Disruption of lipid order by short-chain ceramides correlates with inhibition of phospholipase D and downstream signaling by FcεRI

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Summary

Specialized plasma membrane domains known as lipid rafts participate in signal transduction and other cellular processes, and their liquid-ordered properties appear to be important for their function. We investigated the possibility of using amphiphiles to disrupt lipid rafts and thereby inhibit IgE-FcεRI signaling. We find that short-chain ceramides – C_2-ceramide and C_6-ceramide – decrease plasma membrane lipid order and reduce the extent of fluorescence resonance energy transfer between lipid-raft-associated molecules on intact cells; by contrast, biologically inactive C_2-dihydroceramide does neither. Structural perturbations by these ceramides parallel their inhibitory effects on antigen-stimulated Ca^{2+} mobilization in RBL mast cells in the presence and absence of extracellular Ca^{2+}. Similar inhibition of Ca^{2+} mobilization is caused by n-butanol, which prevents phosphatidic acid production by phospholipase D, but not by t-butanol, which does not prevent phosphatidic acid production. These results and previously reported effects of short-chain ceramides on phospholipase D activity prompted us to compare the effects of C_2-ceramide, C_2-dihydroceramide and C_{16}-ceramide on phospholipase D1 and phospholipase D2 activities in vitro. We find that the effects of these ceramides on phospholipase D1 activity strongly correlate with their effects on antigen-stimulated Ca^{2+} mobilization and with their disruption of lipid order. Our results indicate that phospholipase D activity is upstream of antigen-stimulated Ca^{2+} mobilization in these cells, and they demonstrate that ceramides can serve as useful probes for investigating roles of plasma membrane structure and phospholipase D activity in cellular signaling.

Key words: Ceramides, Lipid rafts, IgE receptors, Phospholipase D, Mast cells

Introduction

Considerable evidence has accumulated for an important role of the plasma membrane structure in signal transduction and other cellular processes. In particular, detergent-resistant domains on plasma membranes, known as lipid rafts, have been implicated in several types of cellular signaling (Simons and Toomre, 2000). These domains are enriched in sphingolipids, cholesterol and glycerophospholipids with saturated acyl chains, as well as glycosylphosphatidylinositol (GPI)-anchored proteins and dually acylated Src-family tyrosine kinases; their liquid-ordered properties are important for their participation in signaling (Brown and London, 2000). As a prototypic example, our laboratory demonstrated that clustering of FcεRI, the high-affinity receptor for IgE, causes coalescence of lipid rafts and consequent coupling with the tyrosine kinase Lyn to initiate mast cell signaling (Field et al., 1997; Sheets et al., 1999; Holowka and Baird, 2001).

Membrane structural heterogeneity is likely to be particularly important for enzymes that have lipids as substrates, including phospholipases. For example, it is well established that defects in membrane bilayers modulate phospholipase A_2 (PLA_2) activity (Burack and Biltonen, 1994; Burack et al., 1997), and a recent study showed that binding of ADP-ribosylation factor 6 (ARF6) to bilayers containing phosphatidylinositol (4,5)-bisphosphate (PIP_2) creates defects that could be involved in the activation of phospholipase D (PLD) (Ge et al., 2001). These lipases generate important lipid second messengers, as well as amphiphilic products that can modulate membrane structure and influence processes such as vesicle budding and exocytosis (Liscovitch et al., 2000; Cohen and Brown, 2001; Ivanova et al., 2001). Ceramides are the products of sphingomyelin hydrolysis by sphingomyelinases, and these endogenous, long-chain neutral lipids have been implicated as signal transduction mediators in certain cell types (Kolesnick et al., 2000). Short-chain, cell-permeable ceramides are commonly used to mimic the mechanisms of action of naturally occurring ceramides in signal transduction, and effects of these ceramides on specific pathways in cell signaling have been described (Mathias et al., 1991; Huwiler et al., 1996; Hannun and Obeid, 2002). Among such studies, Nakamura et al. showed that C_2-ceramide (C_2-cer) inhibits antigen-stimulated PLD activation in RBL mast cells, and they concluded that this results from inhibition of Ca^{2+} influx and consequent inhibition of Ca^{2+}-dependent protein kinase C

(PKC) isozymes upstream of PLD (Nakamura et al., 1996). Other studies have implicated more-direct effects of short-chain ceramides on PLD activity (Singh et al., 2001; Venable et al., 1996). Specific binding of these short-chain ceramides to ion channels or enzymes is commonly suggested to be responsible for the biological effects observed. However, it has been shown that membrane-destabilizing properties of C_2-cer correlate with inhibition of platelet signaling, and it has been suggested that this may be a more general mechanism by which short-chain ceramides affect cell signaling (Simon and Gear, 1998).

In the present study, we evaluated the effects of various ceramides on lipid order in RBL mast cell plasma membranes, and compared these effects with inhibition of signaling mediated by FcεRI in these cells. We find that disruption of lipid order by certain short-chain ceramides is highly correlated with their inhibition of antigen-stimulated Ca^{2+} mobilization and activated PLD1. Our results point to activation of PLD as a critical step for antigen-stimulated release of Ca^{2+} from internal stores, and they indicate that perturbation of lipid order by short-chain ceramides may be a generally useful strategy for investigating the role of membrane structure in cellular signaling.

Materials and Methods

Reagents

C_2-ceramide (C_2-cer), C_2-dihydroceramide (C_2-dhcer) and C_{16}-ceramide (C_{16}-cer) were from Calbiochem (San Diego, CA), and C_6-ceramide (C_6-cer) was from Sigma (St Louis, MO). n-butanol was from Malinckrodt Baker (Paris, KY) and t-butanol was from Fisher Chemicals (Pittsburgh, PA). Alexa Fluor® 488 Protein Labeling Kit, Alexa488-cholera toxin B subunit, indo-1 AM and 2-(3-(diphenylhexatrienyl) propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (DPH-PC) were from Molecular Probes (Eugene, OR). Cy3 monofunctional reactive dye pack was from Amersham Pharmacia Biotech (Piscataway, NJ). Mouse monoclonal IgE specific for 2,4-dinitrophenyl (DNP) (Liu et al., 1980) was purified as previously described (Posner et al., 1992). Purified mouse monoclonal antibody (mAb) AA4, specific for the α-galactosyl derivative of the ganglioside GD_{1b} was a gift from Reuben Siraganian (National Institutes of Health, Bethesda, MD). Purified mouse monoclonal anti-rat Thy-1 Ox-7 (anti-Thy-1), specific for the GPI-anchored protein Thy-1, was from PharMingen (San Diego, CA). The amino-reactive dyes Cy3 and Alexa488 were conjugated to antibodies by incubation at manufacturer recommended ratios for 3 hours at room temperature in PBS (pH 8.5), followed by extensive dialysis against PBS (pH 7.4) to remove unconjugated dye.

Cells

RBL-2H3 cells (Barsumian et al., 1981) were maintained in monolayer culture in Minimum Essential Medium supplemented with 20% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 10 μg/ml gentamicin sulfate. All tissue culture reagents were obtained from Gibco (Grand Island, NY) unless otherwise noted. Cells were harvested 3-5 days after passage.

