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Ceramides Modulate Protein Kinase C Activity and Perturb the Structure of Phosphatidylcholine/Phosphatidylserine Bilayers

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ABSTRACT We studied the effects of natural ceramide and a series of ceramide analogs with different acyl chain lengths on the activity of rat brain protein kinase C (PKC) and on the structure of bovine liver phosphatidylcholine (BLPC)/dipalmitoylphosphatidylcholine (DPPC)/dipalmitoylphosphatidylserine (DPPS) (3:1:1 molar ratio) bilayers using ^{2}H-NMR and specific enzymatic assays in the absence or presence of 7.5 mol % diolein (DO). Only a slight activation of PKC was observed upon addition of the short-chain ceramide analogs (C_2-, C_6-, or C_8-ceramide); natural ceramide or C_{16}-ceramide had no effect. In the presence of 7.5 mol % DO, natural ceramide and C_{16}-ceramide analog slightly attenuated DO-enhanced PKC activity. ^{2}H-NMR results demonstrated that natural ceramide and C_{16}-ceramide induced lateral phase separation of gel-like and liquid crystalline domains in the bilayers; however, this type of membrane perturbation has no direct effect on PKC activity. The addition of both short-chain ceramide analogs and DO had a synergistic effect in activating PKC, with maximum activity observed with 20 mol % C_6-ceramide and 15 mol % DO. Further increases in C_6-ceramide and/or DO concentrations led to decreased PKC activity. A detailed ^{2}H-NMR investigation of the combined effects of C_6-ceramide and DO on lipid bilayer structure showed a synergistic effect of these two reagents to increase membrane tendency to adopt nonbilayer structures, resulting in the actual presence of such structures in samples exceeding 20 mol % ceramide and 15 mol % DO. Thus, the increased tendency to form nonbilayer lipid phases correlates with increased PKC activity, whereas the actual presence of such phases reduced the activity of the enzyme. Moreover, the results show that short-chain ceramide analogs, widely used to study cellular effects of ceramide, have biological effects that are not exhibited by natural ceramide.

INTRODUCTION

PKC is a serine/threonine kinase family involved in many cellular responses, such as cell proliferation (Bishop and Bell, 1988; Nishizuka, 1992; Murray et al., 1993), cell differentiation (William et al., 1990; Aihara et al., 1991; Nishizuka, 1992; Murray et al., 1993), and apoptosis (McConkey et al., 1989; Grant et al., 1992; Lucas and Sánchez-Margalet, 1995; Lavin et al., 1996). Through phosphorylation of other signaling protein intermediates, PKC is involved in a variety of intracellular signal transduction pathways, including the release of the active form of NF-κB (Buchner, 1995), and the activation of Raf-1 (Kolch et al., 1993; Carroll and May, 1994; Zou et al., 1996). To date, at least 12 PKC isozymes have been discovered (Dekker and Parker, 1994). Differences in tissue distribution, intracellular localization, and cofactor requirements among PKC isozymes suggest the possibility that these isozymes may play distinct roles in various cellular responses and in the complex cellular signaling network (Hug and Sarre, 1993).

The activity of the conventional PKCs can be modulated by lipophilic molecules, such as fatty acids (Lester, 1990; Hardy et al., 1994; Goldberg and Zidovetzki, 1997, 1998), cholesterol (Bolen and Sando, 1992), sphingosine (Hannun et al., 1986; Khan et al., 1991), and lysophosphatidic acid (Sando and Chertihin, 1996). A number of studies correlated the perturbation of membrane structure induced by these molecules with their effect on PKC activity (Epand and Lester, 1990; Bolen and Sando, 1992; Sando et al., 1992; Zidovetzki and Lester, 1992; Senisterra and Epand, 1993; Stubbs and Slater, 1996; Zidovetzki, 1997). The mechanism of action of the best-studied PKC cofactor, DAG, includes DAG-induced changes in the lipid membrane structure such as increased tendency to form nonbilayer lipid phases (Epand and Bottega, 1988; Goldberg et al., 1994), changes in the phospholipid headgroup conformation (Goldberg et al., 1995), and the presence of DAG-poor and DAG-rich lipid domains (Dibble et al., 1996; Hinderliter et al., 1997).

