in phospholipid bilayers; (*d*) possess a marked intrinsic negative curvature that facilitates formation of inverted hexagonal phases; (*e*) make bilayers and cell membranes permeable to small and large (i.e., protein-size) solutes; and (*f*) promote transmembrane (flip-flop) lipid motion. Unfortunately, there is hardly any link between the physical studies reviewed here and the mass of biological and clinical studies on the effects of ceramides in health and disease.

Contents

INTRODUCTION

Ceramides are a class of sphingolipids. Sphingolipids are structurally based on sphingosine [(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol] or related bases (e.g., the saturated sphinganine, the 20-carbon-atom eicosasphingosine, the 4-hydroxylated and saturated phytosphingosine, and several others, known globally as the sphingoid bases). Ceramides are the N-acyl amide derivatives of the sphingoid bases (**Figure 1**). Because of the diversity of sphingoid bases and acyl chains, as many as several hundred ceramide species have been identified thus far. Ceramides are usually found in very small amounts [<1 mole percent (mol%)] in cell membranes, although their concentration may increase by tenfold and even more under stress/apoptosis conditions. They exist at high concentrations in the skin stratum corneum, but this aspect is not considered in our review.

Ceramides exhibit a number of unique physical properties, particularly in mixtures with phospholipids. This review presents a concise update of the current studies on those properties. Previously published reviews (24, 39, 40, 50) provide useful insights on the progress of these studies over the past two decades. In our contribution, we focus our attention on work published in the last five years. In this review, the word ceramide refers, unless specified otherwise, to N-palmitoyl (C16:0) ceramide.

CERAMIDE PHYSICAL PROPERTIES: A SUMMARY AND UPDATE

As shown in the early studies by I. Pascher (64), the capacity of ceramides to be simultaneously involved in multiple hydrogen bonds (H-bonds) has been recognized as a typical and important capability of these and other related sphingolipids. Currently, the ceramide H-bonding network is generally understood to be at the basis of most of the unusual physical properties of these biomolecules. Some of these properties, reviewed in References 39–41, are (*a*) ceramide hydrophobicity is extreme, at least for N-acyl chains 12 carbon atoms or longer (89), and thus free ceramides cannot exist in suspension in aqueous media, including the cytosol; (*b*) ceramides give rise to highly

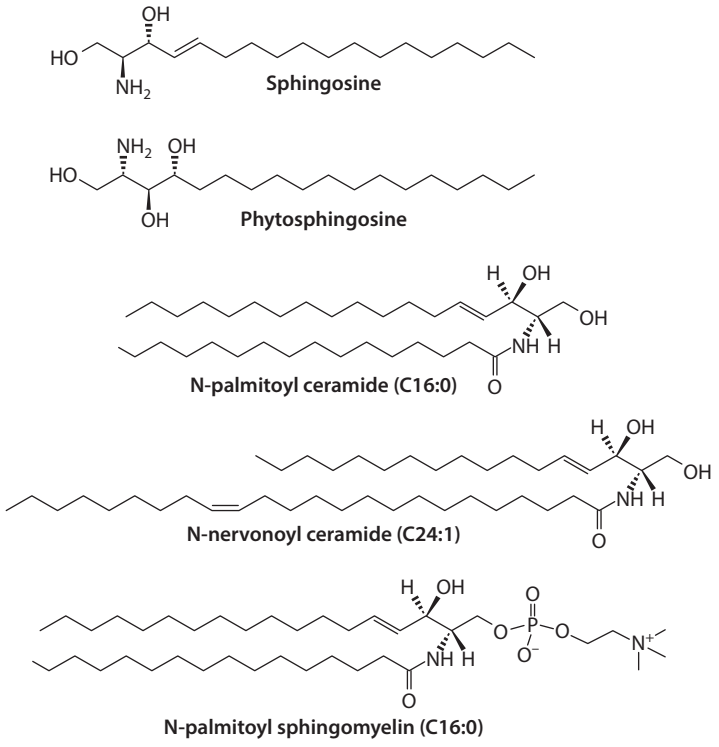

Figure 1

Chemical structures of sphingosine and relevant sphingolipids.

condensed monolayers at the air-water interface (12) and increase the molecular order (rigidity) of phospholipids in membranes (29, 46, 47, 82); (*c*) ceramides, in phospholipid bilayers, give rise to lateral phase separation and domain formation (17, 45, 47, 88, 95; review in 40); (*d*) ceramides possess an intrinsic negative curvature, a property that facilitates formation of inverted hexagonal phases and promotes the lamellar-to-hexagonal transition (89, 95); (*e*) ceramides make bilayers and cell membranes permeable to small and large (i.e., protein-size) solutes (61, 62, 75); and (*f*) ceramides promote transmembrane (flip-flop) lipid motion (5, 21, 23, 55, 72).

Some of these aspects have received renewed attention in recent years and are explained below.

Hydrogen Bonding and Ceramide Hydrophobicity

Gillams et al. (38) examined ceramide headgroup hydration using a combination of experimental and computational techniques: nuclear magnetic resonance (NMR), neutron diffraction, empirical potential structure refinement, and molecular dynamics. When water is added in small amounts to ceramide in chloroform solution, chloroform solvation of the headgroup is disturbed and water is observed to preferentially hydrate the carbonyl oxygen. The strongly solvated amide carbonyl moiety bridges via H-bonding interactions to hydroxyl groups, preferably to the C1 hydroxyl. The intermolecular H-bonding directs the conformation adopted by the ceramide headgroup. Molecular dynamics simulations suggest that the water molecules are highly mobile rather than strongly bound to any particular part of the headgroup. With respect to the hydroxyl groups, the C1 is much more frequently involved in H-bonding than the C3 hydroxyl is and H-bonds either to its neighboring amide group or to a water molecule. The lack of tightly bound (i.e., the

short residence time of) water molecules explains the extreme ceramide hydrophobicity at the macroscopic level and may be critical for allowing ceramides to pack very tightly in membranes or membrane domains (38).

Lateral Phase Separation of Ceramides

The well-known property of the lateral phase separation of ceramides was further studied by Busto et al. (9) using a combination of lipid monolayer- and bilayer-based model systems. These authors explored in detail the interactions between and organization of palmitoyl sphingomyelin and palmitoyl ceramide. Altogether, the presence of at least two immiscible phase-segregated mixtures of different compositions was proposed at high mole fractions ($x_{SM} > 0.7$) of sphingomyelin. A condensed phase, with domains segregated from the liquid-expanded phase, showed enhanced thermodynamic stability near a compositional ratio of 2:1 (sphingomyelin to ceramide). Ceramide phase separation was also explored from a novel point of view by Leung et al. (51). Using deuterium NMR (^{2}H NMR), these authors monitored the thermal properties of perdeuterated-palmitoyl ceramide in mixtures with proton-based palmitoyl sphingomyelin and conversely of perdeuterated-palmitoyl sphingomyelin in mixtures with proton-based ceramide. This allowed the separate observation of sphingomyelin and ceramide N-acyl chain melting in the presence of the second component, meaning that the miscibility of both lipids could be examined over a wide temperature range (25–80°C). The corresponding data confirmed that a ceramide-enriched gel phase formed with the addition of even a small amount of ceramide to sphingomyelin. In addition, they showed that the gel phase formed by sphingomyelin and ceramide was more stable than the pure sphingomyelin gel phase and that there was no ceramide crystal formation up to at least 50 mol% ceramide in the mixture.