Fluorescence anisotropy measurements of lipid order in plasma membrane vesicles

Plasma membrane vesicles were isolated from RBL-2H3 cells by chemically induced cell blebbing and labeled with DPH-PC as described previously (Gidwani et al., 2001). After washing the vesicles to remove unincorporated probe, 2 ml of the membrane suspension (~150 μM in phospholipid) in buffered saline solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 20 mM HEPES, pH 7.4) was placed in a 10×10×40 mm acrylic cuvette and stirred continuously in a thermostatic sample chamber at 37°C. Fluorescence anisotropy measurements were made as described previously (Gidwani et al., 2001) before and after C_6-cer, C_2-cer or C_2-dhcer were added in successive increments from stock solutions in ethanol or dimethyl sulfoxide (DMSO). Control experiments showed that the solvents added (<1% v/v total) had negligible effects on anisotropy values. Mol% ceramide was based on the known amount of ceramide added and the amount of plasma membrane phospholipid determined by an organic phosphate assay as described previously (Gidwani et al., 2001).

Fluorescence resonance energy transfer (FRET) measurements of proximity between cell-surface molecules

For donor labeling, RBL-2H3 cells were harvested in 1.5 mM EDTA, 135 mM NaCl, 20 mM HEPES, pH 7.4, pelleted by centrifugation (200 ***g***, 8 minutes), and washed once in BSS. Cell aliquots at ~5×10^6/ml in BSS were incubated with various FRET donors: Alexa488-IgE (1 hour at 37°C), Alexa488-anti-GD_{1b} (1 hour at 37°C), Alexa488-cholera toxin B (30 minutes at room temperature), FITC-anti-transferrin receptor (3 hours at 4°C) or Alexa488-anti-CD43 (3 hours at 4°C). The cells were pelleted by centrifugation, washed twice in BSS to remove unbound antibodies, and resuspended at 1×10^6/ml in BSS in thermostated cuvettes. Prior to each experiment, donor-labeled samples were examined in a fluorescence microscope to confirm uniform plasma membrane staining. Steady-state donor fluorescence (Alexa488 or FITC; excitation, 490 nm; emission 520 nm) was monitored as a function of time in an SLM 8000C spectrofluorometer; FRET acceptor (Cy3-anti-Thy-1, 1.5 μg/ml final concentration) was added to the cuvette, and time-dependent donor quenching was quantified. Various ceramides were then added to the cuvette from concentrated stock solutions in ethanol or DMSO and changes in donor fluorescence were monitored. Percent disruption of FRET between the cell-surface molecules by the ceramides was calculated according to Eqn 1:

$$\%\ \text{decrease in FRET} = (I_{DC} - I_{DA}) \times 100/(I_D - I_{DA}), \quad (1)$$

where steady-state donor fluorescence values are as denoted in Fig. 3A: I_D, intensity before acceptor addition; I_{DA}, intensity after acceptor addition; and I_{DC}, intensity after ceramide addition. Control experiments were also carried out to correct for small effects of ceramides and their solvents on donor fluorescence in the absence of acceptors.

Ca^{2+} measurements

Cells were harvested as described above and loaded with indo-1, a fluorescent indicator of intracellular free Ca^{2+} ($[Ca^{2+}]_i$), as previously described (Paar et al., 2002), during which time they were also sensitized with excess IgE. Suspensions of indo-1-loaded cells (1×10^6 cells/ml) in BSS with 0.3 mM sulfinpyrazone to minimize leakage of indo-1 were stirred at 37°C in acrylic cuvettes, and $[Ca^{2+}]_i$ was monitored with an SLM 8000C spectrofluorometer (excitation, 330 nm; emission, 400 nm). For measurements in the absence of extracellular Ca^{2+}, indo-1-loaded cells were washed twice and resuspended at 1×10^6 cells/ml in Ca^{2+}-free BSS with sulfinpyrazone. $[Ca^{2+}]_i$ is represented as indo-1 fluorescence intensity, which is nearly proportional to $[Ca^{2+}]_i$ under the conditions of these experiments (Pierini et al., 1997). The Ca^{2+} response was quantified by integrating the area under the antigen response curve from 0 to 10 minutes post-stimulation in the presence of extracellular Ca^{2+} and until the response returned back to baseline level in the absence of extracellular Ca^{2+}. Percent inhibition of Ca^{2+}

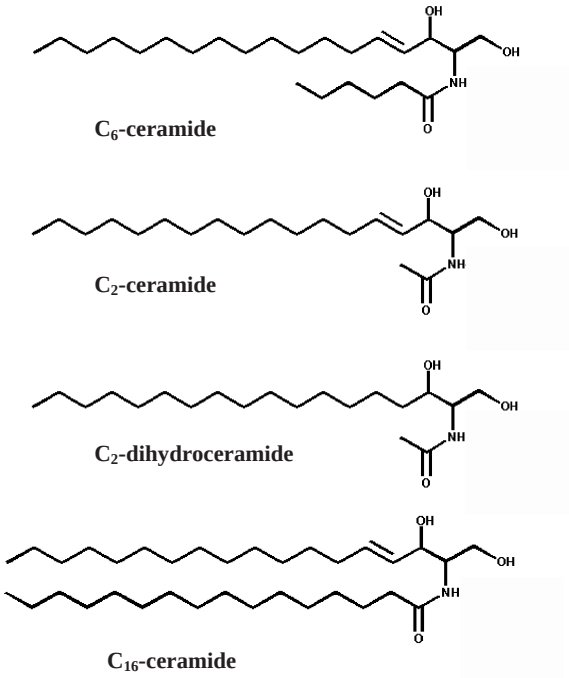

Fig. 1. Structures of ceramides used in this study.

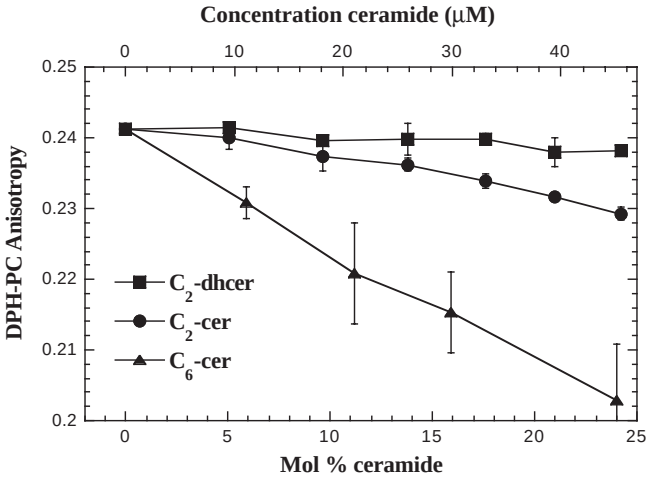

Fig. 2. Short-chain ceramides decrease lipid order as measured by fluorescence anisotropy of DPH-PC in plasma membrane vesicles from RBL cells. Ceramides were added to DPH-PC-labeled vesicles (~150 μM phospholipids), and fluorescence anisotropy was measured subsequent to each addition. ■, C_2-dhcer; ●, C_2-cer; ▲, C_6-cer. Results shown are average values of at least three experiments for each ceramide; bars designating s.e.m. are shown where larger than the width of data points.

mobilization by the ceramides or butanols was calculated using Eqn 2:

$$\%\ \text{inhibition of } Ca^{2+}\ \text{mobilization} = (A_o - A_t) \times 100/A_o\ , \qquad (2)$$

where A_o and A_t are areas under the Ca^{2+} response curves for control and test cuvettes, respectively, after correction for small differences in numbers of cells determined as the ratio of indo-1 intensities for Triton X-100-lysed cells (Field et al., 2000).