Another intracellular second messenger, ceramide, is the product of the hydrolysis of sphingomyelin by sphingomyelinase, initiated by extracellular stimuli including tumor necrosis factor-α (Kim et al., 1991; Kolesnick and Golde, 1994), and interleukin-1 (Mathias et al., 1993). Ceramide is implicated in cell proliferation (Olivera et al., 1992; Sasaki et al., 1995; Auge et al., 1996), cell differentiation (Riboni et al., 1995), and apoptosis (for a review, see Obeid and Hannun, 1995). In addition to activating its intracellular target, ceramide-activated protein kinase (Kolesnick and Hemer, 1990; Mathias et al., 1991; Yao et al., 1995), ceramide also modulates the activity of other enzymes in-

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Abbreviations used: PKC, protein kinase C; BLPC, bovine liver phosphatidylcholine; C_2-ceramide, N-acetylsphingosine; C_6-ceramide, N-hexanoylsphingosine; C_8-ceramide, N-octanoylsphingosine; C_{16}-ceramide, N-palmitoylsphingosine; DAG, diacylglycerol; DO, 1,2-dioleoyl-*sn*-glycerol; DPPC, dipalmitoylphosphatidylcholine; DPPC-d_{62}, diperdeuteriopalmitoylphosphatidylcholine; DPPS, dipalmitoylphosphatidylserine; PC, phosphatidylcholine; PS, phosphatidylserine.

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volved in signal transduction, such as phosphatase (Wolff et al., 1994), phospholipase A_2 (Huang et al., 1996, 1998), and phospholipase D (Venable et al., 1996; Abousalham et al., 1997). Ceramide is also involved in transducing signals toward the cell nucleus via NF-κB activation (Schütze et al., 1994), c-*myc* downregulation (Kim et al., 1991), and c-*jun*/AP-1 activation (Sawai et al., 1995). Ceramide has a structure similar to DAG and induces perturbation of the membrane structure similarly to long-chain DAGs (Huang et al., 1996, 1998; Veiga et al., 1999).

There are few studies and no consensus on the effect of ceramide on PKC. Lozano et al. (1994) reported that PKCζ is activated by natural ceramide in vitro and by treatment with sphingomyelinase in National Institutes of Health-3T3 fibroblasts with subsequent activation of NF-κB. In contrast, PKCα is inactivated by synthetic cell-permeable C_2- and C_6-ceramides in Molt-4 cells; in in vitro systems, C_6-ceramide did not affect PKCα activity (Lee et al., 1996). The translocation of PKCα, but not of PKCϵ, is blocked by treatment with sphingomyelinase or C_2-ceramide in mouse epidermal (HEL-37) and human skin fibroblast (SF 3155) cells (Jones and Murray, 1995). Sawai et al. (1997) suggested that C_2-ceramide induces cytosolic translocation of PKCδ and ϵ, which leads to apoptosis in human leukemia cell lines. Furthermore, Chmura et al. (1996) have suggested antagonistic roles of PKC and ceramide in apoptosis in murine B-cell lymphoma, WEHI-231.

In the present paper we examined the effect of natural ceramide, and its long-chain and short-chain cell-permeable analogs, on the membrane structure and the activity of rat brain PKC in the absence or presence of the PKC cofactor DO. We have found that short-chain ceramide analogs have qualitatively different effects from natural ceramide and C_{16}-ceramide on both PKC activity and lipid bilayer structure. The effects of the ceramides on PKC activity are dependent on chain length and can be correlated with ceramide-induced perturbations in the lipid bilayer structure.

MATERIALS AND METHODS

Materials

Synthetic C_2-ceramide, C_6-ceramide, C_8-ceramide, and C_{16}-ceramide were from Biomol (Plymouth Meeting, PA). BLPC, DPPC, DPPS, DPPC-d_{62}, and DO were purchased from Avanti Polar Lipids (Alabaster, AL). PKC extracted from rat brain was purchased from Calbiochem (La Jolla, CA). Histone 1 (Sigma type III-S), adenosine triphosphate, sphingomyelin extracted from bovine brain, and morpholinopropanesulfonic acid (MOPS) were obtained from Sigma Co. (St. Louis, MO).

