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Lipid Phase Separation and Protein-Ganglioside Clustering in Supported Bilayers Are Induced by Photorelease of Ceramide†

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Abstract

Photolysis of 6-bromo-7-hydroxycoumarinyl-caged ceramide was used to generate ceramide with spatial and temporal control in supported lipid bilayers prepared from mixtures of caged ceramide and phospholipids. The caged ceramide molecules are randomly distributed in fluid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayers, and upon photolysis with long wavelength UV light small ordered ceramide domains are formed that phase separate from the bulk fluid membrane. Irradiation of a spatially restricted area leads to the transient formation of ceramide-enriched gel phase domains that equilibrate via lipid diffusion with the surrounding unirradiated membrane. Photorelease of C16-ceramide in supported bilayers prepared from POPC, caged ceramide and the ganglioside GM1 (90:10:1 molar ratio) results in partitioning of a ganglioside-protein complex into the ceramide-enriched domains, modeling some aspects of ceramide's behavior in cells. The photo-uncaging strategy used here for delivery of ceramide in bilayers provides a novel and useful alternative to the enzymatic generation of ceramide in sphingomyelin-containing membranes. The ability to control membrane phase separation behavior and the clustering of membrane-anchored proteins illustrates the potential of photo-uncaging for studying the compartmentalization of ceramide in cellular membranes.

Introduction

Ceramide (Cer) is an important lipid second messenger and is involved in regulating a number of cellular processes, including programmed cell death, cell growth and differentiation, and cellular responses to stress stimuli.^{1–3} It is a key intermediate in the sphingolipid biosynthetic pathway and its concentration in cells is regulated both by the sphingolipid pathway and by its formation via hydrolysis of sphingomyelin by sphingomyelinases (SMase). Cer is typically present in relatively low concentrations in the plasma membrane but Cer levels increase in response to stress signals, reaching levels as high as ~10% in apoptotic cells.⁴ Many of the biological effects of Cer are linked to its ability to modulate the biophysical properties of membranes. Cer is one of the most hydrophobic and high-melting lipids and can promote phase separation and the formation of ordered gel-phase domains.^{5,6} It is also postulated to play an important role in concentrating signaling molecules in liquid-ordered (raft) domains, providing one mechanism for enhancing the efficiency of cell signaling.^{2,3} Both the role of Cer in sphingolipid

†Electronic supplementary information (ESI) available: Eight figures with additional AFM and fluorescence images documenting phase separation and protein clustering following photorelease of Cer from **1**.

metabolism and its diverse range of biological functions are hypothesized to be regulated by specific biochemical pathways that occur in distinct subcellular compartments.¹ This complexity requires a compartment-specific approach to mechanistic studies.

The effects of Cer have been studied extensively in lipid monolayers and bilayers as a model for the behavior of Cer in cells.^{6–21} These studies have generally used either lipid mixtures containing premixed Cer or *in situ* Cer generation by the enzymatic hydrolysis of sphingomyelin-containing membranes. Although the enzymatic generation of Cer is a better alternative than Cer incorporation into a lipid film prior to hydration for modeling cellular effects, it does not duplicate the highly regulated and localized activity of SMase in cells. Recently, we have reported the synthesis and characterization of a 6-bromo-7-hydroxycoumarinyl-caged Cer that shows efficient photorelease of Cer in solution and is compatible with uptake and Cer release in cells.²² The bromohydroxycoumarin moiety has several advantages, including activation at longer wavelengths than the commonly used α -nitrobenzyl photocage, compatibility with two-photon uncaging, and increased aqueous solubility and membrane permeability due to the presence of the deprotonated phenol at physiological pH.^{23–25} Photolysis of a caged Cer could lead to Cer release with a high degree of spatial and temporal control, providing a potentially useful tool for studying the effects of Cer incorporation in both model and cell membranes. A caged Cer probe can be readily taken up by cells, in contrast to exogenous long-chain ceramides which are not membrane-permeable and are frequently delivered in ethanol/dodecane mixtures.²⁶ The membrane-permeable short-chain ceramides that are used as models for cellular studies have biological effects that differ significantly from those of the natural long-chain analogs.^{27–29}