In vitro assays for PLD activity

Recombinant PLD1, ARF1 and PKCβII were purified as described previously (Walker et al., 2000). For recombinant PLD2, monolayers of *Spodoptera friguperda* 21 (Sf21) cells were infected with baculovirus encoding human PLD2. After 72 hours infection, the cells were harvested, washed twice in 2 ml Solution F (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1 mM DTT, 0.1 mM PMSF, 2.5 mM EDTA), and lysed by nitrogen cavitation at 4°C for 60 minutes at 1100 psi. Membranes and cytosol were separated by spinning at 174000 ***g*** for 1 hour, and the membrane-bound fraction of PLD2 were isolated, resuspended in solution F and aliquoted for assays. Lipid vesicles with [^{3}H]phosphatidylcholine (PC), phosphatidylethanolamine, PIP_2 and 11 mol% cholesterol were prepared as previously described (Brown et al., 1993). To prepare vesicles with ceramide, an appropriate volume of the ceramide stock in ethanol was added to the lipid mixture in chloroform to a phospholipid:ceramide ratio of 2:1; they were then dried, hydrated and sonicated similar to lipids for control vesicles. All assays were conducted at 37°C for 30 minutes as described (Brown et al., 1993). PLD activity was quantified as previously described (Brown et al., 1995).

Results

Short-chain ceramides decrease lipid order in plasma membrane vesicles from RBL-2H3 mast cells

To identify candidate molecules that alter cell signaling by perturbation of plasma membrane structure, we used fluorescence anisotropy measurements with DPH-PC to monitor lipid order in plasma membranes from RBL cells. We previously established this method as a practical and sensitive measure of lipid order in cholesterol-containing membranes (Gidwani et al., 2001). Using this approach, we found that both C_2-cer and C_6-cer (Fig. 1) cause a concentration-dependent decrease in the lipid order of plasma membrane vesicles isolated from RBL-2H3 mast cells. As shown in Fig. 2, C_6-cer is more potent than C_2-cer on a mole percentage basis, possibly because the former has a higher membrane:aqueous partition coefficient (Dobrowsky and Hannun, 1992; Simon and Gear, 1998). In contrast to these ceramides, C_2-dhcer, which lacks the trans double bond between C4 and C5 of the sphingoid backbone (Fig. 1), does not have a significant effect on lipid order at the same concentrations as C_2-cer and C_6-cer (Fig. 2). These results are consistent with those of a previous study that found C_2-cer, but not C_2-dhcer, causes a decrease in anisotropy of DPH in cholesterol-containing model membranes (Simon and Gear, 1998); they demonstrate that short-chain ceramides C_2-cer and C_6-cer perturb the structure of ordered biological membranes, decreasing lipid order. By contrast, long-chain ceramides such as C_{16}-cer, which are more similar to naturally occurring ceramides (Chalfant et al., 1999), have been shown to increase lipid order in model membranes containing cholesterol (Massey, 2001; Wang and Silvius, 2000). Other amphiphilic sphingosine derivatives such as *N*,*N*-dimethyl-sphingosine and galactosylsphingosine have no effect on lipid order at similar mole percentages (A. Gidwani, Membrane structure in FcεRI signaling: Measurement of lipid order and disruption of order and signaling by short chain ceramides, PhD thesis, Cornell University, 2002).

Short-chain ceramides reduce the extent of FRET between lipid raft markers on intact cells

DPH-PC is not suitable for measurements of lipid order in

intact cells, in part because it is difficult to maintain its plasma membrane localization. As an alternative, we used FRET between lipid-raft-associated proteins at the cell surface (Kenworthy and Edidin, 1998; Varma and Mayor, 1998). For our experiments, Alexa488-labeled IgE or anti-ganglioside mAb AA4 serve as the energy transfer donors, and Cy3-labeled anti-Thy-1 bound to Thy-1, an abundant GPI-anchored protein, serves as the energy transfer acceptor. As shown previously, these antibodies label RBL cells uniformly at the plasma membrane and co-redistribute when IgE is crosslinked at 4°C (Holowka et al., 2000). As shown in Fig. 3A for Alexa488-IgE bound to FcεRI on RBL cells, addition of excess Cy3-anti-Thy-1 causes time-dependent donor quenching, which attains a new steady state level within a few minutes at 37°C. This quenching of Alexa488 fluorescence is the result of FRET to Cy3 because it is not observed when Thy-1 is pre-occupied with excess unlabeled anti-Thy-1, and Cy3-anti-Thy-1 is added subsequently (A.G., unpublished). Similar FRET are observed when either Alexa488-cholera toxin B, bound to ganglioside GM_1, or Alexa488-anti-GD_{1b} (AA4), bound to a GD_{1b} ganglioside (Guo et al., 1989), are used as the donor to Cy3-anti-Thy-1. Quenching of donor fluorescence is not observed when either Alexa488-anti-CD43 mAb, bound to CD43 (leukosialin) (Shelley et al., 1989), or FITC-anti-transferrin receptor mAb, bound to transferrin receptor (Testa et al., 1993), are used as the donor, and the same amount of Cy3-anti-Thy-1 is added as acceptor (A.G., unpublished). Both of these donor-labeled cell-surface proteins have been shown to be excluded from lipid rafts (Cinek and Horejsi, 1992; Harder et al., 1998; Melkonian et al., 1999), indicating that FRET detected between acceptor-labeled anti-Thy-1 and donor-labeled IgE, anti-ganglioside, or cholera toxin B is due to enhanced proximity resulting from their mutual preferences for a lipid raft environment.

To evaluate whether short-chain ceramides disrupt lipid raft structure on intact cells using FRET as an indicator, these amphiphiles were added once donor quenching reached a steady state, and the relief of this quenching was monitored. As shown in Fig. 3A, addition of 8 μM C_6-cer causes a rapid, time-dependent increase in Alexa488-IgE fluorescence. This increase in donor fluorescence by C_6-cer addition was not observed in the absence of the bound acceptor, indicting that it is not due to a direct effect on donor fluorescence (A.G., unpublished). A similar reduction in donor quenching by Cy3-anti-Thy-1 is observed if 8 μM C_6-cer is added to cells in which Alexa488-anti-GD_{1b} is employed as the FRET donor, consistent with disruption of lipid raft structure.