When NMR data were combined with differential scanning calorimetry (DSC) of the same samples, it was seen that the DSC signal paralleled the melting of sphingomyelin in mixtures with 10 mol% ceramide, while at 20–30 mol%, the DSC data overlapped the ceramide melting data. A detailed phase diagram was obtained from the combined NMR and DSC data for sphingomyelin-ceramide mixtures containing between 0% and 50% ceramide. The main conclusion was that the two lipids mixed well in the liquid-crystalline state and, for high palmitoyl ceramide contents, also in the gel state (51).

Ceramides and Lipid Polymorphism

The ability of ceramides to promote hexagonal phase formation (95) has been revisited by Doroudgar & Lafleur (26), who combined DSC with ^{2}H- and phosphorous-31–NMR spectroscopy. In mixtures with POPE (1-palmitoyl-2-oleoyl phosphatidylcholine), the presence of ceramide lowered the temperature of (i.e., facilitated) the lamellar-to-inverted hexagonal (L-H_{II}) transition of POPE, in agreement with the previous observations. Ceramide was also seen to increase the gel (L_β) to liquid crystalline (L_α) phase transition. This leads to a large area of L_β/L_α coexistence in the phase diagram and can be related to the above described property of ceramide of rigidifying the phospholipid acyl chains in binary mixtures (46).

An interesting observation of a hexagonal phase was recently made by Dupuy et al. (27) with N-decanoyl ceramide. This lipid exhibits an important chain-length asymmetry between the C18 sphingoid and the C10 N-acyl chains. Using a combination of small-angle X-ray scattering and polarized light microscopy, they were able to establish that, at room temperature, fully hydrated N-decanoyl ceramide gave rise to an inverted hexagonal phase. This H_{II} phase had the unusual peculiarity of containing highly ordered acyl chains, perhaps because chain-length asymmetry reduces the void volumes between the lipid tubules arranged in hexagonal lattice.

Ceramides Markedly Increase Bilayer Permeability

Colombini and coworkers (16, 68; review in 20) have published reports indicating that ceramide increases the permeability of mitochondrial outer membranes. This in itself is confirmatory of the original observation (75) of the permeabilizing properties of ceramides. However, Siskind & Colombini (84) proposed that ceramide-induced permeabilization would occur through the formation of stable ceramide channels that would be, in turn, related to apoptosis. This hypothesis has been criticized on the basis of the highly unfavorable energetics involved and the lack of structural evidence. Samanta et al. (77) published transmission electron microscopy images that they considered consistent with stain-filled pores having a roughly circular profile. However, the staining method that was used can give information of the surface only; thus the dark circles seen could be ceramide-caused membrane invaginations, if not artifacts due to the osmium tetroxide/uranyl acetate treatment affecting differently the control and ceramide-containing samples. In a recent paper addressing the issue of the mechanism of membrane permeabilization by ceramides, Artetxe et al. (4), using assays of release of the fluorescent dye calcein from liposomes induced either by addition of ceramide to preformed liposomes or by in situ generation of ceramide through sphingomyelinase action on sphingomyelin in the bilayers, obtained results contradictory to the channel hypothesis. They were able to distinguish experimentally between "gradual" and "all-or-none" release mechanisms, corresponding, respectively, to all the vesicles leaking part of their aqueous contents and to part of the vesicles leaking out all of their aqueous contents (65). The presumed ceramide channels, typically 10 nm in diameter, which would stay open for tens of minutes (20), could cause only all-or-none leakage. However, Artetxe et al. (4) observed, in general, an overall gradual phenomenon of contents release (**Figure 2**). Only at short times after ceramide addition or with low ceramide concentrations was all-or-none leakage observed—the latter phenomenon being attributed to open, collapsed vesicles produced as a result of changes in membrane surface tension induced by the sphingomyelin-to-ceramide conversion (56). A direct comparison between

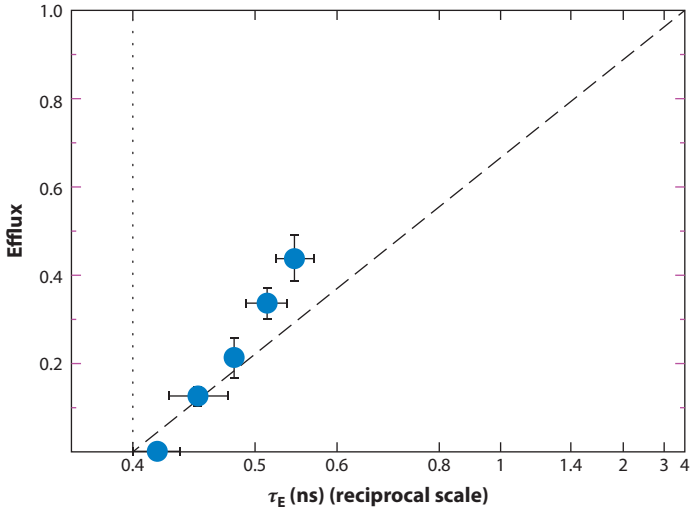

Figure 2

Correlation of calcein efflux with the lifetime of the entrapped dye. Calcein efflux induced by ceramide in sphingomyelin-phosphatidylethanolamine-cholesterol liposomes. The dashed line corresponds to an ideal graded leakage, and the dotted line corresponds to an ideal all-or-none leakage mechanism (65). For methodological details, see Reference 4. Abbreviation: τ_E, fluorescence emission lifetime of the entrapped dye.

Colombini's and our results is difficult, owing to differences in assay techniques and membrane composition. In the absence of specific structural proof of long-lived channels, it is safer to support the alternative, simpler hypothesis—that ceramide-rich islets, formed as a result of the lateral segregation of ceramide (88) in a sea of fluid lipids, would give rise to structural defects at the fluid-solid interface, through which leakage would occur (63).

MECHANICAL PROPERTIES OF CERAMIDES

Studies by Monroy, López-Montero, and coworkers (14, 15, 30, 54) have shed light on the unique mechanical properties of ceramides in monolayers. In general, lipids are considered to have viscous properties but no significant elastic behavior. However, in the case of egg-ceramide monolayers, Espinosa et al. (30), using surface shear rheology measurements at a surface pressure of $\pi = 30$ mN/m and $T = 37°C$, found a high shear modulus ($G' \approx 30$–100 mN/m) typical of two-dimensional solids and a loss modulus of $G'' \approx 25$ mN/m, $G' > G'' > 0$ being expected for solids. More recently, Catapano et al. (14) have expanded our knowledge of ceramide rheology and correlated it with known data of monolayer phase transitions, obtained through a combination of Langmuir balance surface pressure measurements and Brewster angle microscopy observations (32). Catapano et al. (14) describe a solid ceramide phase, at low T (<20–25°C), able to support shear deformations. Above that temperature, ceramides are still solid ($G' > G'' > 0$) but mechanically softened, as both moduli decrease almost linearly with temperature. This so-called soft solid state corresponds to the liquid-condensed phase in most monolayer studies and displays viscoelastic properties. At a given fluidification temperature of 40–50°C, G' vanishes and the ceramide monolayers become liquid (i.e., adopt a liquid-expanded phase that may coexist with microscopic liquid-condensed domains in a composite viscoregulated system). Interestingly, the authors mention the possibility of supercooling effects and a glass transition of ceramide monolayers when T was lowered from 60°C to 45°C. In another study, Catapano et al. (15) measured the shear viscosity of sphingomyelin monolayers and its variation upon addition of sphingomyelinase that degrades sphingomyelin to ceramide. The data show that the surface shear viscosity increases in parallel with ceramide formation, giving rise to a composite material in which a macroscopic fluid coexists with ceramide-rich microscopic condensed domains. Such viscous thickening seems to be due to frictional interactions between solid ceramide domains, correlating with the lattice structuring previously reported by Fanani & Maggio (32). Furthermore, López-Montero et al. (54) reported that ceramide monolayers undergo plastic deformations in response to the applied stress beyond a yield point corresponding to a critical stress of $\sigma_Y \approx 1$ mN/m. This complexity of mechanical properties of ceramides under conditions not far from the physiological ones is probably unparalleled among lipids.