Although photodeprotection of caged biomolecules has been widely applied for the release of small molecule neurotransmitters, nucleotides, peptides, and proteins for mechanistic studies,^{30–32} there are relatively few applications of photocaged lipids.^{30, 33–35} Membrane-permeable caged lipids have the potential to probe lipid metabolism and regulation of cell signaling, to investigate membrane reorganization in cellular and model membranes, and to provide phototunable liposome properties for applications such as drug delivery. Herein we report a detailed correlated fluorescence and atomic force microscopy (AFM) study of the incorporation and photolysis of a caged C16-Cer (**1**, Chart 1) in POPC supported bilayers, demonstrating the capability to generate Cer with spatial and temporal control. We observe the transient formation of Cer-enriched gel phase domains that equilibrate via lipid diffusion with the bulk membrane and the partitioning of a ganglioside-protein complex into these Cer-enriched domains. The ability to control bilayer phase separation and the clustering of proteins provides a new approach for understanding the roles of Cer in biological pathways. Photoactivation offers a level of spatial and temporal control that is not achievable with enzymatic generation or direct incorporation of Cer, an essential step for probing Cer's functions in specific cellular compartments.

Experimental

Chemicals

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), *N*-palmitoyl-*D*-erythro-sphingosine (C16-Cer), and ganglioside GM1 were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. 1,1'-Dieicosanyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C₂₀) was purchased from Molecular Targeting Technologies (Westchester, PA). Recombinant cholera toxin subunit B (Ctx-B) conjugated to Alexa Fluor 647 and Alexa Fluor 488 was acquired from Invitrogen/Molecular Probes (Eugene, OR). (2*S*,3*R*,4*E*)-2-Palmitoylamido-3-hydroxyoctadec-4-enyl (6-bromo-7-hydroxycoumarin-4-yl)methyl carbonate (caged C16-Cer, **1**) was synthesized as described previously.²² Optical adhesive 88 was obtained from Norland Products (Cranbury,

NJ) and used to glue mica onto cover glass (Fisher Scientific, Hampton, NH). All aqueous solutions were prepared using 18.3 MΩ cm *Milli-Q* water. Ctx-B buffer (8 mM Na₂HPO₄, 20 mM NaCl, 2 mM NaN₃, pH 7.4) was used for Ctx-B labeling experiments. All buffered solutions were passed through a 0.22-μm filter (Millipore, Billerica, MA) before being used.

Supported lipid bilayers

Small unilamellar vesicles (SUVs) were prepared as previously described subject to some minor modifications.³⁶ Lipids, **1**, and DiI-C₂₀ were dissolved in chloroform, methanol, ethanol, or mixtures thereof as required. After the solutions were mixed in appropriate amounts, the organic solvents were evaporated under reduced pressure. The POPC/**1** SUVs were prepared with a 9:1 molar ratio unless otherwise noted. The resulting dry lipid films were hydrated in *Milli-Q* water and then sonicated in a bath sonicator (final temperature of 40 °C) to clarity to form SUVs with a final lipid concentration of 0.5 mg/mL. Lipid films were stored for up to 1 week at -20 °C prior to use; however, fresh vesicles were prepared on the day of each imaging experiment.

Planar supported bilayers were formed on mica via vesicle fusion. Freshly cleaved mica disks (15–25 μm thick for fluorescence imaging) were glued onto circular cover glasses. To prepare POPC/**1** or POPC/C16-Cer supported bilayers, 950 μL of 8 mM CaCl₂ was first added to a mica/glass slide clamped in a liquid cell at room temperature. Then 20–50 μL of vesicle suspension was added, and the samples were incubated for 1 h at room temperature. Bilayers were gently washed with *Milli-Q* water or buffer to remove unattached vesicles before imaging. Supported bilayers of POPC/**1**/GM1 with 0.5 mol% DiI-C₂₀ were prepared according to the same procedure. GM1 was labeled with Alexa 488- or Alexa 647-Ctx-B prior or subsequent to photolysis of **1** as follows: the bilayer was first washed with Ctx-B buffer and then incubated for 30 min with labeled Ctx-B (final concentration ~ 3 μg/mL) before a final wash with Ctx-B buffer.

Photolysis and photoproduct analysis

Photolysis was performed in a quartz cuvette containing 3 mL of vesicle solution (POPC/**1** with 0.5 mol% DiI-C₂₀). The samples were irradiated at 350 nm in a Rayonet photochemical reactor (Southern New England Ultraviolet Co., Branford, CT, USA) equipped with four lamps (nominal output of 8 W/lamp, sample centred between lamps at a distance of ~10 cm). Aliquots of 20 μL were removed periodically for analysis. Coumarin 480 (5 μl at 5 μM) was added to each aliquot as an internal standard. Loss of starting material **1** was monitored by reversed-phase HPLC with a SunFire C18 column (Waters Corp., Milford, MA, USA), and eluted with an ethanol/water gradient (75% ethanol increasing to 95% over 3 min) at a flow rate of 0.8 mL/min. Detection was by absorbance at 325 nm and fluorescence at 480 nm.