The effects of different short-chain ceramides on FRET between Alexa488-IgE and Cy3-anti-Thy-1 are summarized in Fig. 3B. 8 μM C_6-cer inhibits FRET by about 25%, and a similar amount of inhibition by C_2-cer required four times more of this derivative, consistent with a lower membrane partition coefficient for this less hydrophobic amphiphile. Similarly, Fig. 2 shows that ~4 times more C_2-cer than C_6-cer is required to achieve the same reduction in membrane order. Higher concentrations of these ceramides (e.g. 16 μM C_6-cer) caused significant changes in donor fluorescence in the absence of acceptors, possibly due to membrane disruption (A.G., unpublished). 32 μM C_2-dhcer inhibits FRET by less than 5% (Fig. 3B), consistent with the lack of effect of this derivative on lipid order measured by DPH-PC anisotropy (Fig. 2). FRET

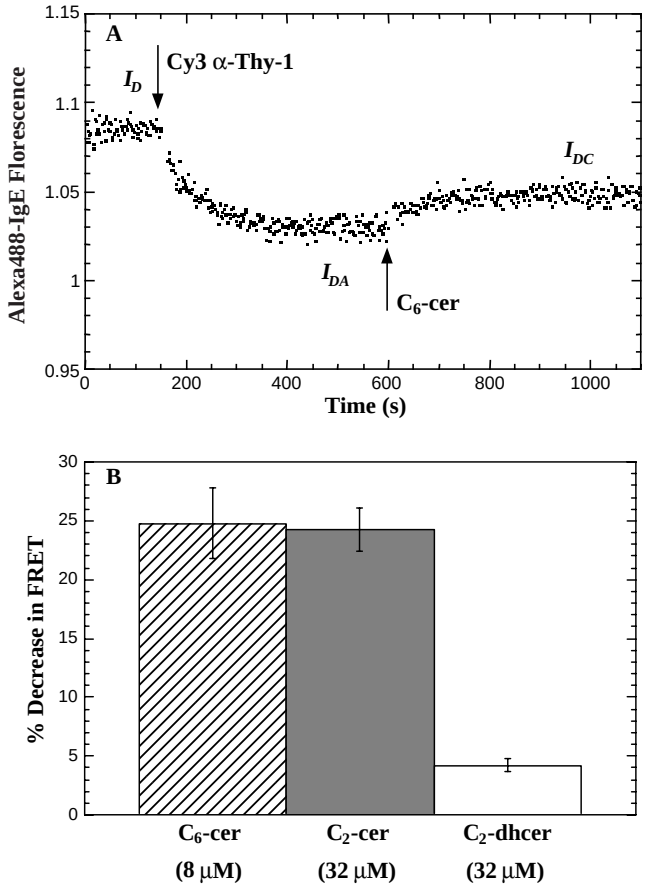

Fig. 3. FRET between Alexa488-IgE (donor) and Cy3-anti-Thy-1 (acceptor) is disrupted by short-chain ceramides. (A) Alexa488-IgE fluorescence monitored as a function of time, showing the effect of sequential addition of Cy3-anti-Thy-1 and 8 μM C_6-cer. Steady-state donor fluorescence intensity values denoted are: I_D, before acceptor addition; I_{DA}, after acceptor addition; I_{DC}, after ceramide addition. Typical transfer efficiency (E) between the donor and acceptor was 0.05-0.10. (B) Percent disruption of FRET between Alexa488-IgE and Cy3-anti-Thy-1 by 8 μM C_6-cer (hatched bar), 32 μM C_2-cer (filled bar) and 32 μM C_2-dhcer (open bar). Percent disruption was calculated according to Eqn 1, and averages of at least three experiments plotted, with bars showing s.e.m.

measurements were also carried out by pretreating donor-labeled cells with ceramide just prior to acceptor addition; the resulting inhibition of FRET for each of these ceramides was found to be similar to that shown in Fig. 3B (A.G., unpublished). These results show that short-chain ceramides reduce proximity between lipid-raft-associated components on intact cells in parallel with their capacity to disrupt lipid order in plasma membrane vesicles as measured by DPH-PC fluorescence anisotropy.

Short-chain ceramides inhibit antigen-stimulated Ca^{2+} mobilization in parallel to disruption of lipid order

A study by Nakamura et al. found that Ca^{2+} mobilization via FcεRI is effectively inhibited by 30 μM C_2-cer in parallel to inhibition of antigen-stimulated degranulation (Nakamura et al., 1996). To investigate whether these effects are mediated by