Preparation of multilamellar vesicles for PKC assay

The multilamellar vesicles had a composition of 3:1:1 (molar ratios) BLPC/DPPC/DPPS with DO and/or ceramides added as mol % to the phospholipids. The vesicles were prepared by mixing all lipids in chloroform. The chloroform was then evaporated under a stream of dry nitrogen and the samples were placed under a vacuum (<1 mtorr) overnight. Each sample was fully hydrated with 75 μL 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 40 μM Ca^{2+}. The hydrated lipids were temperature-equilibrated at 30°C in a water bath.

Preparation of multilamellar vesicles for NMR spectroscopy

The compositions of lipids used in NMR measurements were identical to the PKC samples, except for the substitution of DPPC-d_{62} for DPPC. After mixing the lipids in chloroform, the solvent was evaporated with a stream of dry nitrogen. Lipids were then dissolved in benzene/methanol (20:1, volume ratio), quickly frozen with liquid nitrogen, and lyophilized under a vacuum (<1 mtorr) for at least 8 h. Samples were then fully hydrated with 50 μL 40 mM MOPS (pH 7.4), 65 mM $MgCl_2$ buffer prepared in ^{2}H-depleted H_2O (1:2 w/w lipid/buffer). In order to achieve equivalent saturation of Mg^{2+} binding to PS for NMR samples and PKC samples, NMR samples were prepared with 65 mM Mg^{2+} according to the Mg^{2+}-PS binding constant of 1.6×10^3 M and stoichiometry of 2:1 (PS/Mg^{2+}) (Portis et al., 1979). A uniform lipid suspension was obtained by five freeze-thaw cycles (Westman et al., 1982; Mayer et al., 1985). In some samples 1-palmitoyl, 2-oleoylPC (POPC), perdeuterated at the palmitic chain, was added substituting for 33% of BLPC content. The ^{2}H-NMR results obtained with these samples were identical to those obtained with corresponding DPPC-d_{62}-containing samples, allowing for the obvious difference between the ^{2}H-NMR spectra of POPC, deuterated at only one acyl chain, and DPPC-d_{62}, deuterated at both.

Protein kinase C assay

PKC activity was measured by phosphorylation of the exogenous substrate histone 1 according to Sando and Chertihin (1996), with modifications. Briefly, samples were temperature-equilibrated with substrate and ATP with 0.6 μCi [γ-^{32}P]ATP/sample (Dupont-NEN, Boston, MA) before performing the assay, which was initiated by adding PKC. The reaction was allowed to proceed for 5 min at 30°C, and was stopped by blotting 60 μL of the sample onto Whatman P-81 (Whatman, Fairfield, NJ) cation exchange filter paper. In order to remove unreacted ATP, the filters were washed with 500 mL 50 mM NaCl four times. The reaction kinetics were linear under all conditions of the assay. The amount of ^{32}P transferred to histone was determined by liquid scintillation counting. A sample containing the BLPPC/DPPC/DPPS mixtures and 15 mol % DO was included in every assay as an internal standard, and the results are expressed relative to the activity obtained with this sample, taken as 100% activity.

NMR measurements

^{2}H-NMR spectra were acquired at 11.74 T (corresponding to 76.77 MHz ^{2}H frequency) on a General Electric GN500 spectrometer. ^{2}H-NMR spectra were acquired with a high-power probe using the standard quadrupole echo sequence (Davis et al., 1976). The spectral width was 500 kHz and the refocusing time was 60 μs, with a 90° pulse of 3.5 μs and a recycle time of 200 ms.