Fluorescence microscopy and correlated fluorescence-atomic force microscopy

Fluorescence imaging and correlated fluorescence-AFM were performed on the same experimental apparatus at room temperature (~22 °C). The imaging system consisted of a NanoWizard II BioAFM (JPK Instruments, Berlin, Germany) integrated with an IX81 inverted optical microscope (Olympus Corporation, Tokyo, Japan). Epifluorescence images were obtained using lamp excitation with an Olympus UPlanSAPO 100x, NA = 1.4 oil immersion objective, Cy3 and DAPI filter sets (Chroma Technology, Bellows Falls, VT), and a high resolution CoolSNAP CCD camera (Semrock, AZ). Fluorescence images were scaled, cropped, and correlated with the corresponding AFM scans using Image J freeware (NIH, Bethesda, MD). Samples labeled with Alexa 647 Ctx-B were imaged on an IX81 Olympus microscope equipped for 543 (DiI) and 647 (Alexa 647) nm laser excitation with a

150x UApo oil immersion objective, NA = 1.45, due to availability of filter sets (Cy3 and Cy5, Semrock).

Photolysis experiments for POPC/**1** bilayers were carried out on the integrated AFM-fluorescence microscope using the lamp (X-Cite 120 PC, 120 W focused through the 100x oil immersion objective) and DAPI filter set. For most experiments the adjustable aperture built into the microscope illuminator was used to confine UV irradiation of the sample to a hexagonal area $\sim 25 \mu\text{m}$ in diameter. Irradiations of the entire bilayer were done using a hand-held UV lamp (365 nm, output 6 W; Spectronics Corporation, Westbury, NY) positioned 2 cm under the sample. To assess the ability of the microscope lamp equipped with a 377-nm filter to trigger the photorelease of C16-Cer from **1**, we irradiated an aqueous dispersion of **1** for several minutes on the fluorescence microscope. HPLC analysis of the resulting suspension confirmed that photolysis of the caged lipid had taken place efficiently to afford C16-Cer and 6-bromo-7-hydroxy-4-hydroxymethylcoumarin (data not shown).

AFM images were captured using uncoated silicon nitride DNP-S-10 (Veeco, Camarillo, CA) AFM cantilevers with a typical spring constant of 0.12 N/m. Contact mode topographic images were collected at scan rates of 0.7–1 Hz, and continuous adjustments to the set point kept the force exerted on the sample at a minimum. Scans were collected at 512×512 pixel resolution and were line fitted with first- to third-order polynomials when necessary.

Solid-supported bilayers of the desired composition were prepared, and fluorescence and AFM images of the same sample area were acquired sequentially after first assessing sample homogeneity over several areas by fluorescence. The sample was irradiated briefly (typically 5–60 s) and fluorescence and/or AFM images were used to monitor changes in the bilayer. Typically fluorescence images were acquired before and after AFM scans of the same area to check for changes in membrane morphology occurring during (or resulting from) scanning.

Results

Direct incorporation of C16-Cer in POPC bilayers

The direct incorporation of Cer in POPC bilayers was examined using a combination of AFM and fluorescence microscopy prior to investigating Cer release by photolysis of **1**. It is known from the literature phase diagram for POPC/Cer/SM mixtures and from previous results with supported bilayers and giant unilamellar vesicles (GUVs) that POPC membranes containing as little as 4–5 mol% Cer have a mixture of coexisting gel and fluid phases.^{11, 14, 19} A fluorescence image of a supported bilayer prepared from 9:1 (molar ratio) POPC/C16-Cer vesicles containing 0.5 mol % DiI-C₂₀ showed small dark dye-excluded domains (Fig. 1A). An AFM scan of the same bilayer region (Fig. 1B) demonstrated that most of the dye-excluded features corresponded to raised domains that were 1.2–1.5 nm above the surrounding membrane. It is straightforward by AFM to distinguish these raised domains from occasional membrane defects, which also appear as dye-excluded regions in the fluorescence image (see arrows in Fig. 1A). A second fluorescence image (Fig. 1C) acquired after the AFM scan indicated that some of the defects had started to fill in, presumably an effect of the scanning tip. The small raised domains that exclude dye are assigned to a Cer gel phase, consistent with literature results^{11, 14, 19} and with the known propensity of DiI to partition strongly into disordered membrane domains.³⁷ A control experiment showed that UV irradiation had no effect on the morphology of POPC/C16-Cer bilayers (Fig. S1, Supporting Information).