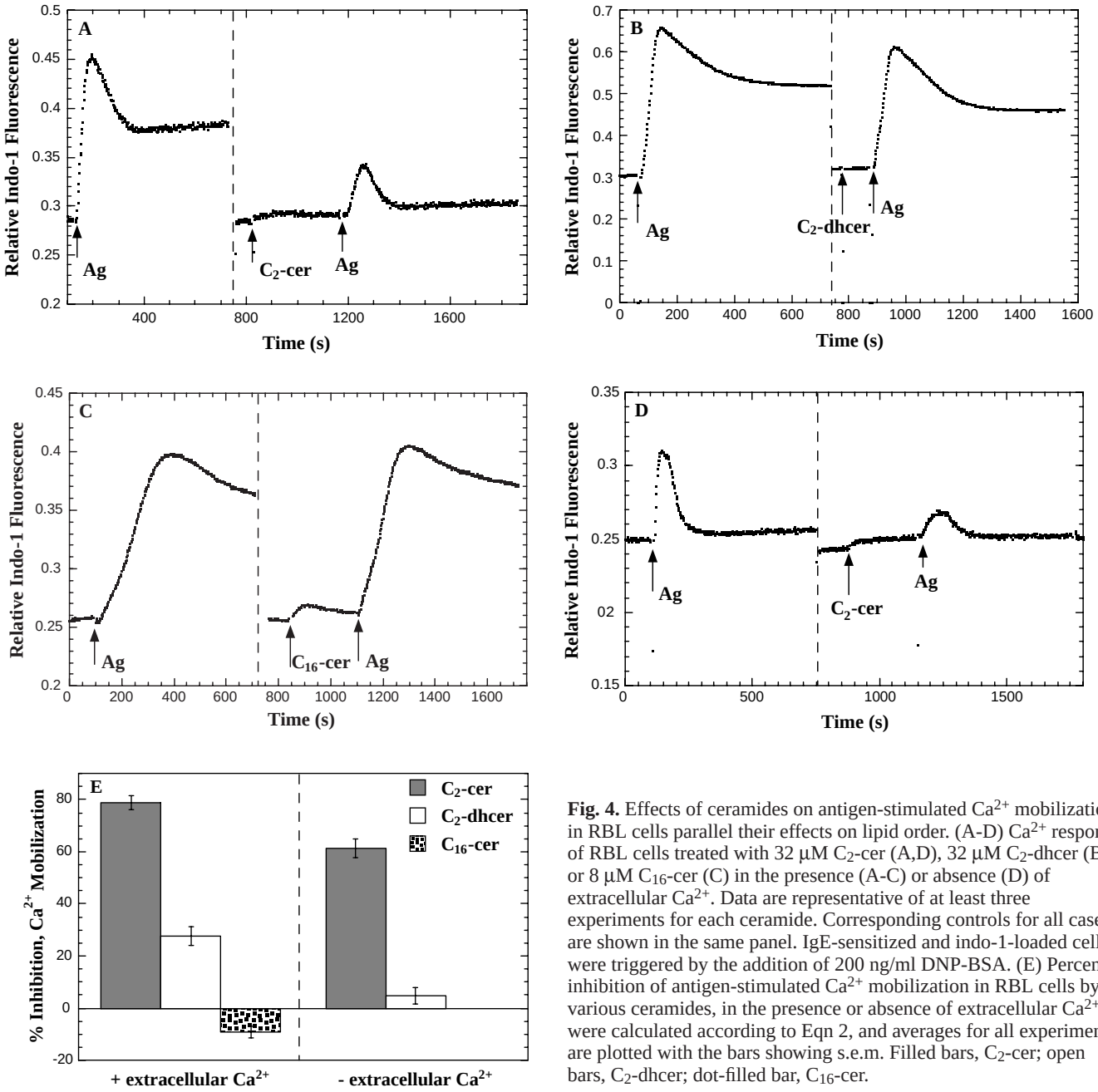

Fig. 4. Effects of ceramides on antigen-stimulated Ca^{2+} mobilization in RBL cells parallel their effects on lipid order. (A-D) Ca^{2+} response of RBL cells treated with 32 μM C_2-cer (A,D), 32 μM C_2-dhcer (B) or 8 μM C_{16}-cer (C) in the presence (A-C) or absence (D) of extracellular Ca^{2+}. Data are representative of at least three experiments for each ceramide. Corresponding controls for all cases are shown in the same panel. IgE-sensitized and indo-1-loaded cells were triggered by the addition of 200 ng/ml DNP-BSA. (E) Percent inhibition of antigen-stimulated Ca^{2+} mobilization in RBL cells by various ceramides, in the presence or absence of extracellular Ca^{2+}, were calculated according to Eqn 2, and averages for all experiments are plotted with the bars showing s.e.m. Filled bars, C_2-cer; open bars, C_2-dhcer; dot-filled bar, C_{16}-cer.

disruption of lipid order, we compared the effects of different ceramides on Ca^{2+} mobilization in RBL cells to their effects on lipid order. As shown in first half of Fig. 4A, crosslinking IgE-FcεRI with multivalent antigen leads to a typical biphasic Ca^{2+} response. In the second trace in Fig. 4A, addition of 32 μM C_2-cer causes little perturbation of the basal Ca^{2+} level, but substantially inhibits both the initial and the sustained phases of the response to antigen. For the integrated Ca^{2+} response over 10 minutes of stimulation, this represents an average of 80% inhibition for three separate experiments (Fig. 4E). As for the energy transfer results, 8 μM C_6-cer inhibits Ca^{2+} mobilization to a similar extent as 32 μM C_2-cer, but the addition of C_6-cer causes a significant increase in [Ca^{2+}] that complicates quantitation of its inhibitory effects on antigen-stimulated responses (A.G., unpublished). In contrast to these ceramides, 32 μM C_2-dhcer causes much less inhibition of this response (Fig. 4B). Long-chain C_{16}-cer at 8 μM final concentration has a small potentiating effect on antigen-stimulated Ca^{2+} mobilization, and it causes a small transient response itself when added to the cells (Fig. 4C). These results are summarized for multiple experiments in Fig. 4E. The relative potencies of these ceramides parallel their effects on lipid order detected by anisotropy measurements on plasma membrane vesicles (Fig. 2) or on model membranes for C_{16}-cer (Massey, 2001), and they also parallel effects on FRET on the RBL cell surface (Fig. 3B).

Because the initial phase of the Ca^{2+} response was inhibited by C_2-cer almost as effectively as the sustained phase (Fig. 4A), we tested the effect of this ceramide on the Ca^{2+} response observed in the absence of extracellular Ca^{2+}. Under these

conditions, Ca^{2+} influx does not occur, and only a transient response due to Ca^{2+} release from intracellular stores is triggered by stimulated inositol (1,4,5)-trisphosphate (IP_3) production (Meyer et al., 1988; Smith et al., 2001). As shown in Fig. 4D, substantial inhibition of this transient antigen-stimulated response by C_2-cer is observed, indicating an effect of C_2-cer at, or upstream of, Ca^{2+} release from stores. As summarized for multiple experiments in Fig. 4E, 32 μM C_2-cer inhibits this transient Ca^{2+} response by ~60%, whereas 32 μM C_2-dhcer does not inhibit this response. These results show that a principal step in Ca^{2+} mobilization inhibited by short-chain ceramides is at, or upstream of, Ca^{2+} release from stores.

PLD is upstream of antigen-stimulated Ca^{2+} release from stores in RBL-2H3 cells

Previously, our laboratory demonstrated that cholesterol-dependent lipid order is important for initiation of signaling via FcεRI (Field et al., 1997; Sheets et al., 1999). Therefore, we investigated whether C_2-cer inhibits antigen-stimulated tyrosine phosphorylation under conditions in which these short-chain ceramides inhibit Ca^{2+} mobilization. Consistent with a previous report (Nakamura et al., 1996), we could not detect significant inhibition of tyrosine phosphorylation with 32 μM C_2-cer in whole cell lysates (A.G., unpublished), possibly because of the relatively small reductions in membrane order that these amphiphiles cause compared with that caused by cholesterol depletion (Fig. 2) (Gidwani et al., 2001).

In previous studies, C_2-cer was reported to inhibit in vitro PLD activity (Singh et al., 2001) as well as antigen-stimulated PLD activity in RBL cells in vivo (Nakamura et al., 1996). To evaluate whether PLD might be involved in antigen-stimulated Ca^{2+} mobilization in RBL cells, we investigated the effects of n-butanol, a substrate for transphosphatidylation by PLD that prevents phosphatidic acid (PA) production and inhibits stimulated degranulation in RBL cells (Lin and Gilfillan, 1992; Cissel et al., 1998). As shown in Fig. 5A,B, n-butanol inhibits Ca^{2+} mobilization in RBL cells by ~60% at a final concentration of 0.5% (v/v). By contrast, t-butanol, which does not undergo this transphosphatidylation reaction, inhibits the Ca^{2+} response by <10% at the same concentration (Fig. 5A,B). In the absence of extracellular Ca^{2+}, transient Ca^{2+} mobilization is also inhibited by n-butanol, but not by t-butanol (Fig. 5B), similar to the effects of C_2-cer and C_2-dhcer (Fig. 4E). These results suggest that stimulated production of PA by PLD is an important step in the antigen-stimulated release of Ca^{2+} from intracellular stores.