Phase composition analyses

Spectra exhibiting the coexistence of liquid-crystalline bilayer and nonbilayer phases were analyzed by spectral subtraction similar to the method of Morrow and Davis (1988) as previously described (Goldberg and Zidovetzki, 1998). All spectra were scaled to contain the same spectral area, and then pure-phase spectra were digitally subtracted from a multiple-phase spectrum. The amount of subtraction necessary to remove the component from the multiple-phase spectrum indicated the fraction of lipid occupying that phase. No simulated spectra were used. Pure nonbilayer phase spectra were obtained from samples exhibiting L_α/nonbilayer phase components, and digitally subtracting out the unwanted phase. In order to compensate for differences in the width of isotropic peaks and changes in

quadrupole splittings of L_α phase between samples, pure spectra could be modulated by a width constant (keeping total spectral area fixed) to best match the spectrum being analyzed.

RESULTS

The effects of natural and the synthetic ceramides on PKC activity in the absence or presence of DO are shown in Fig. 1. The activity was normalized relative to the activity exhibited with the samples containing 15 mol % DO included in each assay as internal standards. In the absence of DO, the control BLPC/DPPC/DPPS samples supported only 9% of the PKC activity observed in these DO-containing samples. Addition of natural ceramide resulted in a concentration-dependent decrease of the activity down to 3% in the presence of 25 mol % natural ceramide (Fig. 1 *A*). In the presence of 7.5 mol % DO, the addition of natural ceramide resulted in a decrease of PKC activity from 35% to 16% (Fig. 1 *A*). In the case of C_{16}-ceramide the observed tendency for decrease in PKC activity was not significant in the absence of DO; a small but significant reduction in DO-enhanced activity was observed at the highest tested C_{16}-ceramide concentration of 25 mol % (data not shown).

A different picture was observed in the cases of the short-chain ceramide analogs (Fig. 1, *B* and *C*). In the absence of DO, C_2-ceramide did not affect PKC activity up to 25 mol % (Fig. 1 *B*), whereas at 25 mol % C_6-ceramide increased PKC activity from 9% to 52% (Fig. 1 *C*), and C_8-ceramide to 40% (data not shown). A dramatic effect of the short-chain ceramides was observed in the presence of 7.5 mol % DO. Each ceramide induced concentration-dependent increases of PKC activity from 30% activation in the absence of the ceramides to 68%, or 123% in the presence of 25 mol % C_2- or C_6-ceramide, respectively (Fig. 1, *B* and *C*) and to 144% in the presence of C_8-ceramide (data not shown). The effects were already significant at 5 mol % ceramide.

The effects of the acyl chain length of the synthetic ceramide analogs (at 15 mol %) are summarized in Fig. 2. In the presence of 7.5 mol % DO, short-chain (C_2-, C_6-, C_8-) ceramides synergistically increased DO-induced PKC activation. The effect was most pronounced in the cases of C_6- or C_8-ceramide, which increased DO-enhanced PKC activity by more than threefold. Similarly to natural ceramide, C_{16}-ceramide did not affect PKC activity.

We further investigated the synergistic effect of DO and C_6-ceramide on PKC activity by varying concentrations of both DO and C_6-ceramide (Fig. 3). C_6-ceramide alone showed only a minor activation of PKC, which agrees with the results shown in Fig. 1. As expected, DO alone activated PKC in a concentration-dependent manner reaching a maximum at 20 mol %. At lower DO concentrations (5–15 mol %), C_6-ceramide synergistically enhanced the effects of DO in a concentration-dependent manner, reaching the maximum observed activation at 15 mol % DO/20 mol % C_6-ceramide. Further increase in DO and/or C_6-ceramide concentration led to a decrease in PKC activity (Fig. 3).

