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ARTICLE

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Ceramides perturb the structure of phosphatidylcholine bilayers and modulate the activity of phospholipase A_2

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Abstract The effects of a series of ceramide analogs with acyl chain lengths of 2, 6, 8 and 16 on the structure of dipalmitoylphosphatidylcholine (DPPC) bilayers and cobra venom phospholipase A_2 (PL-A_2) activity were studied using ^{2}H-NMR and specific enzymatic assays. C_2-ceramide did not induce a significant effect on the structure of DPPC bilayers and did not alter PL-A_2 activity. C_6- and C_8-ceramides increased the ordering of the DPPC acyl chains, correlating with the inhibition of PL-A_2 activity which was probably due to the increased lateral surface pressure. The long-chain C_{16}-ceramide induced lateral phase separation of the bilayers into gel and liquid crystalline domains and activated PL-A_2, as does natural ceramide (Huang et al. 1996). Taken together, the results strongly suggest a correlation between membrane defects induced by ceramide analogs and their effects on phospholipase A_2 activity. Furthermore, the effects of short-chain ceramides on PL-A_2 are different from those of natural ceramide, indicating that the cell-permeable short-chain ceramide analogs, widely used to study the sphingomyelin-dependent cellular signal transduction pathway, may not completely mimic the natural product.

Key words Ceramides · ^{2}H-NMR · Membrane structure · PL-A_2

Abbreviations *DAG* diacylglycerol · *DPPC* dipalmitoylphosphatidylcholine · *DPPC-d_{62}* diperdeuteriopalmitoylphosphatidylcholine · *NBD-PC* 1-palmitoyl-2[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-phosphatidylcholine · *PL-A_2* phospholipase A_2 · *diC_8* 1,2-*sn*-dioctanoylglycerol · *diC_6* 1,2-*sn*-dihexanoylglycerol · *C_2-ceramide* N-acetylsphingosine · *C_6-ceramide* N-hexanoylsphingosine · *C_8-ceramide* N-octanoylsphingosine · *C_{16}-ceramide* N-palmitoylsphingosine

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Introduction

A recently discovered sphingomyelin-dependent pathway of transmembrane signal transduction involves a stimulus-induced production of ceramide as an intracellular second messenger (Kolesnick and Clegg 1988). This novel signaling pathway is triggered by several extracellular stimuli, including tumor necrosis factor-α (Kim et al. 1991), γ-interferon (Kim et al. 1991), and interleukin-1 (Mathias et al. 1993). Ceramide is involved in cell proliferation (Olivera et al. 1992; Sasaki et al. 1995; Auge et al. 1996), cell differentiation (Kim et al. 1991; Riboni et al. 1995) and apoptosis (see Obeid and Hannun, 1995, for a review). Ceramide is implicated in the modulation of several signaling pathways including ceramide-activated protein phosphatase (Dobrowsky et al. 1992; Dobrowsky and Hannun 1993; Wolff et al. 1994), protein phosphorylation via a ceramide-activated protein kinase (Kolesnick et al. 1990; Mathias et al. 1991; Yao et al. 1995), inhibition of phospholipase D (Venable et al. 1996; Abousalham et al. 1997), NF-κB activation (Machleidt et al. 1994; Schutze et al. 1994), *c-myc* downregulation (Kim et al. 1991), and other effects (see Hannun 1994; Kolesnick and Golde 1994; Obeid and Hannun 1995 for reviews). Ceramide is also one of the main components of the uppermost layer of the mammalian epidermis, the stratum corneum (Yardley and Summerly 1981; Lampe et al. 1983).

Ceramide is composed of a fatty acyl chain esterified to sphingosine. Natural ceramide from brain tissue consists of a mixture of species with long chain fatty acids (≥16 carbons) of various lengths; however, in brain tissue about 85% are saturated fatty acids (manufacturer information). Because the long-chain saturated fatty acids make natural ceramide impermeable to the cell, short-chain cell-permeable ceramide analogs are generally used for studying intracellular effects of this second messenger. Most commonly used ceramide analogs include, N-acetylsphingosine (C_2-ceramide), N-hexanoylsphingosine (C_6-ceramide), and N-octanoylsphingosine (C_8-ceramide); however, the difference in structure between the short and long chain ceramides makes

it likely that they will induce different perturbation in lipid membrane structure and, consequently, different biological effects.