Photo-uncaging of **1** in POPC bilayers

The UV-vis absorption spectrum of **1** in PC SUVs at pH 7.5 has a characteristic peak at 370 nm due to the deprotonated phenol, whereas at pH 5.5 the maximum is shifted to 340 nm (protonated phenol) with a shoulder at 370 nm.²² Photolysis of **1** in POPC vesicles (9:1 and 98:2 **1**/POPC) at 350 nm resulted in rapid formation of the product coumarin, as assessed by HPLC (Fig. S2). Caged Cer **1** was incorporated in POPC vesicles (9:1 molar ratio) containing 0.5 mol% DiI-C₂₀, and supported bilayers were prepared by vesicle fusion on mica. Fluorescence microscopy showed the formation of a uniformly fluorescent bilayer (Fig. 2A), indicating that the caged Cer was homogeneously distributed throughout the bilayer, at least at the resolution of optical microscopy. Some fluorescence images had occasional bright features which are due to adsorbed vesicles that are frequently difficult to remove completely by rinsing the sample. Correlated AFM and fluorescence imaging confirmed an absence of small nanoscale bilayer domains (Fig. S3). *In situ* irradiation of the supported bilayer was carried out using the microscope lamp through a λ 377 $\pm$ 25 nm filter, which will excite both protonated and deprotonated coumarin efficiently. The bilayer was irradiated for 5 s with the microscope aperture adjusted to give an $\sim$ 25 μ m illumination area and then reimaged (Fig. 2B, C) with the aperture opened. The post-UV image revealed a number of small dye-excluded features within the area defined by the aperture. These features had completely disappeared by 10 min after irradiation (Fig. 2D), with the dark domains closest to the edge of the irradiated area defined by the aperture disappearing more rapidly than those in the centre. A second irradiation through the aperture regenerated a similar pattern of small dye-excluded domains (Fig. 2E) that also equilibrated to give a homogeneous bilayer within $\sim$ 10 min. Comparable behavior with a well-conserved pattern of domains in the irradiated area was observed following a third (not shown) and fourth (Fig. 2F) 5 s period of UV irradiation. Examination of the pattern of domains generated by repeated irradiations showed two interesting features. First, as shown in Fig. 2C, E and F, many of the domains appeared in the same location after each irradiation, although there were also differences in the domain pattern from one image to the next. Second, as shown in Fig. 2F, the area covered by domains had increased significantly by the fourth irradiation.

A correlated imaging experiment was performed for the bilayer shown in Fig. 2. Fig. 3A, B show fluorescence and AFM images obtained immediately after the fourth 5 s irradiation. The dark domains observed by fluorescence corresponded to raised features in the AFM image, consistent with their assignment to a Cer-enriched gel phase. A second fluorescence image measured immediately after the AFM image (and $\sim$ 13 min after irradiation) indicated that many of the initial dark domains had disappeared and that domain loss was more pronounced in the region scanned by AFM. These observations suggest that domain disappearance is enhanced by AFM scanning and that the domains disappear more slowly after the same region of the bilayer has been irradiated multiple times.

Dependence of bilayer restructuring on irradiation time and area

In order to further examine the equilibration of the Cer-enriched domains generated by photo-uncaging of **1**, well-separated regions of a POPC/**1** bilayer were irradiated for different periods of time, and imaged by fluorescence only to eliminate changes induced by the AFM tip. The area covered by domains increased significantly for longer irradiation times (Fig. 4), although the individual domains did not increase in size; the area covered by domains was $\sim$ 25 μ m in diameter (the aperture size) for a short 5 s irradiation but increased to $\sim$ 80 μ m after a 1 min irradiation. This effect is much too large to be caused by effects of weaker illumination at the edges of the aperture. Fluorescence images of the individual irradiated areas at various times after UV photolysis indicated that the pattern of domains disappeared independent of the irradiation time (Fig. S4), although it took roughly twice as long for this to occur for the larger pattern created by a 60 s irradiation (panel E) compared

to the domains generated by a 2.5 s irradiation (panel A). As illustrated in Fig. S4, a significant fraction of the domain pattern remained after 5 min for the longer irradiation times (30 s and 1 min).