CERAMIDE INTERACTION WITH CHOLESTEROL

Cholesterol not only is a very important molecule from a functional point of view but also has very peculiar structural properties. This very rigid, planar molecule cohabits in membranes, often at concentrations above 10 mol%, with the flexible, long chains of phospholipids and sphingolipids. Studies by Slotte & Bierman (85) had shown that when human fibroblasts were treated with natural sphingomyelinase, leading to in situ production of ceramide, cholesterol moved rapidly away from the plasma membrane to some intracellular pool. This was early proof of the interactions of sphingolipids and cholesterol in membranes. Ceramide and cholesterol are both highly hydrophobic molecules, with small headgroups. This probably explains their common tendency to intercalate between phospholipid acyl chains, sometimes occupying the same space, and perhaps competing

for it, mutually displacing each other. This was first described by Megha, Bakht, and London (60). Later, Taniguchi et al. (94) demonstrated that sphingomyelinase activity caused disassembly of the cholesterol-enriched liquid-ordered phase, and they also described the ensuing displacement of cholesterol from the ordered, segregated phase to the disordered, continuous phase.

The observation that, in the presence of phospholipids and cholesterol, the existence of ceramide-enriched domains was highly dependent on cholesterol concentration suggested the idea of a critical balance between the two molecules that could act as a modulator of cell processes (e.g., signaling or apoptosis). The coexistence of two distinct phases, cholesterol-enriched and ceramide-enriched, was demonstrated by fluorescence, electron spin resonance, and atomic force microscopy (AFM) methods (19, 81, 90, 96). Alanko et al. (2) showed that ceramide displaces cholesterol from sphingomyelin if the concentration of both lipids is in the 10–20 mol% range. When this happens, the gel phase formed by ceramide and sphingomyelin has a higher packing density than the original liquid-ordered phase; thus its nanomechanical properties and thickness are strongly affected (17, 18, 36, 91–93). Another interesting feature of the ceramide-enriched gel phase in binary systems (phospholipid-ceramide, without cholesterol) is that it seems to be stoichiometrically constant, as a higher content in ceramide does not affect nanomechanical resistance or thickness but only domain size (36).

In turn, when the lipid system is saturated in cholesterol, ceramide is not able to displace the sterol. Moreover, in systems highly saturated in both cholesterol and ceramide (10, 11), cholesterol prevents ceramide-enriched domain segregation into fluid (13, 83) and nonfluid areas. This demonstrates that ceramide/cholesterol displacement at high concentrations is not a matter of a special preference or interaction of any of them toward any phospholipid but rather a matter of the relative ratio of both molecules, as both have a tendency (owing to their hydrophobicity) to occupy the spaces between the lipid acyl chains of phospholipids.

POPC: 1-palmitoyl-2-oleoyl phosphatidylcholine

SM: sphingomyelin

DOPC: dioleoylphosphatidylcholine

DPPC: dipalmitoyl phosphatidylcholine

PSM: palmitoyl sphingomyelin

PCer: palmitoyl ceramide

CERAMIDES IN TERNARY OR MORE COMPLEX MIXTURES

An early, and very interesting, study of ceramides in POPC (1-palmitoyl-2-oleoyl phosphatidylcholine)-sphingomyelin (SM)-ceramide (Cer) ternary mixtures performed by Boulgaropoulos et al. (7) included a rather detailed phase diagram along the (SM + Cer) 50 mol% line of the complete ternary phase diagram. A further study of ternary and quaternary mixtures was published by Pinto et al. (69). In addition to writing a very instructive methodological section, these authors described the behavior of mixtures of increasing complexity, from binary [DOPC (dioleoylphosphatidylcholine)-DPPC (dipalmitoyl phosphatidylcholine), POPC-PSM (palmitoyl sphingomyelin), POPC-PCer (palmitoyl ceramide)] to ternary (POPC-PSM-cholesterol), and to quaternary (POPC-PSM-cholesterol-Cer). The ternary mixtures were designed to mimic lipid rafts, while the quaternary ones were intended to model the ceramide platforms. These authors were perhaps the first to underline the prime importance of the ceramide-to-cholesterol ratio to analyze the behavior of the quaternary mixtures, although the phenomenon had already been hinted at by Sot et al. (90), Castro et al. (13), Silva et al. (83), and Busto et al. (11). Pinto et al. (69) found, comparing a series of quaternary mixtures, that the ceramide-enriched gel domains became less tightly packed as the cholesterol fraction was increased. Cholesterol also decreased the extent of the gel phase formed in these mixtures. The data were interpreted in terms of an increased solubilization of ceramide in the cholesterol-rich liquid-ordered phase when cholesterol levels were high.

Shortly afterward, Busto et al. (10) described and characterized lamellar gel (L_β) phases of ternary lipid composition containing ceramide and cholesterol. Until then, L_β phases had been found only in single- or two-lipid systems. With a combination of X-ray scattering, confocal fluorescence microscopy, AFM, and DSC, these authors were able to characterize gel phases of ternary

PC: phosphatidylcholine

lipid compositions in the presence of cholesterol, palmitoyl ceramide, and saturated phospholipids that could be either DPPC or palmitoyl sphingomyelin at a 23:23:54 ceramide-to-cholesterol-to-DPPC or PSM mol ratio. These phases were stabilized by direct cholesterol-ceramide interactions (small changes in the chemical groups of either lipid prevented gel phase formation) and exhibited intermediate properties between the liquid-ordered (e.g., DPPC-cholesterol) and the conventional gel (e.g., pure DPPC below 35°C) phases. The calorimetric and X-ray data were characteristic of a gel (L_β) phase, with a gel-fluid transition temperature at a melting temperature (T_m) of 54°C. However, force spectroscopy measurements (based on AFM tip-mediated indentation experiments) revealed, at least for the sphingomyelin-based mixtures, intermediate breakthrough forces between the sphingomyelin-cholesterol and the sphingomyelin-ceramide systems. The study by Busto et al. (10) provided novel evidence for a chemically defined, multicomponent lipid system that could support the formation of ceramide- and cholesterol-rich domains or platforms in cell membranes.

A more detailed study of the ternary phase by AFM-surface topology and force spectroscopy was performed by García-Arribas et al. (36). Starting with binary phospholipid-palmitoyl ceramide mixtures at $x_{PCer} < 0.33$, they observed that increasing ceramide concentrations led to an increased area of ceramide-rich, micron-sized domains. However, the AFM-derived parameters of these domains did not vary with ceramide mole fraction, indicating that the domains increased in size while keeping a constant phospholipid-ceramide stoichiometry. Comparing sphingomyelin-ceramide and DPPC-ceramide bilayers, they could observe that the sphingomyelin-ceramide domains were thinner than the surrounding sphingomyelin-rich phase, while the opposite occurred in DPPC-based bilayers. A higher breakthrough phospholipid-ceramide force was measured for sphingomyelin-ceramide than for DPPC-ceramide domains, suggesting a preferential interaction of ceramide with its cognate sphingolipid sphingomyelin. When cholesterol was incorporated into the mixtures, all binary domains disappeared; instead, bilayers with nanomechanical and topological properties different from any of the pure or binary systems were observed. No displacement of cholesterol by ceramide or vice versa was detected under these conditions. Instead, the data were interpreted in terms of a preferential interaction between ceramide and cholesterol.