Membrane structure has been demonstrated to modulate the activities of a number of membrane-associated enzymes, including protein kinase C (see Zidovetzki and Lester 1992 for a review), CTP: phosphocholine cytidyltransferase (Arnold & Cornell 1996), and phospholipase A_2 (PL-A_2) (see Burack and Biltonen 1994; Zidovetzki 1997 for reviews). In our previous studies we have demonstrated that the second messenger, diacylglycerol (DAG), modulates the activity of phospholipase A_2 and correlated this effect with the DAG-induced perturbations in lipid bilayer structure (De Boeck and Zidovetzki 1989, 1992; Zidovetzki et al. 1992). As DAGs and ceramides are structurally similar, it could be expected that they have similar effects on membrane structure and thereby modulate PL-A_2 in a similar manner. Indeed, we have found that, like dipalmitin, ceramide extracted from bovine brain activates cobra venom PL-A_2 by inducing lateral phase separation of gel and liquid-crystalline domains in dipalmitoylphosphatidylcholine (DPPC) lipid bilayers (Huang et al. 1996).

In the present paper we investigated the effects of ceramide analogs C_2-, C_6-, C_8- and C_{16}-ceramides on the structure of DPPC bilayers and the enzymatic activity of cobra venom PL-A_2, using ^{2}H-NMR and specific enzymatic assays. We have found that ceramide analogs induce perturbations into membrane structure which correlate with their modulation of PL-A_2 activity. Moreover, both the effects of the short-chain ceramides on bilayer structure and PL-A_2 activity differ from those of natural ceramide.

Materials and methods

Materials

Diperdeuteriopalmitoylphosphatidylcholine (DPPC-d_{62}), dipalmitoylphosphatidylcholine (DPPC), and 1-palmitoyl-2[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-phosphatidylcholine (NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL), NBD-hexanoic acid was from Molecular Probes (Eugene, OR). C_2-, C_6-, C_8- and C_{16}-ceramides were from Biomol (Plymouth Meeting, PA). Phospholipase A_2 (*Naja mocambique mocambique*) was obtained from Sigma Chemical Co. (St. Louis, MO).

^{2}H-NMR spectroscopy

Multilamellar lipid dispersions were prepared by dissolving the DPPC-d_{62} or the DPPC-d_{62}/ceramide mixture in chloroform. The solvent was then evaporated with a stream of dry nitrogen, and the samples were placed under a vacuum (<1 mtorr) for 8 h. The dried lipids were hydrated with 250 mM Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] (pH 8.0) buffer solution with 10 mM Ca^{2+}, prepared in ^{2}H-depleted H_2O (Sigma). The samples were always fully hydrated and were typically 1:10 (w/w) in lipid to water. A uniform lipid suspension was obtained by five freeze-thaw cycles (Westman et al. 1982; Mayer et al. 1985). Lipid extraction measurements showed quantitative partition of the short-chain ceramides into lipids (unpublished observations, and Bielawska and Obeid, personal communication).

^{2}H-NMR spectra were acquired at 11.74 T (corresponding to 76.78 MHz ^{2}H frequency) on a General Electric GN500 spectrometer using a high-power, broad line ^{2}H-NMR probe (Doty Scientific, Columbia, SC) and the standard quadrupole echo sequence (Davis et al. 1976). The spectral width was 500 kHz, refocusing time 60 μs, 90° pulse 3.5 μs and typically 16,000 scans per spectrum.

Lipid phase composition analyses

The spectra exhibiting the coexistence of liquid-crystalline and gel phase were analyzed by spectral subtraction similar to the method of Morrow and Davis (1988). All spectra were normalized to contain the same spectral area. The amount of subtraction necessary to remove a component from the multiple-phase spectrum indicated the fraction of lipid occupying the removed phase. Pure-phase spectra for liquid-crystalline phase were obtained from samples exhibiting only L_α phase, and pure gel phase spectra were obtained from samples exhibiting only the L_β component. In order to compensate for changes in quadrupole splittings of L_α phase among samples, pure spectra could be modulated by a width constant (keeping total spectral area fixed) to match the spectrum being analyzed. This was accomplished by multiplying the frequency axis of the pure spectra components by an expansion factor (E) until it matched the corresponding component of the spectrum being analyzed. To compensate for the change in area that this introduces, intensity in the pure spectrum was divided by the same factor (E).