We hypothesized that the formation and disappearance of domains resulted from the generation of a high local Cer concentration that was above the threshold for domain formation, followed by lipid diffusion which diluted the newly generated Cer into the bulk bilayer. The effects of irradiation time on the area covered by the domains and on their rate of disappearance suggested the formation of an expanding region of a Cer-enriched bilayer due to Cer photorelease and lipid diffusion. This hypothesis was tested with two additional experiments. First, bilayers prepared from **1**/POPC (5/95 molar ratio) also produced small dye-excluded islands upon photolysis, whereas bilayers prepared with a lower concentration of **1** (2 mol %) failed to produce dye-excluded domains (Fig. S5, S6). These results are consistent with the reported 4–5% threshold for phase separation in C16-Cer/POPC bilayers.¹¹ Second, an entire bilayer was irradiated using a small lamp mounted under the sample, rather than irradiating a small region through the microscope aperture. The bilayer was irradiated for a longer time period (30 min) to ensure complete photolysis of **1** using the lower intensity lamp and different irradiation geometry. A combination of fluorescence and AFM images showed that small dye-excluded (fluorescence) and raised (AFM) features formed. These features were stable over a period of >45 min, and were unaffected by AFM scans (Fig. S7). This supports the hypothesis that the domains disappear by equilibration of the initial Cer-enriched areas formed within a limited area with the bulk membrane; the equilibration is driven by the concentration gradient created by photo-uncaging of **1** in a spatially restricted region of the bilayer. The sizes of the domains were similar independent of whether the entire bilayer or a small localized region was irradiated. However, the domains generated by photolysis of **1** were smaller than those formed when C16-Cer was incorporated directly in the bilayer (Fig. 1).

Protein localization in ceramide-enriched domains formed by photo-uncaging of **1**

To examine whether membrane-bound proteins partitioned into the ceramide-enriched domains, we monitored the distribution of the ganglioside GM1/cholera toxin B subunit (Ctx-B) complex in POPC/**1**/GM1 bilayers before and after photochemical Cer generation. Bilayers prepared from POPC/**1**/GM1 (90/10/1) formed small dark-dye-excluded domains that rapidly disappeared in the dark, but could be regenerated upon repeated UV irradiation (Fig. S8), behavior that is qualitatively similar to that observed in the absence of GM1. In order to assess the partitioning of GM1 between coexisting gel and fluid phases, a POPC/**1**/GM1 bilayer was irradiated over its entire area for 30 min to form persistent Cer-rich domains. The bilayer was then incubated with Alexa 647-labeled Ctx-B and washed and imaged (Fig. 5). The DiI channel showed typical small dark domains, whereas the Alexa 647 channel showed small bright domains that corresponded to the dye-excluded areas in the DiI channel, indicating partitioning of GM1 into the Cer-enriched domains. This is clearly illustrated in the false color-merged image which shows strong enrichment of Ctx-B (red) in the Cer domains that exclude DiI (green). Note that Förster resonance energy transfer between DiI and Alexa 647 may change the ratio of DiI fluorescence in the fluid vs gel phase from that observed in the absence of Ctx-B. The Ctx-B labeling experiment was also repeated using Alexa 488 labeled protein, with qualitatively similar results (data not shown). In this case, energy transfer from Alexa 488 to DiI in the POPC phase (where the DiI concentration is higher) will result in an overestimation of the amount of Ctx-B in the gel domains vs the fluid POPC bilayer. Nevertheless, the combination of the results in the presence and absence of the two labeled proteins clearly demonstrated enrichment of GM1 in the ordered Cer domains.

We next investigated whether preformed ganglioside-protein complexes showed similar partitioning into Cer-enriched domains. A POPC/1/GM1 (90/10/1) bilayer stained with DiI-C₂₀ showed uniform fluorescence in the DiI channel and no signal in the Alexa 647 nm channel prior to incubating with Ctx-B (Figure 6A). After incubation with Ctx-B and rinsing, the bilayer still showed reasonably uniform fluorescence in the DiI channel and diffuse fluorescence with some brighter features in the Alexa 647 channel (Figure 6B), consistent with GM1-Ctx-B distribution throughout the bilayer. After UV irradiation of the entire bilayer for 30 min small dye-excluded features appeared in the DiI channel and overlapped well with bright domains observed in the Alexa 647 channel (see merged image, Figure 6C). This indicates that photo-uncaging of **1** results in partitioning of GM1/Ctx-B into ordered Cer-enriched domains. An additional experiment in which a POPC/1/GM1/Ctx-B bilayer was irradiated through the microscope aperture for short time periods (ranging from a few seconds to 10 min) indicated that Cer-enriched domains formed more slowly than for samples in the absence of protein. In this case, domains took ~ 1 min to form whereas the domains were readily detectable after a 5 s photolysis for samples in the absence of protein. Furthermore, in the presence of GM1/Ctx-B, the Cer-enriched domains generated by irradiation of a small region of the bilayer were stable for a period of at least 1 h after their formation.