Cholesterol-ceramide interactions in DPPC or sphingomyelin bilayers were also studied using a novel technique—positron annihilation lifetime spectroscopy—that allows the detection of free volume voids in lipid bilayers (35). The data showed a different behavior of cholesterol and ceramide in binary mixtures with phospholipids. While cholesterol decreased the average void size as compared with the pure phospholipids in the fluid phase, ceramide increased the mean void volume with respect to pure sphingomyelin, particularly above 30°C. In the same temperature range, the mixture phospholipid-ceramide-cholesterol (54:23:23) exhibited a sharper increase in free volume size in the same temperature range, which provided new evidence pointing toward the formation of a specific phase by the ternary composition. Companion molecular dynamics simulations also indicated direct cholesterol-ceramide interactions in the ternary phase, as ceramide molecules appear to be displaced by 1–2 Å toward the polar region of each leaflet to accommodate cholesterol in order to optimize the interaction.

Slotte et al. (86) have examined, using mainly ^{2}H NMR, mixtures of ceramide and cholesterol with either DOPC or POPC. They found that the ordering effect of the sterol on the ceramide acyl chain, both in the fluid and the gel phase, was markedly influenced by the acyl chain composition of the fluid PC. These results were interpreted as suggesting that ceramide did not display a high affinity toward the sterols.

The most complex ceramide-enriched system studied up to now consists of human red blood cell lipid extracts to which different concentrations of palmitoyl ceramide were added (34). The mixtures were examined by AFM, in both imaging and force spectroscopy modes. The lipid extracts contained ~45 mol% cholesterol, which explains why ceramide concentrations up to 10%

did not give rise to any apparent lateral heterogeneity. However, the presence of ceramide at 30 mol% gave rise to a clearly distinguishable segregated phase with a nanomechanical resistance sevenfold higher than the surrounding continuous phase. Furthermore, cholesterol depletion of the bilayers using cyclodextrin caused domain generation in the originally homogeneous samples, and cholesterol-depleted domain stiffness significantly increased with increasing amounts of ceramide. These results indicate that, in contrast with the idea that ceramide-rich domains are tightly packed gel phases that exclude cholesterol, with constant stoichiometry and properties independent of ceramide concentration, ceramide can induce, in the presence of cholesterol, multiple-component gel-like phases depending on the lipid proportions, with biophysical properties intermediate between the phospholipid-ceramide gel and the phospholipid-cholesterol liquid-ordered phases. These complex gel-like phases (whose precise ceramide-to-cholesterol ratios are still poorly defined) could be fine-tuned by the cell metabolic machinery to exert a role in sphingolipid signaling, membrane platform formation, and intracellular membrane traffic.

Studies with Very Long Acyl Chain Ceramides

The above, in common with most biophysical studies on ceramides, were performed with the palmitoyl (C16:0) species. However, the very long chain ceramide N-nervonoyl (C24:1) sphingosine is relatively abundant in many mammalian tissues (M. Manni, unpublished data). To explore the effects of using this very long chain ceramide, researchers studied both C24:1 and C16:0 ceramides in mixtures with DOPC, sphingomyelin (either C24:1 or C16:0), and cholesterol (37). Confocal fluorescence microscopy, DSC, and AFM (imaging and force measurements) were used. In general, C24:1 ceramide appeared to have a lower stiffening effect than C16:0 ceramides did, and C24:1 sphingomyelin fluidized the mixture and decreased the T_m gel-fluid transition temperature in every case. Maté et al. (57) had shown that the 24:1 fatty acid (in sphingomyelin) prevented phase separation of cholesterol-rich liquid-ordered domains but acted differently with ceramide-rich gel phases, as both C16:0 and C24:1 ceramides gave rise to segregated gel domains. C16:0 sphingomyelin was able to accommodate both ceramides in a single phase of intermediate properties, while C24:1 sphingomyelin was less effective in this respect. The preference that both ceramides appeared to have for C16:0 sphingomyelin was confirmed in mixtures containing both C16:0 and C24:1 sphingomyelin. Interestingly, the imperfect capacity of C24:1 sphingomyelin to accommodate the ceramides gave rise to different gel phases; each of the different gel phases was enriched in a different ceramide. The ceramide-rich domains in mixtures containing both sphingomyelins exhibited a higher nanomechanical resistance (i.e., were stiffer) than those in mixtures with a single ceramide, suggesting some kind of interceramide cooperation mediated by C24:1 sphingomyelin, as this effect was not seen with the C16:0 species. In summary, a complex variety of phase separation effects could be observed: (*a*) With C16:0 sphingomyelin, a continuous phase enriched in DOPC and cholesterol was formed, coexisting with a liquid-ordered domain (C16:0 sphingomyelin, cholesterol) and a gel subdomain (C16:0 sphingomyelin + cholesterol); (*b*) with C24:1 sphingomyelin, the continuous phase coexists with two different gel domains, each of them enriched in one of the ceramides; and (*c*) when both sphingomyelins were present in equimolar amounts, a continuous phase (DOPC, C24:1 sphingomyelin, cholesterol) coexisted with a gel domain (C16:0 sphingomyelin + both ceramides). The possibility of chain interdigitation between monolayers should be taken into account in the C24:1-containing samples; García-Arribas et al. (37) mentioned this possibility, although they could not test it experimentally. These findings show the sharp increase in complexity when membranes exhibit different sphingolipids of varying N-acyl chains, as is probably the case in the cell membrane environment.

EFFECT OF CHAIN LENGTH AND UNSATURATION

A number of studies have been dedicated to study the effect of systematically changing the structure of either the N-acyl or the sphingoid chain. Note in this context the review by Grösch et al. (42) on the chain length–specific properties of ceramides at the cellular level.

Sphingoid Chain Structure

Slotte and coworkers have studied this important matter. In a 2012 study, Maula, Slotte, and coworkers (59) described the synthesis of ceramides consisting of a C12-, C14-, C16-, C18-, or C20-sphingosine analog N-coupled to palmitic acid. The ceramides were studied in mixtures of POPC and/or C16:0 sphingomyelin, in some cases also containing cholesterol. Ordered- or gel-phase formation was monitored through changes in *trans*-parinaric acid lifetimes. Ceramides with C12- and C14-sphingoid bases failed to increase the order of POPC bilayers, but ordering and domain segregation was observed with the longer-chain derivatives. All the analogs, however, were able to stabilize the gel phase of sphingomyelin, increasing its gel-fluid transition temperature in a chain-length-dependent way. An interesting observation, based on sterol partitioning experiments, was that the C18 (native) and C20 ceramides formed, with sphingomyelin, gel phases that excluded cholesterol, while the ceramide containing the C16-sphingosine analog had an intermediate effect. These results complement previous data by Fyrst et al. (33), obtained in *Drosophila*, and underline the importance of sphingoid-base length in the biological context.