Phospholipase A_2 assay

The lipids for the PL-A_2 assays were prepared identically to the NMR samples, except non-deuterated DPPC was used at 5 mM, and NBD-PC was added as a fluorescent probe with molar ratios of 10^{-5}–10^{-3}:1 to DPPC. Ceramide concentrations ranged from 0 to 25 mol%.

Phospholipase A_2 activity was determined according to the method of Stubbs et al. (1988) with the following modifications: the lipids were hydrated in 250 mM Tris buffer with 10 mM Ca^{2+}, and phospholipase A_2 (0.5 μg) was added to start the reaction. The total volume of reaction mixture was 0.5 mL and the reaction time was 10 min at 45 °C. Kinetic experiments established that under these conditions the reaction progressed linearly, and the amount of hydrolyzed lipids did not exceed 10% of the total. The reaction was stopped by the addition of 1.875 mL of chloroform-methanol (1:2, v/v), and the mixture was separated into aqueous and non-aqueous phases by the addition of 625 μL each of chloroform and water. An aliquot

of the aqueous phase was removed, and the fluorescence intensity at 530 nm was determined with 470 nm excitation in a Perkin-Elmer MPF-66 spectrofluorometer. A standard curve was obtained using known quantities of NBD-hexanoic acid. Background fluorescence was determined from the identical measurement in the absence of PL-A_2.

Results

The spectra of DPPC-d_{62} in the absence or presence of 25 mol% C_2-, C_6-, C_8-, or C_{16}-ceramides at 45 °C are shown in Fig. 1. The spectrum of the control DPPC-d_{62} sample exhibits the typical shape for the liquid crystalline phase of a lipid bilayer at 45 °C (Fig. 1A). An ^{2}H-NMR spectrum of DPPC-d_{62} is the superposition of axially averaged powder patterns that corespond to the deuterons at different positions along the lipid acyl chains. The quadrupole splittings ($\Delta\nu^i$), the distances between the pairs of symmetric peaks, are related to the order parameters S^i_{CD} of the various CD_2 acyl chains segments and the terminal CD_3 segment according to the equation:

$$\Delta\nu^i = 3/4\ (e^2Qq/h)\ S^i_{CD}$$

where e^2Qq/h is the quadrupole coupling constant, 167 kHz (Burnett and Muller 1971). Addition of 25 mol% C_{16}-ceramide results in the appearance of a broad component in the ^{2}H-NMR spectrum, superimposed with a spectrum that corresponds to the L_α phase (Fig. 1B). This additional component is characteristic of lipids in the gel phase. The coexistence of gel-like and liquid crystalline phases in the spectrum indicates that C_{16}-ceramide induces lateral phase separation in the DPPC-d_{62} bilayers. An analysis of the spectrum in Fig. 1B showed that 41 ± 2% of the spectral intensity was associated with the gel phase. Lateral phase separation was also observed in our previous studies on natural ceramide (Huang et al. 1996) and long-chain saturated DAGs (De Boeck and Zidovetzki 1989, 1992). In the cases of C_2-, C_6-, or C_8-ceramide, the shape of each DPPC-d_{62} spectrum was similar to DPPC-d_{62} alone (Fig. 1C, D, E), indicating that the phase of the DPPC bilayers at 45 °C was not affected by the addition of the short chain ceramides.

The effects of ceramide analogs on the order parameters (S^i_{CD}) of the DPPC-d_{62} acyl chains are summarized in Fig. 2. The peak assignment of the ^{2}H-NMR spectra was done according to Davis, (1979). C_2-ceramide did not show a significant effect on the order parameters of DPPC-d_{62}. Both C_6- and C_8-ceramides increased the order parameters of DPPC-d_{62} (Fig. 2). The effects were most prominent in the regions adjacent to the phospholipid headgroups (segments 2–9) and gradually declined toward the end of the acyl chain (Fig. 2). C_{16}-ceramide also increased the order parameter of DPPC-d_{62}, however, this effect was more prominent for the segments in the bilayer interior (Fig. 2). Similar preferential ordering of the bilayer interior was observed by long-chained DAGs (Goldberg et al. 1994; Goldberg and Zidovetzki 1997), phosphatidylethanolamine, cholesterol,