The relationship between PKC activity and membrane physical properties was studied by ^{2}H-NMR using DPPC-

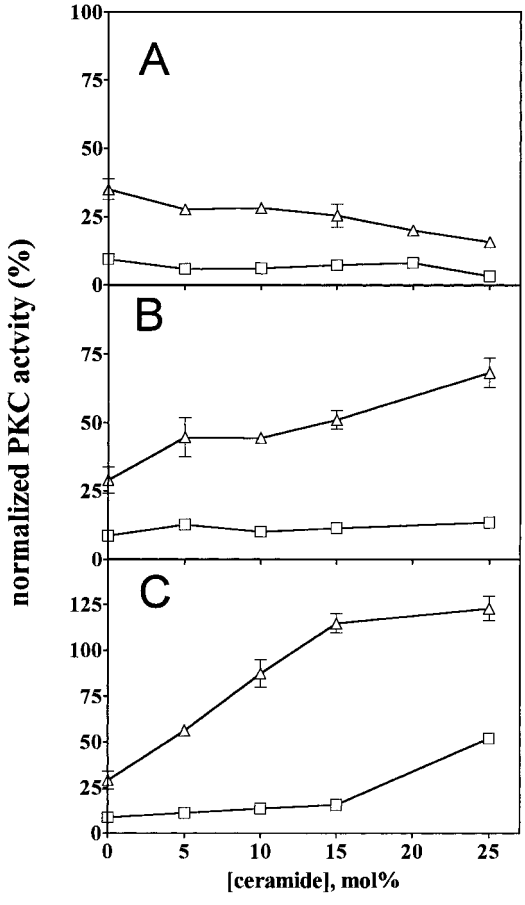

FIGURE 1 Dependence of PKC activity on the concentration of ceramides in the absence (□) or presence (△) of 7.5 mol % DO in BLPC/DPPC/DPPS bilayers. 100% PKC activity was defined as the activity in the BLPC/DPPC/DPPS samples with the addition of 15 mol % DO, included in every assay as an internal standard. The samples were run in triplicates at 30°C. The error bars correspond to SEM. (*A*) natural ceramide; (*B*) C_2-ceramide; (*C*) C_6-ceramide.

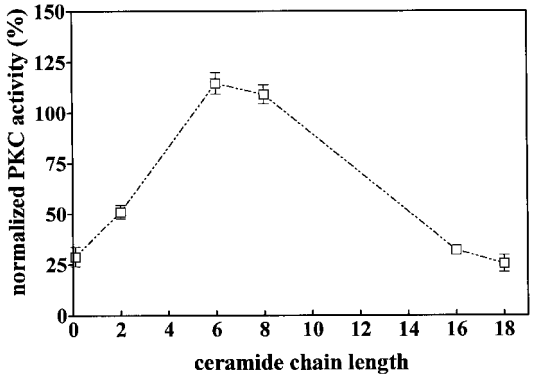

FIGURE 2 Dependence of PKC activity on synthetic ceramide side-chain length at 15 mol % ceramide in the presence of 7.5 mol % DO in PC/PS bilayers. The leftmost point corresponds to the normalized activity of PKC without ceramides. The rightmost point corresponds to the natural ceramide.

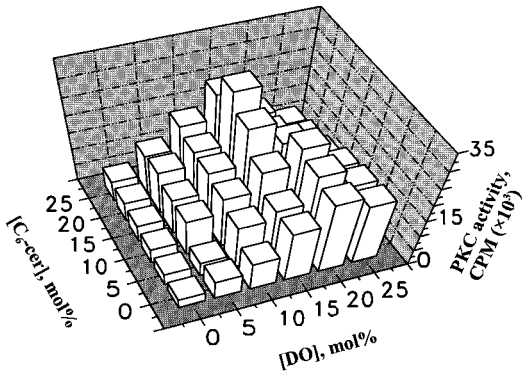

FIGURE 3 Dependence of PKC activity on the concentrations of DO and/or C_6-ceramide.