Differential inhibition of in vitro PLD activities by ceramides

Parallel inhibition of antigen-stimulated Ca^{2+} mobilization by n-butanol and C_2-cer suggested that the effects of C_2-cer on this response might be mediated by direct inhibition of stimulated PLD activity. Therefore, we systematically investigated the effects of C_2-cer, C_2-dhcer and C_{16}-cer on the enzyme activity of both the known isoforms PLD1 and PLD2 (Colley et al., 1997; Hammond et al., 1995) in a previously established in vitro assay (Brown et al., 1993). Sonicated phospholipid/cholesterol vesicles containing ^{3}H-PC, with

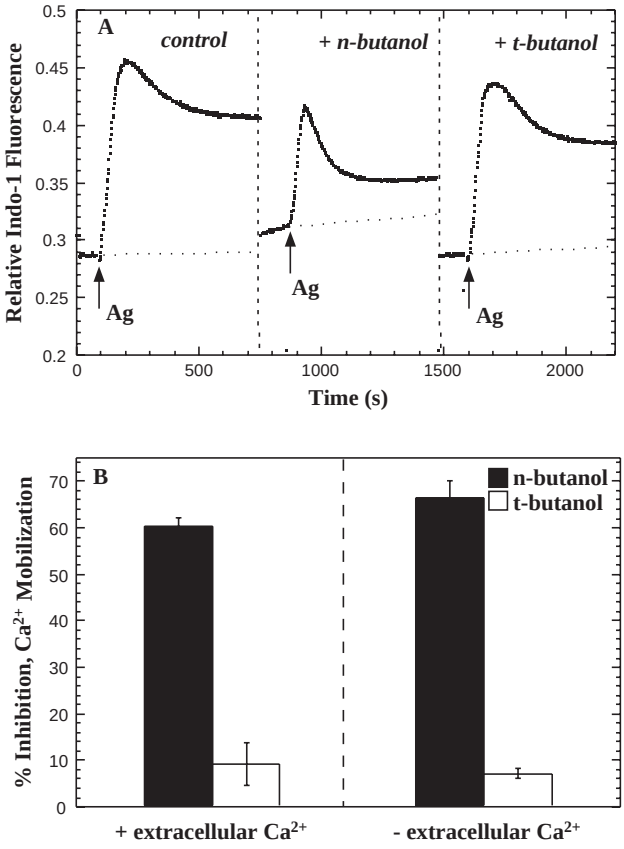

Fig. 5. Transphosphatidylation of PA by n-butanol inhibits antigen-stimulated Ca^{2+} mobilization in IgE-sensitized and indo-1-loaded RBL cells. (A) Ca^{2+} response of RBL cells in the presence of extracellular Ca^{2+}: control cells (first trace), cells pretreated with 0.5% (v/v) n-butanol for 2 minutes (second trace), or pretreated with 0.5% (v/v) t-butanol for 2 minutes (third trace). (B) Percent inhibition of Ca^{2+} mobilization in RBL cells by n-butanol (dark bars) and t-butanol (open bars), in the presence and absence of extracellular Ca^{2+}. IgE-sensitized and indo-1-loaded cells were evaluated for antigen-stimulated Ca^{2+} response with or without butanol treatment, and percent inhibition calculated according to Eqn 2. Results shown are averages of three experiments and bars show s.e.m.

or without various ceramides at a mole ratio of 2:1 phospholipid:ceramide, were used as substrates for preparations of PLD1 and PLD2 with or without their activators. PLD2 does not usually respond to small GTPases or PKC in the exogenous substrate assay (Colley et al., 1997), although relatively minor stimulation by ARF has been reported (Lopez et al., 1998). Fig. 6A shows relative enzyme activity for PLD2 in the presence of ARF1; C_2-cer inhibits enzyme activity by ~50% compared with control vesicles. By contrast, C_2-dhcer and C_{16}-cer enhance PLD2 activity by ~1.6-fold and ~2.7-fold, respectively, relative to the control. Similar trends were observed in the absence of ARF1 (A.G., unpublished).

Fig. 6B shows the effects of ceramides on PLD1 activity in the presence of its activators ARF1 and PKCβII. C_2-cer very effectively inhibits PLD1 activity, to the extent of 85%, whereas C_2-dhcer and C_{16}-cer have very small effects. Under these conditions, ARF1 and PKCβII activate PLD1 by 6-8 fold.

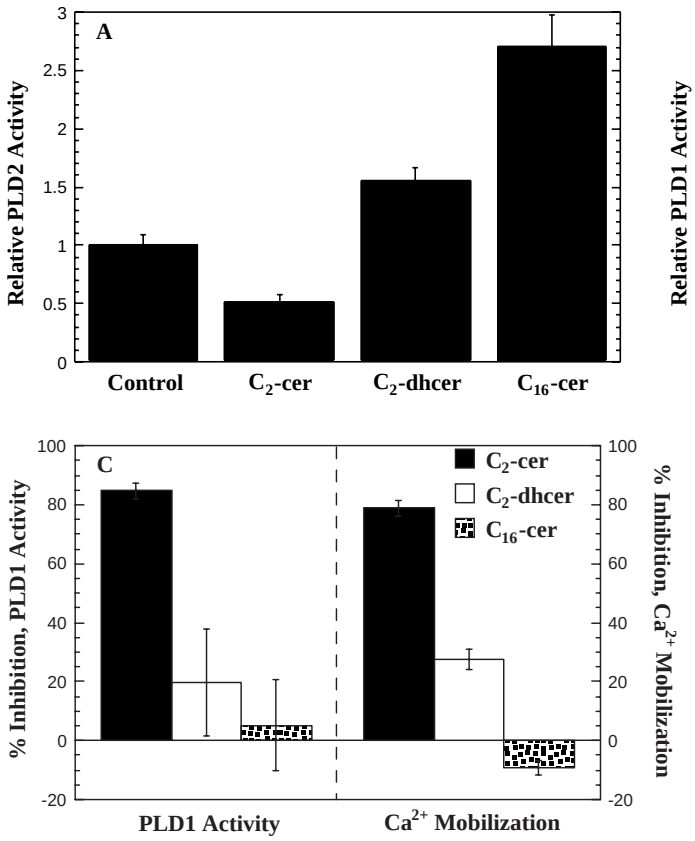

Fig. 6. Ceramides differentially inhibit in vitro enzyme activity of activated PLD2 (A) and activated PLD1 (B). Representative absolute activities for enzymes and control vesicles were: 50 pmole PC hydrolyzed/30 minutes at 37°C for PLD2 plus activators, and 250 pmole PC hydrolyzed/30 minutes at 37°C for PLD1 plus activators. Results are normalized with respect to control vesicles, and represent the average values for at least four experiments with error bars indicating s.e.m. (C) Percent inhibition of PLD1 activity by ceramides compared with percent inhibition of Ca^{2+} mobilization in RBL cells. Filled bars, C_2-cer; open bars, C_2-dhcer; dot-filled bars C_{16}-cer.

PLD1 in the absence of these activators is also inhibited by C_2-cer to a similar extent (~90% inhibition; A.G., unpublished). As represented in Fig. 6C, the pattern of inhibition of activated PLD1 by the various ceramides is highly correlated with the inhibition profile for Ca^{2+} mobilization in RBL cells, consistent with the butanol results that implicate activated PLD as being upstream of antigen-stimulated Ca^{2+} release from stores in RBL cells. Together, these results provide evidence that short-chain ceramides exert primary effects on signaling by inhibition of PLD1 via perturbation of membrane structure.