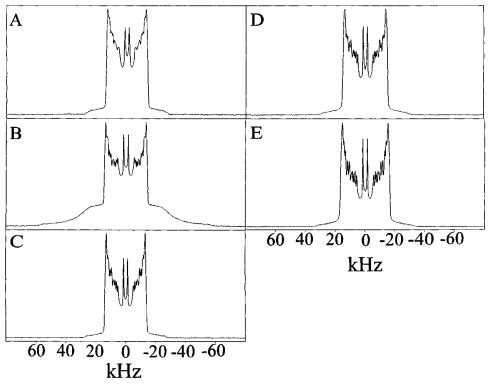

Fig. 1A–E ^{2}H-NMR spectra of DPPC-d_{62} in the presence or absence of 25 mol% ceramides at 45 °C. **A** DPPC-d_{62}; **B** DPPC-d_{62} with C_{16}-ceramide; **C** DPPC-d_{62} with C_2-ceramide; **D** DPPC-d_{62} with C_6-ceramide; **E** DPPC-d_{62} with C_8-ceramide

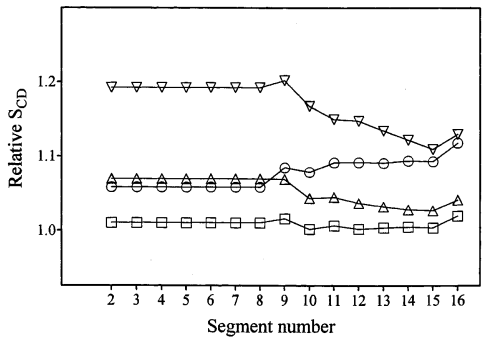

Fig. 2 Effect of 25 mol% ceramides on the order parameter profile of DPPC-d_{62} bilayers at 45 °C. Relative S_{CD} is the ratio of a segmental order parameter in the presence of ceramide to the corresponding value for DPPC-d_{62} alone. The experimental errors are within the symbols. □ DPPC-d_{62} with C_2-ceramide; △ DPPC-d_{62} with C_6-ceramide; ▽ DPPC-d_{62} with C_8-ceramide; ○ DPPC-d_{62} with C_{16}-ceramide

or decreased temperature (Lafleur et al. 1990). The effect is probably due to the lower sensitivity of the tightly packed acyl chains near the bilayer/water interface to these perturbants (Cheng et al. 1994).

The values of the quadrupole splittings of the plateau regions ($\Delta\nu_{plateau}$) provide information on the effects of ceramides on the surface area per lipid molecule in the bilayer. The surface area per phospholipid in the bilayer (A) can be calculated from the surface density, σ, which is the ratio of the surface area per phospholipid in the bilayer (A) to that in the crystal ($A_0 = 40.8$ Å^2) (De Young and Dill 1988). The surface density per phospholipid molecule (σ) is related to the quadrupole splittings of the outermost peaks by the following equation (De Young and Dill 1988):

$$\sigma = (16/9)\ [\Delta\nu_{plateau}/167\ \text{kHz}] + (1/3)$$

The assumptions involved in this treatment are given in De Young and Dill (1988). Figure 3 shows the effect of ceramides on the surface area per phospholipid molecule in the bilayer. All ceramides, except C_2-ceramide, decreased the lateral surface area per DPPC-d_{62} molecule with the greatest effect exhibited by C_8-ceramide.

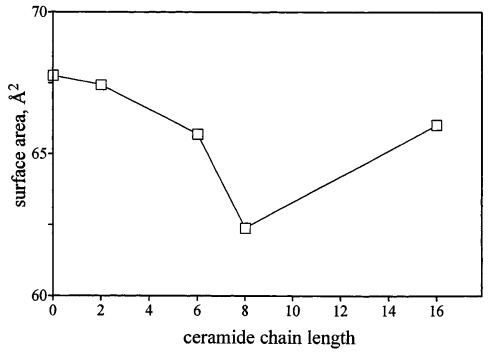

Fig. 3 Dependence of the area per phospholipid molecule in the chain lengths of added 25 mol% ceramides at 45 °C. The leftmost point corresponds to DPPC-d_{62} in the absence of ceramides