In a more recent report from Slotte's group (1), the subject was revisited in further detail, using sphingoid bases with 16, 17, 18, 19, and 20 carbon atoms in N-palmitoyl ceramide mixed in POPC bilayers. The longer the sphingoid chain, the less ceramide was needed for the onset of lateral segregation and ceramide-rich domain formation. Moreover, a series of ceramides based on the C18 sphingoid base (sphingosine) containing N-linked acyl chains of 14, 16, 17, 18, 19, and 20 carbon atoms, saturated, exhibited a similar but much weaker trend. The authors also compared two ceramides containing in total 33 carbon atoms each, one with a 16C sphingoid base and a 17-carbon-atom fatty acid and one with a 20-carbon-atom base and a 13-carbon-atom acyl chain. They could observe that the one with a longer sphingoid base segregated to a ceramide-rich phase at lower concentrations than the one with the 16-carbon-atom base and that the gel phase was more thermally stable in the former case. The prime importance of the sphingoid phase in directing the stability of the resulting gel phases was confirmed by ^{2}H-NMR data of C18:0 ceramide in POPC that showed that the sphingoid chain (selectively deuterated at C12) was more ordered than the acyl chain (selectively deuterated at C10) at comparable chain positions and temperatures up to ~40°C (i.e., in the gel phase), above which data from both lipids overlapped.

A further possible change in the sphingoid-base long chain is unsaturation. Maula et al. (58) prepared a ceramide in which a second *cis*–double bond had been introduced in C14 of the sphingosine. Binary mixtures of palmitoyl sphingomyelin with the C14 unsaturated-base ceramide exhibited a T_m lower than those with the *cis*–double bond at the corresponding bilayer depth—in other words, C18:1$^{\Delta 12c}$ (unsaturation in the fatty acyl chain). Furthermore, the melting of the sphingomyelin mixture with sphingodiene-base ceramide was less cooperative than that with C18:1$^{\Delta 12c}$ ceramide. Maula et al. (58) suggested that the *cis*–double bond in C14 of the sphingoid base could introduce orientational disorder about the C4 *trans*–double bond, an important factor for close packing of the ceramide acyl chains and for stabilizing the intermolecular H-bond network within ceramides (8, 53). Studies involving quenching of cholestatrienol fluorescence (cholestatrienol being a fluorescent cholesterol analog) in POPC-sphingomyelin-ceramide-cholestatrienol quaternary mixtures revealed that more sterol was incorporated into the gel domains with the sphingadiene-base ceramide than with the C18:1$^{\Delta 12c}$ ceramide.

N-Acyl Chain Structure

Little attention has been paid to this aspect of ceramide biophysics until recently, aside from brief comments in, for example, the review by Kolesnick et al. (50) or the work by Holopainen et al. (44)—an early example of a comparative study of C16:0 and C24:1 ceramides. Also, Sot et al. (87) examined the effects of short-, medium-, and long-chain ceramides on the phase transition of DEPE and built the corresponding phase diagrams. This paucity of physical studies is in contrast to the extensive biochemical studies that are beyond the limits of this review and that had described long ago a family of six ceramide synthases, each of which uses a relatively restricted subset of acyl coenzyme A's for sphingosine N-acylation (52). Examples are known of the exquisite specificity of N-acyl chain length for certain sphingolipid-protein interactions in cells (22).

In more recent years, Pinto et al. (71), using defined model systems, studied binary mixtures of POPC and ceramide, in which the N-acyl chain was either long chain (C16, C18), very long chain (C24), or unsaturated (C18:1, C24:1). Fluorescence spectroscopy and microscopy showed that the rigidifying and phase separation properties of ceramides were more marked in the saturated than in the unsaturated species (C18:1 ceramide did not form domains at 37°C) and that very long chain ceramides formed tubular structures that were related to their ability to form interdigitated phases.

A number of interesting papers on the subject of ceramide chain length appeared in 2014. Jiménez-Rojo et al. (48) performed a rather detailed study of the thermotropic properties of ceramides with N-acyl chains of lengths C6, C12, C16, C18, C24, C24:1, and natural egg ceramide, either pure or in mixtures with sphingomyelin of equally varied N-acyl lengths. Even in the pure state, some ceramides originated complex DSC thermograms, including exothermic signals in certain cases. Bilayers containing separate sphingomyelin-rich and ceramide-rich domains were formed in binary mixtures containing the sphingomyelins and 10 mol% ceramide. In some cases, ceramide was generated in sphingomyelin-containing vesicles by sphingomyelinase treatment, and the usual vesicle aggregation and contents efflux were observed. In general, the individual ceramides appeared to have their own specific properties, often unrelated to the changes in N-acyl chain.

A somewhat similar study was performed by Maula et al. (58) using C16, C18:0, C24, C18:1$^{\Delta 9c}$, C18:1$^{\Delta 12c}$, and C24:1$^{\Delta 15c}$ ceramides in binary mixtures with palmitoyl sphingomyelin. Phase transitions of the mixtures were studied by calorimetric and fluorescence methods. Results with saturated ceramides were essentially the same as those recorded by Jiménez-Rojo et al. (48), with increases in N-acyl chain length failing to increase the gel-fluid T_m of the mixtures. Both C18:1$^{\Delta 9c}$ and C24:1$^{\Delta 15c}$ ceramides containing a *cis*–double bond in the acyl chain, in binary mixtures with palmitoyl sphingomyelin, lowered the T_m with respect to their saturated counterparts. The effect was position-dependent, T_m reduction caused by Δ9c (a *cis*–double bond in C9) in C18 ceramide being larger than that caused by Δ15c in C24 ceramide (~23°C versus ~9°C), with the latter being located closer to the membrane hydrophobic core, where the disruption in cell packing is less likely to have marked effects.

The behavior of unsaturated N-acyl ceramides in fluid POPC bilayers was also studied by Maula et al. (58). All the unsaturated ceramides tested were able to form laterally separated domains that melted at a given temperature, usually lower than that of the saturated counterparts. The T_m for the gel-like domains in POPC decreased in the order C24:1 > C18:1$^{\Delta 12c}$ > C18:1$^{\Delta 9c}$. An interesting observation is that, according to *trans*-parinaric acid lifetimes, at 10°C, when the gel-like phases exist for both unsaturated and saturated phospholipids, either in POPC or in sphingomyelin, similar degrees of chain packing exist in all samples.

A useful instrument in the study of the physical properties of lipids is the Langmuir balance, in which lipid monolayers are formed at the air-water interface, and measurements of

surface-pressure changes provide information on the surface behavior of pure and mixed lipids. Dupuy & Maggio (28) used this technique to study sphingomyelin-ceramide mixtures, either of equal chain length (C16-C16), different lengths (C16-C24, C16-C10), or highly mismatched ones (C12-C24, C24-C10). Surface-pressure measurements were complemented with monolayer imaging using Brewster angle microscopy. Favorable sphingomyelin-ceramide interactions were detected that occurred as a consequence of complementary lateral packing and increased acyl chain ordering, in turn dependent on composition even in the presence of considerable hydrocarbon chain mismatch. The data show that results obtained with C16 N-acyl mixtures cannot be extrapolated to all types of ceramide mixtures. Total immiscibility was formed only with C12 sphingomyelin (in the fully expanded state) and C24:0 ceramide (fully condensed monolayer). Long-chain ceramides (C24:0 and C24:1) can form mixed expanded phases with different sphingomyelins as a consequence of the acyl chain disorder induced by the chain asymmetry. The latter, novel overall phase state challenges the common idea that ceramides always give rise to solid domains.