Discussion

Ceramides have been found to influence a wide variety of biological processes (Kolesnick and Kronke, 1998; Hannun and Obeid, 2002) but specific mechanisms for these diverse effects have been difficult to elucidate. Among the complications in these efforts are the observations that short-chain ceramides added exogenously often do not mimic the effect of sphingomyelinase, which usually produces long-chain ceramides when acting on biological membranes (Hofmann and Dixit, 1998). Although several specific protein targets for ceramides have been identified (Mathias et al., 1991; Dobrowsky and Hannun, 1992; Huwiler et al., 1996), binding domains for ceramides on these have been difficult to identify, and the roles of these specific targets in the plethora of biological processes affected by ceramides are still poorly understood. The lipophilic nature of short-chain ceramides, as well as long-chain ceramides, makes it highly likely that these amphiphiles alter biological processes as components of lipid bilayers (Venkataraman and Futerman, 2000; van Blitterswijk et al., 2003), and results indicate that short-chain ceramides inhibit signaling in platelets by decreasing membrane stability (Simon and Gear, 1998).

Our findings are consistent with these latter results and support the hypothesis that short-chain ceramides inhibit signaling by disruption of cholesterol-dependent lipid order in biological membranes. In addition, our results indicate that PLD is a particularly sensitive molecular target for these order-disrupting effects, and that this may be relevant to the effects of short-chain ceramides in many biological processes. PLD1 and PLD2 have been implicated in a wide variety of signaling and vesicle transport events (Exton, 1999), including antigen-stimulated degranulation in RBL mast cells (Brown et al., 1998; Way et al., 2000; Choi et al., 2002), but the lack of potent pharmacological agents and useful dominant-negative constructs have made it difficult to define the roles for PLDs in many of these studies. ARF-dependent PLD activation has been implicated in COPI-mediated coated vesicle budding from Golgi cisternae (Ktistakis et al., 1995; Chen et al., 1997), and the inhibition of membrane protein maturation in the Golgi by C_6-cer (Rosenwald and Pagano, 1993) may also reflect a role for ordered lipids in this process. Additionally, PA has been shown to be an important co-factor for phosphatidylinositol 4-phosphate 5-kinase [PI(4)P5K] α in the synthesis of PIP_2 (Honda et al., 1999), and this provides one example of a PLD-dependent signaling pathway that is potentially relevant to many biological processes.

Cockcroft and colleagues have described evidence for a role for ARF-dependent activation of PLD in the regulation of PIP_2

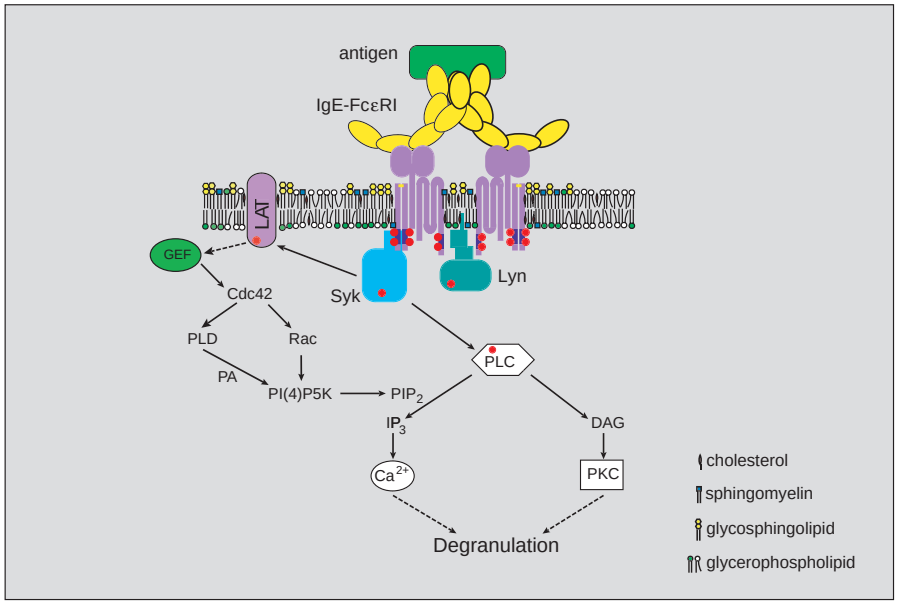

Fig. 7. Proposed signaling scheme shows PLD involvement in stimulated PIP_2 production upstream of IP_3-mediated Ca^{2+} release from stores in RBL cells. See text for details. Also indicated are the minimal steps required for FcεRI-mediated exocytosis: PLCγ activation leading to sustained Ca^{2+} mobilization initiated by IP_3-mediated Ca^{2+} release from stores, together with diacylglycerol (DAG)-mediated activation of PKC.

synthesis in stimulated RBL cell exocytosis (Way et al., 2000) and ruffling (O'Luanaigh et al., 2002). Consistent with these studies, our recent characterization of a mutant RBL cell line that is defective in antigen-stimulated Cdc42 and Rac activation (Field et al., 2000) led us to postulate that Rho-family-dependent synthesis of PIP_2 is necessary for sustained IP_3 production and Ca^{2+} mobilization that leads to exocytosis in these cells (Hong-Geller et al., 2001). The model in Fig. 7 summarizes this hypothesis, and indicates how activation of PLD could play an important role in Ca^{2+} mobilization via stimulated PIP_2 synthesis. In this model, Syk tyrosine kinase-dependent phosphorylation of the adaptor protein LAT leads to the activation of a guanine nucleotide exchange factor (GEF) for Cdc42 that promotes GTP binding and leads to the production of PA and PIP_2. Consistent with this model, the mutant RBL cells defective in Cdc42 activation are also defective in antigen-stimulated PA production (Field et al., 2000).

Our fluorescence anisotropy measurements of lipid order in plasma membrane vesicles from the RBL cells show that biologically active short-chain ceramides decrease cholesterol-dependent lipid order in these membranes in proportion to the amount of these ceramides added up to 25 mole percent, the highest value tested (Fig. 2). In these measurements, C_6-cer is 4-5 times more effective than C_2-cer on a per mole basis, and this is consistent with the ~fourfold greater hydrophobic partition coefficient expected for the longer acyl chain of this ceramide (Tanford, 1980). We used FRET between lipid raft components as a measure of the amount of lipid order in intact cells, and we found that C_2-cer and C_6-cer reduced FRET between FcεRI and Thy-1 in proportion to their expected membrane partition coefficient. Although FcεRI does not fractionate with lipid raft components in sucrose gradients in the absence of crosslinking (Field et al., 1995; Field et al., 1997), it has a detectable association with lipid rafts in intact cells, indicated by its co-redistribution with crosslinked Thy-1 (D.H., unpublished) (Holowka and Baird, 2001). Similar FRET results for Alexa488-anti-GD_{1b} as the donor probe and Cy3-anti-Thy-1 as the acceptor, together with the lack of detectable FRET with non-raft donors anti-transferrin receptor mAb or anti-CD43 mAb, support these conclusions. For both the anisotropy and FRET measurements, C_2-dhcer has little or no effect on lipid order, and this correlates with the lack of biological activity of this ceramide, despite its very similar chemical structure to C_2-cer (Fig. 1).