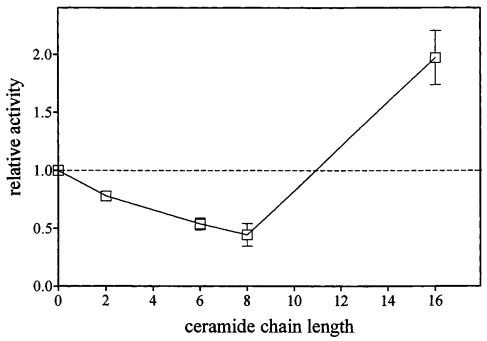

Fig. 5 Dependence of the activity of PL-A_2 on the ceramide side-chain length at 10 mol% of ceramides. The leftmost point corresponds to the relative activity of PL-A_2 in the absence of ceramides

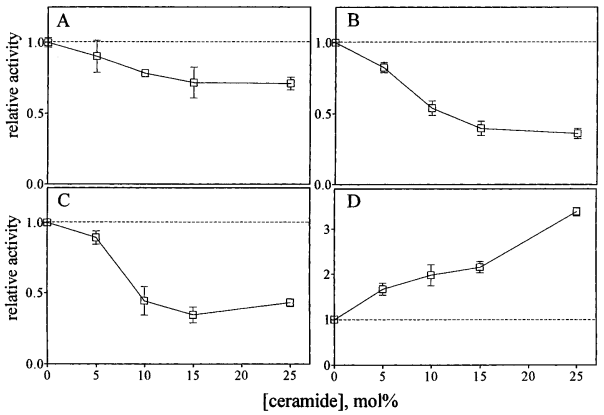

Fig. 4A–D Dependence of PL-A_2 activity on the concentration of ceramides in DPPC bilayers. The assays were performed in triplicates at 45 °C, for 10 min. The error bars correspond to SEM. **A** C_2-ceramide; **B** C_6-ceramide; **C** C_8-ceramide; **D** C_{16}-ceramide

Because perturbations in the lipid bilayer are known to affect the enzymatic activity of phospholipase A_2, we examined the effects of ceramides on the activity of cobra venom PL-A_2 with DPPC as the substrate at 45 °C. The results are summarized in Fig. 4. C_2-ceramide induced a decrease in PL-A_2 activity, which was 29±4% at 25 mol% (Fig. 4A), and this decrease was probably due to the substrate-dilution effect. C_6- and C_8-ceramides significantly inhibited PL-A_2 already at 10 mol% and reached a maximum inhibition (64±4%, 66±6%) at 25 and 15 mol%, respectively (Fig. 4B, C). In contrast to the short chain ceramides, C_{16}-ceramide increased the activity of cobra venom PL-A_2 in a concentration-dependent manner; the increase reached ~1.5 fold of the control by 5 mol% and ~3 fold at 25 mol% (Fig. 4C). The dependence of PL-A_2 activity on fatty acid chain length in the presence of 10 mol% ceramides is shown in Fig. 5.

Approximately 22±3% inhibition of PL-A_2 was observed in the presence of 10 mol% C_2-ceramide, whereas 46±5% and 56±10% inhibition was achieved by C_6-ceramide and C_8-ceramide, respectively (Fig. 5). The long-chain C_{16}-ceramide, however, induced ~2 fold activation of the enzyme (Fig. 5).

Discussion

The present work was initiated to examine the effects of ceramides on PL-A_2 activity and to elucidate the mechanisms of these effects. The previous studies in our laboratory on DAG- or natural ceramide-induced membrane perturbations have established a correlation between the specific types of membrane perturbations induced by DAGs or natural ceramide and the effects of these reagents on PL-A_2 activity (De Boeck and Zidovetzki 1989, 1992; Zidovetzki et al. 1992; Huang et al. 1996).