Discussion

Photolysis of caged Cer **1** in POPC bilayers produces small raised domains that are similar to those observed for supported bilayers prepared from POPC vesicles containing the corresponding C16-Cer. These domains are assigned to a Cer-enriched gel phase, consistent with the phase diagram for ternary POPC/Chol/C16-Cer mixtures derived from fluorescence measurements in GUVs; in the absence of cholesterol, the formation of gel phase domains requires only 4 mol% Cer.¹¹ The formation of Cer domains is also consistent with Cer's high melting temperature ($T_M = 90^\circ\text{C}$ for C16-Cer)³⁸ and its well-documented propensity to form ordered domains in vesicles and supported monolayers and bilayers.^{8, 9, 11, 13, 19, 20, 36} Supported bilayers prepared from 9:1 POPC/1 are initially homogeneous as judged by both AFM and fluorescence, indicating that the caged Cer is unable to organize in tightly packed gel domains and is solubilized in the fluid POPC membrane. The relatively large and polar bromohydroxycoumarin caging group which is partially deprotonated at neutral pH presumably interferes with lipid packing, preventing formation of a Cer-enriched gel phase. Similarly, bulky dyes attached to lipid headgroups have been shown to modify lipid packing and partitioning between ordered and disordered phases.³⁷

Irradiation of a spatially restricted area of the bilayer demonstrates that Cer-enriched domains can be generated with both spatial and temporal control. Domains appear following very short irradiation periods (as little as 5 s), consistent with rapid photorelease of Cer and domain nucleation, followed by lipid diffusion to form domains that are large enough to be visualized by fluorescence microscopy. This requires that the Cer concentration generated in the irradiated area be above the ~4% threshold for gel/fluid coexistence.¹¹ For short irradiation times the initial domains are confined to approximately the area defined by the microscope aperture. Although domains formed by irradiating the entire bilayer are stable over a period of 1 h or more, domains formed within a restricted bilayer region are dynamic and disappear within minutes. The disappearance of the domains is consistent with lipid diffusion leading to equilibration of Cer with the bulk membrane and the concomitant reduction of its concentration below the threshold for domain formation. This is analogous to a fluorescence recovery after photobleaching experiment in which dyes are bleached in a spatially confined region and the recovery of fluorescence due to lipid diffusion is monitored. Note that diffusion coefficients for lipids in mica-supported DOPC bilayers are approximately a factor of 2 lower than those for free-standing bilayers (3.1 vs $7.8\mu\text{m}^2/\text{s}$).³⁹

Domains can be regenerated by additional short irradiations of the same bilayer region; in this case there is a “memory effect” with some (but not all) domains appearing in the same position as those produced in the previous irradiation. There are two possible origins for this effect. First, small Cer clusters may persist after the domains have disappeared (in the fluorescence images) and then serve as nucleation sites for domains upon repeat irradiation. A second possibility is that defects in the underlying support may cause localized lipid pinning, thus providing nucleation sites for the Cer domains. Since domains can be repeatedly regenerated by continued light/dark cycles we conclude that both Cer and **1** must equilibrate rapidly in the supported bilayer.

Although the area covered by Cer domains corresponds to the size of the aperture used to restrict the irradiated area for initial short irradiations, we observed an enlarged domain pattern when the irradiation time was increased or when multiple irradiation/dark equilibration cycles were carried out. The rate of domain disappearance also decreased with increasing size of the irradiated area. These results are consistent with the generation of a Cer concentration gradient as Cer diffuses out of the initial irradiated area. With longer irradiation times, lipid diffusion results in an above-threshold Cer concentration outside the irradiated area, leading to domain formation in an increasingly larger area of the membrane. Thus, the observed domain pattern results from a competition between the rate of Cer generation by photolysis of **1**, diffusion of Cer out of the irradiated area, and replenishment of **1** by diffusion into the irradiated area. AFM imaging leads to more rapid disappearance of domains in the scanned region, presumably due to disruption of the bilayer by sample-tip interactions. The transient domain patterns produced by photolysis of **1** have some analogies to the domain evolution that has been observed upon enzymatic Cer generation in SM monolayers.⁴⁰ In this case rapid photorelease of Cer produced domains that initially had a higher proportion of Cer than the equilibrium composition and evolved in shape and size due to competing effects of line tension and dipolar repulsion.