Ceramide synthase 2 is involved in the synthesis of very long acyl chain sphingolipids (longer than 22 carbon atoms). Silva et al. (80) explored the biophysical consequences of depleting cells of very long chain sphingolipids by using a ceramide synthase 2–null mouse: A variety of effects were detected, from changes in membrane fluidity to marked alterations in membrane morphology and trafficking. These effects were not equally manifest in liver and brain, perhaps owing to a tissue-specific distribution of the synthase (**Figure 3**). In any case, the observed changes were due to the absence of the ensemble of sphingolipids of longer than 22 carbon atoms; thus specific effects of ceramides could not be detected. Specific ceramide effects were, however, shown by the same authors (70) when they treated ceramide synthase 2–deficient cells with bacterial sphingomyelinase so that sphingomyelin was converted into ceramide in an experiment reminiscent of Maxfield and coworkers' in 1998 (98). Pinto et al. (70) found that, shortly after sphingomyelinase addition, the order of the plasma membrane lipids increased, presumably because of ceramide generation. After a certain amount of time (15–45 min according to sphingomyelinase dose), lipid order did not increase any further, but gel-like ceramide-rich domains were formed after that time. It should be noted that bacterial sphingomyelinase would act with similar affinity irrespective of the substrate N-acyl-length; thus the observed ceramide effects correspond to mixed N-acyl chain species, except the ones with fatty acids longer than 22 carbon atoms, in which these cells are deficient.

A unique case of very long chain ceramides is offered by the C28-C32 polyunsaturated N-acyl chain–containing species that are formed in the mammalian testis and generated from the corresponding sphingomyelins during the acrosomal reaction. The fatty acyl chains contain four or five double bonds and may be either 2-hydroxylated or nonhydroxylated. Peñalva et al. (67) isolated these ceramides and subjected them to study in the Langmuir balance. In comparison with the previously studied species of ceramides, these very long species showed a much larger mean molecular area in liquid-expanded phases, suggesting that the unsaturated parts of the chains bend and become partially hydrated. The presence of the 2-OH group induced a closer molecular packing relative to the nonhydroxylated species. Otherwise, all these ceramides showed liquid-expanded to liquid-condensed phase transitions at room temperature, with domain segregation in the monolayers as observed by Brewster angle microscopy.

Peñalva et al. (66) further observed that the abundant 2-OH (2-hydroxy sphingosine) C28:4 exhibited a highly compressible liquid-condensed phase, which the authors interpreted in terms of a high conformational freedom for the ceramide molecules, together with the low diffusional properties that are characteristic of the liquid-condensed phase (**Figure 4**). In mixtures of the (2-OH) C28:4 ceramide and the corresponding sphingomyelin, the components mixed favorably in the liquid-expanded phase, and ceramide-rich domains were formed both in films formed by the ceramide-sphingomyelin mixture and in pure sphingomyelin films treated with sphingomyelinase.

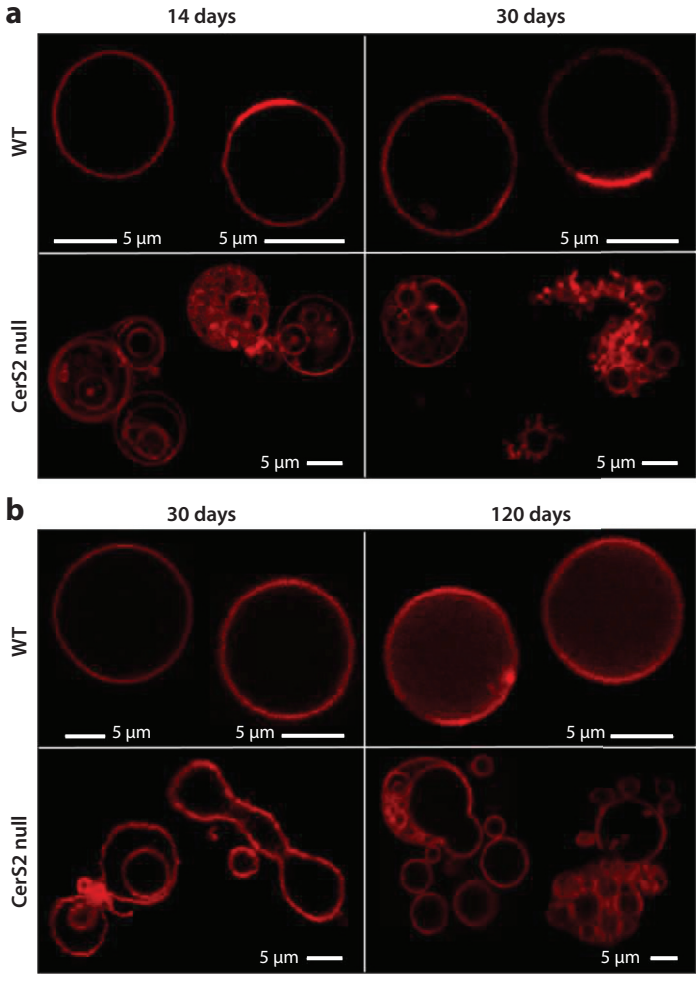

Figure 3

Effect of ceramide synthase 2 (CerS2) ablation on membrane morphology. Confocal fluorescence microscopy of giant unilamellar vesicles (GUVs) prepared from microsomal lipids from (*a*) brain or (*b*) liver of control (*upper panels*) and CerS2 null (*lower panels*) mice of different ages. GUVs were labeled with rhodamine-phosphatidylethanolamine. Adapted from Reference 80. Additional abbreviation: WT, wild type.

In the acrosomal reaction, the acrosomal membrane is believed to fuse with the sperm plasma membrane so that the acrosomal contents are released. Ceramides are known to facilitate membrane fusion (6, 76) owing to their negative intrinsic curvature. As discussed above, the polyunsaturated, very long chain ceramides that are formed in the acrosomal reaction would have a very highly negative intrinsic curvature, with the average area of the chains moiety being much wider than the polar head area; thus they would be particularly potent in promoting fusion (66).

THE INTERFACIAL REGION

After our rather comprehensive discussion of the influence of the sphingoid and acyl chains on the physicochemical properties of ceramides, a shorter section on the importance of the polar