Short-chain C_2-ceramide did not affect DPPC bilayer structure nor PL-A_2 activity. With C_6- or C_8-ceramide, we observed an increase of the order parameters of DPPC acyl side chains, the effect being most pronounced in the regions adjacent to phospholipid headgroups (Fig. 2). A similar type of perturbation was observed in the cases of two short-chain DAGs, 1,2-*sn*-dihexanoylglycerol (diC_6), or 1,2-*sn*-dioctanoylglycerol (diC_8), (De Boeck and Zidovetzki 1992). These DAGs induced a transverse perturbation into DPPC bilayers, which resulted in an increase of the order parameters of DPPC acyl chains in the regions near the phospholipid headgroups, but a decrease of the ordering toward the middle of the bilayer resulting from the free volume created by these DAGs. The transverse character of the bilayer perturbations induced by C_6- or C_8-ceramides was less pronounced than for the corresponding DAGs, which was probably due to the presence of one short and one long chain in the former case. The increase in the order parameters of DPPC acyl chains near the phospholipid headgroups corresponds to the decrease in the lateral surface area per phospholipid molecule (Fig. 3), reflecting an increase of lateral surface pressure in DPPC bilayers. The surface pressure used here is defined at the level of the top of the lipid chains below the glycerol backbone. Such an increased surface pressure may prevent dissociation of the substrate molecules (DPPC) from the bulk lipids or, alternatively, prevent the penetration of PL-A_2 into the bilayers (Zidovetzki et al. 1992). Indeed, inhibitory effects on the activity of PL-A_2 were observed upon the addition of C_6-, or C_8-ceramide to DPPC bilayers (Fig. 4). Substitution of bovine liver PC for DPPC modified the effects of the short-chain ceramides: the ceramide-in-

duced increase of the order parameters in the region adjacent to the phospholipid headgroups was smaller, whereas the disordering of the bilayer interior was larger than with DPPC. Correspondingly, both C_6- and C_8-ceramide slightly activated PL-A_2 in this system (unpublished observations). Thus the net result of the transverse bilayer perturbation in the case of bovine liver PC substrate is increased probability for a single phospholipid molecule to dissociate from the bulk phospholipids and bind to the PL-A_2 catalytic side, leading to the observed increase of the enzymatic activity. Similar results were obtained by us previously with the short-chain DAGs (Zidovetzki et al. 1992).

The long-chain C_{16}-ceramide also decreases DPPC surface area (Fig. 3); however the PL-A_2-inhibiting effect of this decrease is insignificant comparing with the strong PL-A_2-activating effect of lateral phase separation of the lipid bilayers into the domains of gel-like or liquid crystalline phases, induced by this ceramide. Similarly to the DPPC system, C_{16}-ceramide-induced lateral phase separation and PL-A_2 activation were also observed with bovine liver PC (unpublished observations) eliminating the proximity to gel to liquid crystalline phase transition temperatures as the cause of the observed results. Activation of PL-A_2 by long-chain amphiphilic molecules that induce lateral phase separation in the phospholipid substrate was observed in the cases of natural ceramide (Huang et al. 1996), saturated fatty acid (Jain et al. 1989), long-chain saturated DAGs (Zidovetzki et al. 1992; Bell et al. 1995), saturated lysophospholipid (Burack et al. 1993), and triglycerides (Bell et al. 1995). Jain and Zakim (1987) suggested that lateral phase separation induces the formation of long-lived boundary regions (defects) between the gel and liquid crystalline phases, wherein the association between the substrates is weakened facilitating the binding of the lipid substrate to the catalytic site of PL-A_2. Similar interpretation of the observed activation of pig pancreatic PL-A_2 by PC-cholate mixtures was used by Gheriani-Gruszka et al. (1988). The effects of lateral phase separation on the activities of the membrane-associated enzymes have been recently reviewed (Zidovetzki 1997). Presence of laterally separated domains was also reported in biological membranes: (Karnovsky et al. 1982; Schroeder 1983; Yechiel and Edidin 1987; Wolf et al. 1988; Mateo et al. 1991), and it has been suggested that lateral heterogeneity of the biological membranes regulates activities of exogenously added PL-A_2s (Shukla and Hanahan 1982; Purdon et al. 1987; Wang et al. 1987).

Among the synthetic ceramides investigated in the present paper, only C_{16}-ceramide, which is not cell-permeable nor used in in vivo studies, mimics natural ceramide. Cell-permeable short-chain ceramide analogs have a different effect on the lipid bilayer, resulting in a significant inhibition of PL-A_2 activity which is opposite to the effect of natural ceramide (Huang et al. 1996). Thus, caution should be exercised in the use of the short-chain ceramides in cellular studies as a substitute for the natural ceramide.

The present work, combined with our previous studies, strongly suggests a correlation of the membrane perturbations induced by the lipid-derived second messengers, DAG and ceramide, with their modulations of PL-A_2 activity. Also, our studies of C_{16}-ceramide and natural ceramide as activators of PL-A_2 propose an additional biological effect of ceramide and indicate possible cross-talk between the PL-A_2 and sphingomyelinase signal transduction pathways.

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