The physical basis for this dramatic difference in effects on lipid order and biological activity for C_2-cer and C_2-dhcer is not fully understood, but it has been suggested (Simon and Gear, 1998) that more-favorable hydrogen bonding for C_2-cer due to the allylic double bond (Fig. 1) cause formation of two rigid planes of H-bonded atoms that results in a bulkier, more-rigid headgroup structure. This configuration would yield a cone-shaped lipid that could disrupt the packing of saturated phospholipids and cholesterol in a liquid-ordered bilayer. The propensity for these uncharged, short-chain ceramides to flip rapidly from the outer to inner leaflet of the plasma membrane is high (Bai and Pagano, 1997), and this is probably important for their effects on enzymes such as PLD1, which act at the inner leaflet. Thus, the unique physical properties of C_2-cer and C_6-cer make them particularly effective as membrane perturbants with substantial functional consequences. In contrast to these, long-chain ceramides such as C_{16}-cer are more cylindrical in shape and should pack well into a liquid-ordered membrane, much like sphingomyelin. Thus, long-chain ceramides naturally produced by the action of sphingomyelinases would not be expected to cause the same functional effects as short-chain ceramides in situations where perturbation of lipid order is the basis for these effects.

As summarized in Fig. 6C, the inhibitory effects of C_2-cer on antigen-stimulated Ca^{2+} mobilization and on in vitro PLD1 activity are not observed with C_2-dhcer or C_{16}-cer, and thus conform to the criteria for functional inhibition by membrane perturbation. For measurement of Ca^{2+} responses, it is clear that the concentration of C_2-cer used (32 μM) to obtain ~60-80% inhibition in the presence or absence of extracellular Ca^{2+} does not cause significant leakiness in the cells, as judged by the minimal increase in indo-1 fluorescence observed upon addition of this ceramide (Fig. 4A,D). Furthermore, the rapid onset of changes in FRET (Fig. 3A) and the short incubation time with C_2-cer prior to antigen stimulation (Fig. 4A,D) make it unlikely that the effects observed are due to a metabolic derivative of this ceramide. Although we do not have a direct measure of the incorporation of C_2-cer, C_2-dhcer and C_{16}-cer into the cells, all three exhibit significant effects on the Ca^{2+} response, and the previous

study by Simon and Gear showed that radiolabeled C_2-cer and C_2-dhcer both undergo efficient incorporation into platelets at similar concentrations of ceramides as those used in our experiments (Simon and Gear, 1998). Furthermore, the transient increase in intracellular Ca^{2+} that we observe upon addition of C_{16}-cer (Fig. 4C) and the small potentiation of antigen-stimulated Ca^{2+} mobilization observed due to this ceramide (Fig. 4C,E) indicates that it is also incorporated significantly into the cells.

Our evidence for the involvement of PLD1 activation in the initiation of antigen-stimulated Ca^{2+} mobilization is based on the differential sensitivity of Ca^{2+} mobilization to inhibition by n-butanol but not by t-butanol (Fig. 5), and on its sensitivity to inhibition by ceramides with the same structural discrimination as PLD1 activity in in vitro assays (Fig. 6C). By contrast, PLD2 activity under similar assay conditions is less sensitive to inhibition by C_2-cer, and shows marked enhancement by C_2-dhcer and C_{16}-cer (Fig. 6A). This pattern is less consistent with that predicted for inhibition by disruption of lipid order. However, it is possible that these ceramides have multiple effects on the activity of PLD2 under these in vitro assay conditions, such that the net effects include a component of inhibition that is due to disruption of lipid order. Using a mixed-micelle assay system, it has been found that PLD2 activity is effectively inhibited by C_2-cer (Singh et al., 2001). However, they also found that C_{16}-cer inhibits PLD2 activity in this assay; it is unclear to what extent these effects depend on the presence of detergents in their assay. Both PLD1 and PLD2 have been implicated in antigen-stimulated degranulation in RBL cells (Choi et al., 2002), and PLD1 has been shown to be recruited to the plasma membrane due to antigen stimulation (Brown et al., 1998), whereas PLD2 is constitutively localized there (Choi et al., 2002). Our results are most consistent with a role for PLD1 in the initiation of antigen-stimulated Ca^{2+} mobilization, but we cannot exclude a role for PLD2 in this process as well. As depicted in Fig. 7, we suggest that generation of PA by activated PLD is involved in stimulated PIP_2 synthesis, which provides a pool of this substrate for sustained production of IP_3 by PLCγ, a requirement for stimulated exocytosis (Beaven and Kassessinoff, 1997; Scharenburg and Kinet, 1998). Other roles for PA, such as a direct effect on PLCγ activity (Jones and Carpenter, 1993), cannot be excluded by our results.

In the study by Nakamura et al., inhibition of antigen-stimulated Ca^{2+} mobilization and degranulation by C_2-cer was found to correlate with inhibition of Ca^{2+} influx (Nakamura et al., 1996). In a separate study, Mathes et al. reported that C_2-cer inhibits the calcium release-activated calcium current (I_{CRAC}) activated by IP_3 in patch-clamped RBL cells (Mathes et al., 1998). These studies indicate that C_2-cer can inhibit store-operated Ca^{2+} influx, but it is unclear whether this effect of C_2-cer is due to disruption of lipid order and/or inhibition of PLD. Comparing the effects of butanols, C_2-dhcer and C_{16}-cer on IP_3-activated I_{CRAC} should help to answer this question. Such comparisons may have general utility in providing evidence for the involvement of PLD in diverse cellular processes.

Although PLD activity is frequently reported to be a target for inhibition by short-chain ceramides, the mechanism for this effect remains incompletely defined. Our results indicate that this inhibition correlates with disruption of lipid order, and Powner et al. recently reported that PLD1 in stimulated RBL cells is Triton X-100 insoluble, suggesting possible association with lipid rafts (Powner et al., 2002). In our in vitro assays, PLD1 activity is sensitive to C_2-cer in the absence of activators ARF1 and PKCβII, indicating that activation per se is not the ceramide-sensitive step. By analogy to the importance of membrane defects for the catalytic activity of PLA_2 (Burack and Biltonen, 1994), we propose that PLD might also depend on membrane defects for its catalytic activity. Boundary regions between more-fluid and less-fluid membrane domains have been shown to provide defects for PLA_2 (Burack et al., 1997) and, similarly, the interface between liquid-ordered and fluid regions of biological membranes could provide such defects for PLD catalytic activity. Furthermore, the study by Ge et al. indicates that ARF GTPases can contribute to defect formation (Ge et al., 2001). In this context, short-chain ceramides would inhibit PLD activity by reducing order and thereby reducing potential interfaces. Future systematic studies with purified PLD isoforms and model membranes of well-defined composition will be necessary to test this hypothesis. Regardless of the molecular mechanism for these effects on PLD, our present results show that long- and short-chain ceramides, by virtue of their differential effects on membrane lipid order, can serve as useful probes for evaluating the role of plasma membrane structure and heterogeneity in cellular signaling.

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