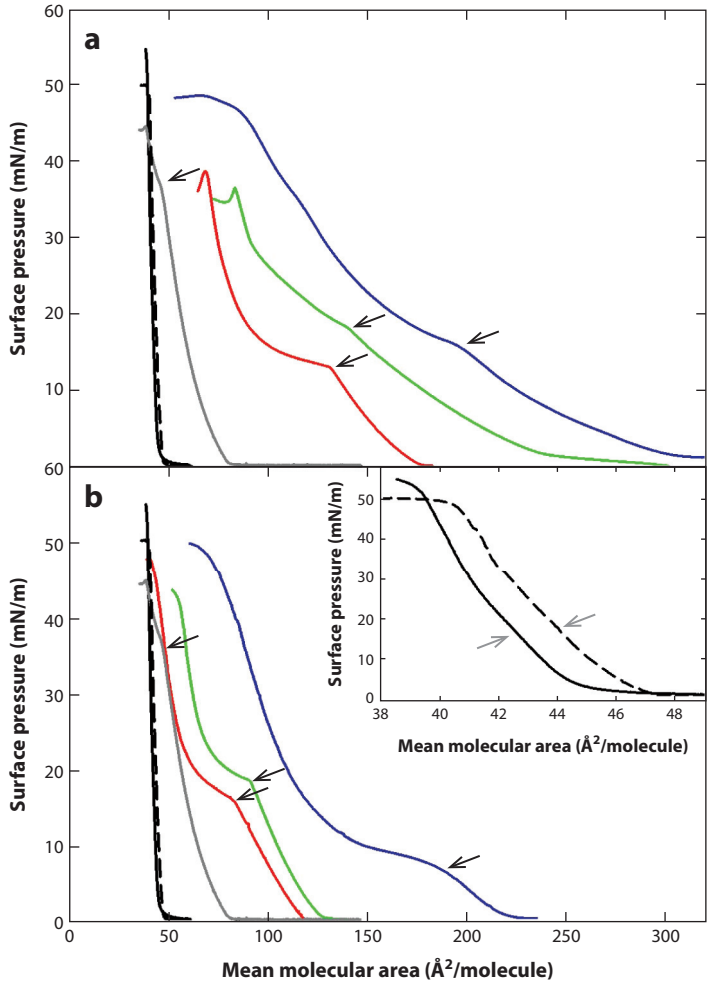

Figure 4

Compression isotherms of (*a*) nonhydroxy and (*b*) 2-hydroxy very long chain, polyunsaturated ceramide species. Red: C28:4; green: C30:5; blue: C32:5. For comparison, isotherms are shown of C24:1 (*black dashed line*), C16:0 (*solid black line*), and C18:1 (*gray line*). Black arrows: liquid-expanded to liquid-condensed phase transition; gray arrows: liquid-condensed to solid phase transition (66).

headgroup, or interfacial region, is appropriate. A number of important contributions have been performed by J.P. Slotte and his coworkers.

It is widely accepted that the sphingomyelin-ceramide complex is stabilized at least in part by the so-called umbrella effect, in which the small polar headgroup of ceramides is not enough to shield its hydrophobic moiety from interactions with water, but together with the large phosphocholine headgroup of sphingomyelin, an effective protection is achieved for all four long alkyl chains. To elucidate the role of the sphingomyelin headgroup in the sphingomyelin-ceramide interaction, Artetxe et al. (3) reduced the size of the choline group by removing one, two, or three methyl groups or removed the entire choline moiety. The authors found that the size of sphingomyelin headgroup had no marked effect on the thermal stability of the rigid domains formed between the various sphingomyelin derivatives and ceramide. Thus the association between sphingomyelin

and ceramide appears to be mainly stabilized by van der Waals forces between the alkyl chains and by H-bonding between both sphingosyl polar groups, irrespective of the choline group and in agreement with the observations by Dupuy & Maggio (28).

Phytosphingosine is a minor saturated sphingoid base in mammals that contains a third OH group in C4 (4-D-hydroxy-sphinganine). Ceramides containing a phytosphingosine backbone generally display higher gel-fluid transition temperatures in the pure state than the corresponding sphingosine analogs (73) by about 20°C for the C18-sphingoid bases. This has been attributed to an enhanced H-bonding capacity resulting from the additional –OH, but infrared studies (73) indicate that phytosphingosine chains are poorly packed in comparison with the sphingosine ones. In general, it appears that the looser chain packing and the denser H-bonding jointly determine the overall properties of phytosphingosine (58).

α-Hydroxylation (C2-OH) of the N-acyl chain stabilizes the packing of ceramides relative to their nonhydroxylated counterparts (64); however, Maula et al. (58) observed that, in binary mixtures with palmitoyl sphingomyelin, α-hydroxylation of the ceramide had hardly any effect on the T_m of the system (**Figure 5**). The same authors found that α-hydroxylation in phytoceramide causes a reduction in T_m and destabilization of chain interactions, possibly because of the weakening of the amide H-bonding, together with steric hindrance for acyl chain packing due to introduction of the 2-OH group (58, 73). In mixtures of increasing complexity (i.e., with POPC, with POPC + palmitoyl sphingomyelin, and with POPC + palmitoyl sphingomyelin + cholesterol), the melting temperatures for the ceramide-rich domains were virtually similar for C16 ceramide and for C16 phytoceramide, and also for C18 ceramide and for C18 (2-OH) ceramide (**Figure 4**) (58). The above results indicate that α-hydroxylation in the acyl group or 4-hydroxylation in the sphingoid base may have small or large effects on the properties of the pure ceramide without these changes being necessarily transferred as such into other, closely related sphingolipid species or into mixtures of several sphingolipid species (58).

1-Deoxyceramides

1-Deoxyceramides are a special case of polar headgroup modification. The advent of lipidomics led to the discovery of the family of 1-deoxyceramides, which contain sphingoid bases lacking the 1-hydroxyl or 1-hydroxymethyl groups. These lipids arise from serine palmitoyltransferase, the main enzyme in sphingosine biosynthesis, accepting also as substrates alanine or glycine in addition to the natural substrate serine. These are not trace lipids but are rather found in substantial proportions in mammalian cells. Three main subfamilies of 1-deoxyceramides are known: the 1-deoxy, 1-deoxydihydro, and 1-deoxymethyldihydro ceramides. Note that the lack of 1-hydroxy group precludes their metabolism to the bioactive 1-phosphates and all the other sphingolipids. 1-Deoxyceramides are elevated in sensory neuropathies and diabetes, among other pathological conditions (25, 74, 78).

Perhaps the only report on the biophysical properties of 1-deoxyceramides is the one by Jiménez-Rojo et al. (49) in which the behavior of 1-deoxyceramides with different N-acyl chain lengths (C12, C16, C24:1) was studied alone or in mixtures with sphingomyelin. The gel-fluid T_m transition temperatures of the pure ceramides increased in the order 1-deoxy < so-called canonical ceramide ≈ 1-deoxydihydro < 1-deoxymethyldihydro ceramide for a given chain length. The 1-deoxyceramides mixed poorly with sphingomyelin in comparison with the canonical ones. 1-Deoxymethyl dihydroceramides were the most hydrophobic among them, not being capable of forming monolayers at the air-water interface. These lipids deserve further study, considering their relative abundance and their implications in human health issues.