d_{62} incorporated into PC/PS bilayers. The ^{2}H-NMR spectra of DPPC-d_{62} in PC/PS bilayers in the absence or presence of C_6-ceramide and/or DO are shown in Fig. 4. An ^{2}H-NMR spectrum of DPPC-d_{62} is the superposition of axially averaged powder patterns that correspond to the deuterons at different positions along the DPPC-d_{62} acyl chains (Davis, 1979). The control spectrum of BLPC/DPPC-d_{62}/DPPS (3:1:1 molar ratio) multilamellar vesicles at 30°C shows a typical liquid crystalline bilayer profile (Fig. 4 *A*). At 60°C, the lipids remained in the liquid crystalline phase (Fig. 4 *B*). The presence of 15 mol % DO or 20 mol % C_6-ceramide, the concentrations that correspond to the maximum PKC activation (see Fig. 3), did not induce nonbilayer lipid phases at temperatures up to 60°C (Fig. 4, *C–F*). However, the addition of *both* 15 mol % DO and C_6-ceramide resulted in the induction of 21% of the isotropic lipid phase at 60°C (Fig. 4 *H*). This indicates that the corresponding bilayers have an increased propensity to form such a phase already at 30°C. A different picture was obtained upon increasing both DO and C_6-ceramide concentrations to 25 mol %, corresponding to the decreased PKC activity (cf. Fig. 3). Addition of 25 mol % DO to the PC/PS bilayers resulted in the formation of nonbilayer phases, comprising 12% of the ^{2}H-NMR intensity at 30°C (Fig. 5 *A*), whereas no nonbilayer phases were induced by 25 mol % C_6-ceramide (Fig. 5 *B*). In the presence of *both* 25 mol % C_6-ceramide and 25 mol % DO at 30°C, the proportion of the lipids in nonbilayer phases increased to 42% (Fig. 5 *C*), demonstrating the synergistic effect of these two reagents in inducing nonbilayer lipid phases. In all cases, identical results were obtained using DPPS-d_{62} as an ^{2}H-NMR label, indicating that DO and ceramides do not preferentially affect PC or PS lipid components (data not shown). In contrast to the effect of C_6-ceramide, natural ceramide and DO did not induce nonbilayer phases at either 30 or 60°C (Fig. 6). The broad component observed at 30°C in the spectrum containing natural ceramide without (not shown) or with DO (Fig. 6 *A*) corresponds to the bilayers in the gel phase, superimposed with the narrower spectrum of the lipids in the liquid crystalline (L_α) phase. Such a superposition is indicative of coexistence of the laterally separated lipid domains with different fluidities, and was described by us previously as an effect of long-chain and natural ceramides on BLPC and DPPC bilayers (Huang et al., 1996, 1998).

DISCUSSION

The purpose of the present work was to investigate the effect of ceramide analogs on modulating PKC activity and to examine the relationship between this effect and the membrane perturbations induced by these ceramides. According to the model of the activation of Ca^{2+}-dependent PKC proposed in many studies (Hannun and Bell, 1986; Stabel and Parker, 1991; Bell and Burns, 1991; Zidovetzki and Lester, 1992), the release of a micromolar level of Ca^{2+} triggers the binding of an inactive form of PKC to membrane bilayer, and the subsequent binding to PS and DAG facilitates PKC to undergo a conformational change and form an active form of the enzyme. Based on this model, extensive studies have suggested that local alterations of the physical properties of the membrane bilayers play a vital role in modulating the activity of PKC (Epand and Lester, 1990; Bolen and Sando, 1992; Zidovetzki and Lester, 1992; Senisterra and Epand, 1993; Slater et al., 1994). It has been reported that several lipophilic molecules, such as chloroform (Lester and Baumann, 1991), phosphatidylethanolamine (PE) (Epand and Bottega, 1988), unsaturated phospholipid (Bolen and Sando, 1992), cholesterol (Bolen and Sando, 1992), lysophosphatidic acid (Sando and Chertihin, 1996), and unesterified fatty acids (Goldberg and Zidovetzki, 1997; 1998) modulate the enzyme's activity. Furthermore, DAG-induced membrane perturbations play an important role in the mechanism of PKC activation (Epand, 1985; Das and Rand, 1986; Goldberg et al., 1994; see Zidovetzki, 1997, for a review). Among different types of membrane perturbations induced by DAGs, the promotion of nonbilayer lipid phases has been long proposed to be associated with the activation of PKC. For example, lowering the liquid-crystalline bilayer phase (L_α)-to-hexagonal phase (H_{II}) transition temperature (T_H) by the addition of certain lipid molecules, such as DO (Epand et al., 1988) or 1-oleoyl, 2-docosahexaenoylPE, into the lipid system leads to an increase in PKC activity (Giorgione et al., 1995). Our previous studies also observed a correlation between PKC activation and the increased tendency to form nonbilayer lipid phases (Goldberg et al., 1994; Goldberg and Zidovetzki, 1998). Epand et al. (1988) have suggested that uncharged zwitterionic hexagonal phase promoters are generally PKC activators, while bilayer stabilizers are inhibitors of the enzyme. In the present study we have also shown a similar relationship between the synergistic effect of DO and C_6-ceramide on PKC activity and the increased tendency of forming nonbilayer phases induced by DO and C_6-ceramide (Figs. 3 and 4). The synergistic effect of DO and C_6-ceramide on PKC is dependent on the concentration of both lipid molecules before reaching the maximum PKC activity that occurred at the combination of 15 mol % DO