Irradiation of POPC/**1** bilayers containing the ganglioside GM1 in both the presence and absence of Ctx-B (Fig. 5 and 6) demonstrates that photorelease of Cer triggers clustering of a membrane-anchored protein in Cer-enriched domains. The choice of GM1/Ctx-B to investigate protein clustering was based on a previous study that used a combination of AFM and fluorescence to show that >80% of GM1-bound Ctx-B partitioned into Cer-rich domains in DOPC/sphingomyelin/cholesterol/Cer bilayers.^{10, 13} We first showed that incorporation of 1 mol% GM1 does not change the behavior of POPC/**1** bilayers, as assessed by DiI-C₂₀ fluorescence prior to or following photochemical generation of Cer. Labeling experiments with Alexa 647 Ctx-B after photolysis confirmed that GM1 concentrates in Cer-rich domains that are distinct from the slightly heterogeneous distribution of GM1 observed by protein labeling prior to Cer generation. Previous studies have shown that GM1 aggregation occurs even in fluid PC bilayers.^{41, 42} Irradiation of POPC/**1** bilayers containing GM1-bound Ctx-B also leads to formation of Cer/GM1 domains, albeit on a longer timescale than in the absence of Ctx-B. The slower appearance of domains may be a result of reduced diffusion due to the presence of GM1/protein complexes in the membrane. It has been reported that the diffusion coefficient, *D*, for GM1 is similar to other dye-labeled lipid probes in DOPC bilayers ($\sim 4 \mu\text{m}^2/\text{s}$)^{43, 44} but decreases by a factor of 6 when bound to Ctx-B,⁴³ consistent with the pentameric structure with five ganglioside-binding sites for the B subunit of this protein.⁴⁵ Interestingly, several lipid probes and GM1/Ctx-B complexes show very slow diffusion in Cer-enriched domains ($D \ll 0.01 \mu\text{m}^2/\text{s}$).¹³ Although we have not examined this in detail, the domains produced in the presence of GM1/Ctx-B are more stable with respect to equilibration with the bulk membrane, presumably due to the presence of GM1/Ctx-B complexes and/or clusters.

The bilayers studied here are much simpler than cellular membranes and we have also examined the generation of Cer from **1** in ternary lipid mixtures that have co-existing liquid-ordered and liquid-disordered phases, as a more relevant model system.⁴⁶ The photochemical generation of Cer resulted in a reduction in the size of the liquid-ordered domains, an effect that we attributed to the expulsion of cholesterol from the domains. However, a third Cer-enriched gel phase was not observed, by contrast to our earlier study of the direct incorporation and enzymatic generation of Cer in the same lipid mixture.³⁶ These differences were hypothesized to result from problems with incorporating >5 mol% **1** in the bilayers prepared from the ternary lipid mixture, since ~8% Cer is required for formation of the third Cer-enriched gel phase.

Finally, it is interesting to compare the present results for controlling phase separation and protein clustering with earlier reports of photoinduced changes in lipid organization in bilayer membranes. Several studies have employed the photoactivation of photoswitchable surfactants and amphiphiles to modulate the permeability of liposomal membranes, a potentially useful approach for drug delivery.^{47–50} A trans-to-cis photoisomerization of an azobenzene moiety attached to the hydroxyl group of cholesterol was shown to produce shape changes in GUVs and to disrupt liquid-ordered domains, leading to their disappearance or to the incorporation of fluid phase regions.⁵¹ More recently, an azobenzene-substituted amphiphile was used to promote lateral phase separation in ternary lipid mixtures for compositions that were close to a miscibility boundary between the one- and two-phase regions.⁵² The ability to trigger phase separation was explained on the basis of the large change in line tension that occurs when the trans azobenzene (which is localized in the headgroup region of the bilayer) is converted to the more bulky cis isomer. Photoswitching required high concentrations of the amphiphile (>~30 mol %), a significant limitation for biological studies. Photocontrolled domain formation has also been achieved by lipid photooxidation, although this is unlikely to be a generally useful approach since it leads to decomposition of membrane components.⁵³ By contrast, photoinduced lipid uncaging can be used not only to induce lateral membrane segregation, but also to enhance the concentration of a lipid-anchored protein in ordered domains.