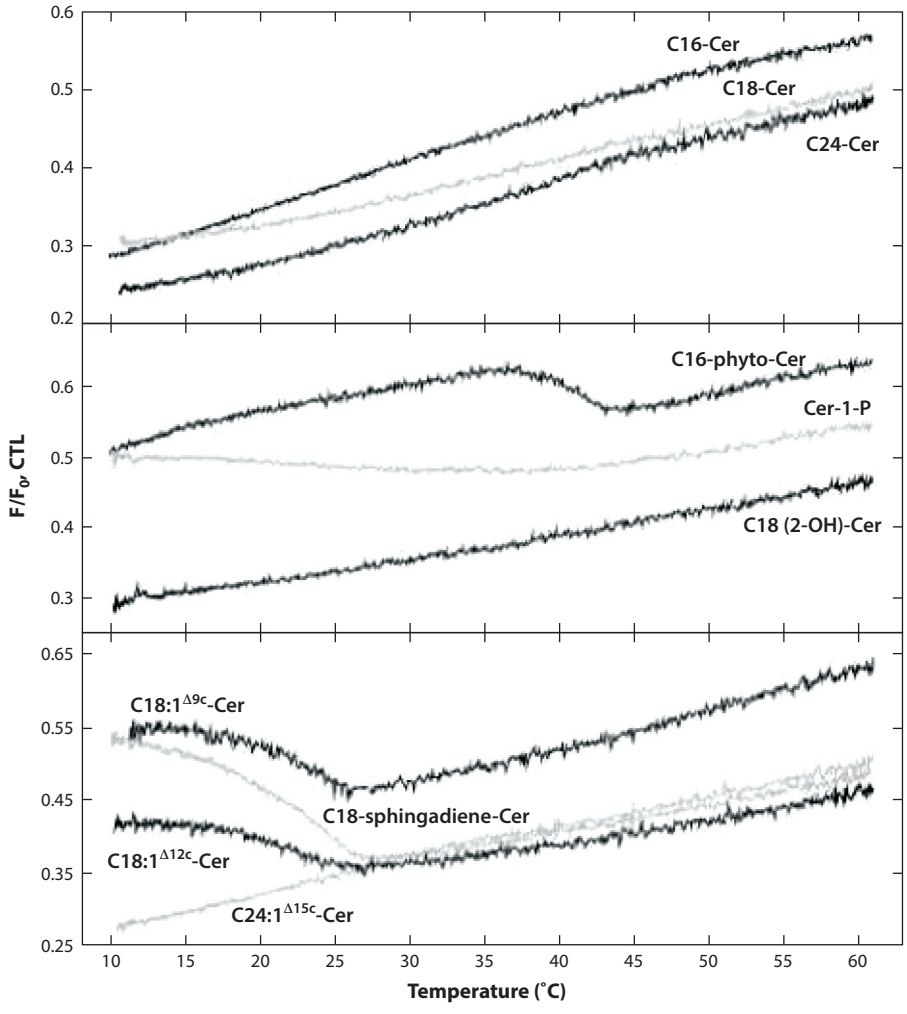

Figure 5

Effect of α-hydroxylation (2-hydroxylation) and other chemical modifications of ceramides on the gel-fluid transitions in POPC-PSM-Cer-Chol bilayers. The data correspond to quenching of cholestatrienol fluorescence by a spin-labeled PC in the quaternary-mixture bilayers containing various ceramides. F/F_o is the fraction of unquenched fluorescence (58). Abbreviations: 1-P, 1-phosphate; 2-OH, 2-hydroxy sphingosine; Cer, ceramide; Chol, cholesterol; CTL, cholestatrienol; PC, phosphatidylcholine; phyto, phytosphingosine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; PSM, palmitoyl sphingomyelin.

Dihydroceramides

Dihydrosphingolipids lack the C4 = C5 double bond in sphingosine. Very little is known of their physical properties. Vieira et al. (97) found that, in phospholipid-sphingolipid-cholesterol mixtures, dihydrosphingolipids gave rise to more rigid mixtures than the corresponding 4-unsaturated compounds. There are several observations on the biological relevance of the dihydroceramides (31, 79). Hernández-Tiedra et al. (43) combined molecular and cellular techniques to confirm that the rigidifying effect of dihydroceramide was higher than that of ceramide and demonstrated that dihydroceramide was also more potent in increasing membrane permeability than the

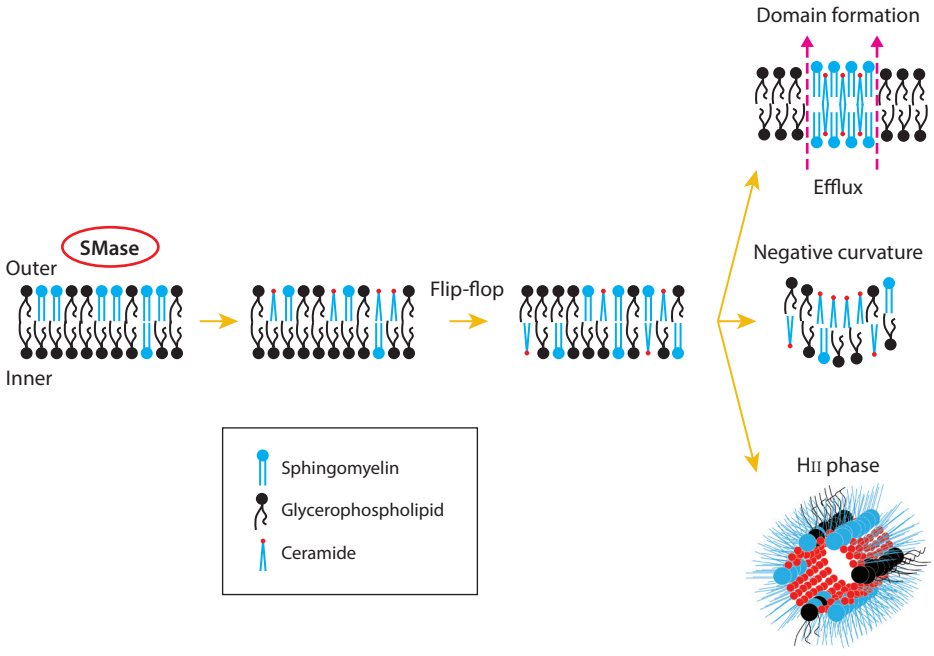

Figure 6

A schematic representation of some physical effects of ceramides on membrane bilayers. Ceramides are presented as resulting from the degradation of sphingomyelin by sphingomyelinase at the membrane outer monolayer. Figure adapted from graphics by J. Sot. Abbreviations: H_{II}, inverted hexagonal; SMase, sphingomyelinase.

unsaturated analog. Interestingly, Contreras et al. (21) found that, unlike the 4-unsaturated ceramides, dihydroceramides did not induce transbilayer (flip-flop) lipid motion in membranes.

THE UNFORTUNATE GAP BETWEEN PHYSICS AND BIOLOGY

In the above pages, the main known physical properties of ceramides have been described and are summarized in **Figure 6**. Some of them are unique to this group of molecules (e.g., their extraordinary hydrophobicity, viscoelastic properties, or capacity to induce flip-flop lipid motion in bilayers). However, the vast majority of published papers on ceramides, at least by one order of magnitude, describe cellular or organismal effects of ceramides, many of them associating the presence/absence of ceramide to certain pathologies. A second significant observation is the complete absence of relationships between the pathophysiological observations and the physicochemical data. More specifically, the cell biological and pathological observations all but totally ignore the biophysical work, and biophysical studies do not go beyond paying token attention to physiology/pathology. The two sets of papers appear to correspond to two groups of researchers who mutually ignore their existence, or at least their publications. This situation is not a healthy one for the progress of knowledge in either of the areas involved.

To mention but a few examples, a large body of evidence now connects ceramides with obesity, type 2 diabetes, and the so-called metabolic syndrome. Other extensive investigations relate ceramides to autophagy. Ceramide also emerges as a potent regulator of cancer. In these extensive areas of scientific literature, there is hardly a reference to the molecular mechanisms of ceramide

effects. At most, vague mentions to "specific receptors" are made. In an early review (50), we distinguished between effects due to ceramide binding to specific proteins and effects due to ceramide changing the physical properties of the membrane. We proposed then, and we still do here, that it would be extremely unlikely that such potent ceramide activities as, for example, membrane permeabilization or transbilayer flip-flop lipid motion did not find a pathophysiological correlate. It is a very clear rule that nature makes good use of any chemical or physical property of a biomolecule for some adaptive or evolutionary purpose. Why should the remarkable physical properties of ceramides be an exception?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors thank the continuous support over the years of the University of the Basque Country, Diputación Foral de Bizkaia, Basque government, Spanish Ministry of Economy, and European Union. Preparation of this manuscript was supported by grants from FEDER and the Spanish Ministry of Economy (BFU 2015–66306-P) and the Basque government (IT849–13 and IT838–13).

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