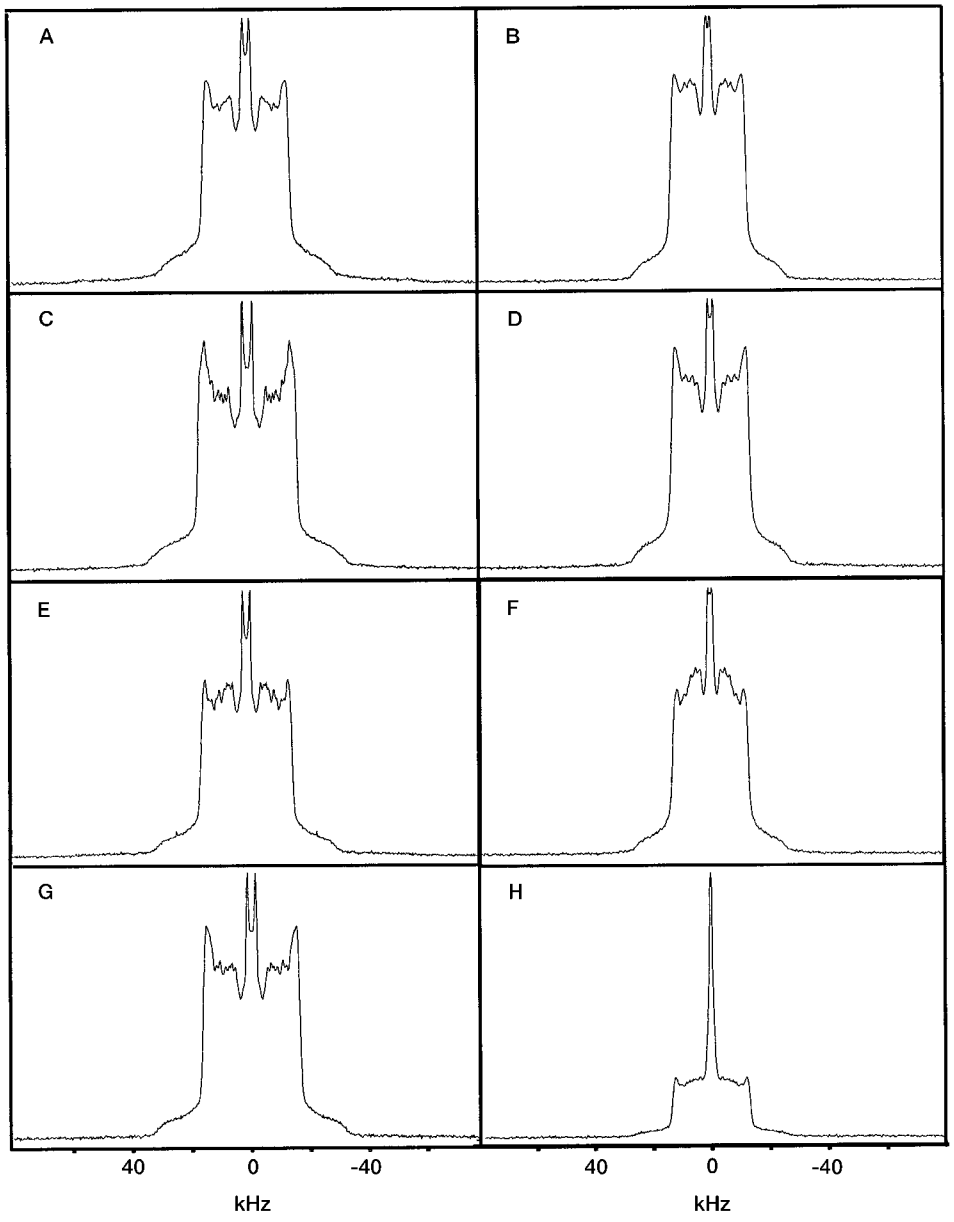

FIGURE 4 ^{2}H-NMR spectra of DPPC-d_{62} in BLPC/DPPC-d_{62}/DPPS mixtures at 30°C (*left*) or 60°C (*right*). (*A, B*) control; (*C, D*) with 15 mol % DO; (*E, F*) with 20 mol % C_6-ceramide; (*G, H*) with 15 mol % DO and 20 mol % C_6-ceramide.

and 20 mol % C_6-ceramide. Based on our NMR results, in the presence of 20 mol % C_6-ceramide and 15 mol % DO, no nonbilayer phases were present at the temperature of PKC activity assays (30°C). However, increasing the temperature to 60°C resulted in the presence of nonbilayer lipid phases, demonstrating the increased tendency of the bilayers with this composition to form such phases. A further increase in DO and/or C_6-ceramide concentration(s) resulted in the presence of nonbilayer lipid phases at the temperature of PKC assays and a decrease of the PKC activity, consistent with the notion that the actual presence of such phases is detrimental for PKC activity. Lee et al. (1996) have reported that C_6-ceramide at concentrations of up to 200 mol % to PS, and in the presence of 6.5 mol % DO, has no effect on PKCα activity. Lee et al. (1996), however, used a mixed micelle assay for measuring PKC activity, thereby eliminating the effects of C_6-ceramide on lipid bilayer structure.