Photorelease of Cer has a number of potential applications, some of which we are currently exploring. The generation of Cer with both spatial and temporal control in cells will be particularly useful for understanding the biological roles of different molecular species of Cer and the role of compartmentalization in regulating Cer activity.¹ More generally, photocaged lipids may find applications for triggering protein clustering that is driven by lipid organization, as for example in transient focal adhesions or in protein assemblies involved in the immunological synapse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A POPC/C16-Cer (9:1 molar ratio) supported bilayer labeled with 0.5 mol % DiI-C₂₀ and imaged by correlated fluorescence and AFM shows dye-excluded, raised features that are assigned to ceramide-rich domains. (A) bilayer visualized by fluorescence before AFM scan; (B) AFM reveals domains that are ~1.5 nm taller than the POPC-rich phase (cross section below image) and membrane-spanning defects (dark regions; arrows in panel A mark two of the defects observed in B); (C) a post-AFM fluorescence image indicates that some bilayer defects have started to fill in. Scale bar = 5 μm.

Fig. 2.

Photorelease of Cer in a POPC/1 supported bilayer labeled with 0.5 mol % DiI-C₂₀. (A) T_{0 min} before UV irradiation the membrane appears uniform by fluorescence, with no visible phase separation; (B) the area that will be irradiated is indicated by the aperture; (C) following 5 s of UV exposure small, dye-excluded, Cer-rich domains are observed in the irradiated region; (D) T_{10 min} the dark domains have disappeared, beginning at the periphery of the irradiated region and moving progressively toward the center; (E) a second 5 s photolysis recreates a similar pattern, where the size and position of most domains correspond to those observed in C. The membrane re-equilibrates within minutes to give a uniform bilayer (not shown); (F) at T_{29 min} after the third and fourth 5 s photolyses, a larger area is covered by domains. Conserved (blue) and non-conserved (red) domains are indicated on images C, E, and F. Scale bar = 5 μm .

Fig. 3. Correlated fluorescence and AFM imaging of a POPC/1 supported bilayer labeled with 0.5 mol % DiI-C₂₀ following localized photolysis confirms that the dye-excluded features seen by fluorescence are Cer-rich domains. (A) The bilayer region shown in Figure 2E, prior to AFM scanning; (B) AFM shows domains that are ~1.5 nm higher than the bulk membrane; (C) a fluorescence image acquired post-AFM (13 min after image A) indicates the disappearance of some domains, primarily those in the AFM-scanned region. The dotted lines in the two fluorescence images mark the region scanned by AFM. Scale bar = 5 μ m.

Fig. 4. Effects of irradiation time on the formation of dye-excluded domains. Several regions of the same POPC/1 bilayer (+ 0.5 mol % DiI-C₂₀) were irradiated through the aperture for varying time intervals: (A) 2.5 s; (B) 5 s; (C) 15 s; (D) 30 s; (E) 1 min. Scale bar = 10 μm .

Fig. 5.

Photorelease of C16-Cer from **1** triggers GM1 partitioning into the Cer-rich domains. An entire POPC/**1**/GM1 bilayer (90/10/1 + 0.5 mol % DiI-C₂₀) was UV irradiated for 30 min in buffer (pH 7.4) and subsequently incubated in a 2 μg/mL solution of Alexa 647-labeled Cholera toxin B subunit (Ctx-B) for 30 min, and then gently rinsed. Fluorescence images were acquired in both DiI-C₂₀ (A) and Alexa 647 (B) channels. (C) A false-color merged image shows GM1-enrichment in the gel-phase domains (red) with respect to the POPC-rich bulk phase (green). Scale bar = 2 μm.

Fig. 6.

Ctx-B GM1 accumulates in photochemically generated Cer-rich domains. A supported bilayer of POPC/1/GM1 (90/10/1 + 0.5 mol % DiI-C₂₀) was incubated in Alexa 647 labeled Ctx-B for 30 min in pH 7.4 buffer, rinsed, and UV-irradiated for 30 min. Fluorescence was recorded in both DiI and Alexa 647 channels before and after photolysis. (A) T_{0 min} prior to Ctx-B incubation the bilayer is uniform; (B) T_{30 min} pre-UV small Ctx-B clusters are randomly distributed throughout the bilayer; (C) T_{60 min} immediately after UV areas of higher Alexa 647 nm Ctx-B fluorescence correlate with dye-excluded gel phase domains. Scale bar = 2 μ m.

Chart 1.