Both natural ceramide and C_{16}-ceramide analog induced lateral phase separation of gel-like and liquid crystalline phases into the PC/PS bilayers without a significant effect on the activity of PKC, confirming that this type of membrane perturbation is not relevant for PKC activity (Snoek et al., 1988; Zidovetzki and Lester, 1992; Senisterra and Epand, 1993; Goldberg et al., 1994). We have previously shown that such ceramide-induced lateral phase separation plays an important role in activating another membrane-associated enzyme, extracellular phospholipase A_2 (Huang et al., 1998). PKC activity can also be modulated by the coexistence of the domains with different lipid compositions, such as DO-rich and DO-poor domains (Dibble et al., 1996).

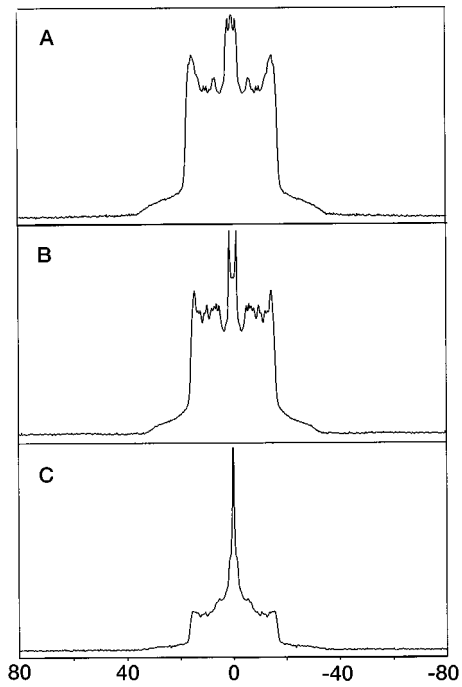

FIGURE 5 ^{2}H-NMR spectra of DPPC-d_{62} in BLPC/DPPC-d_{62}/DPPS mixtures at 30°C. (*A*) with 25 mol % DO; (*B*) with 25 mol % C_6-ceramide; (*C*) with 25 mol % DO and 25 mol % C_6-ceramide.

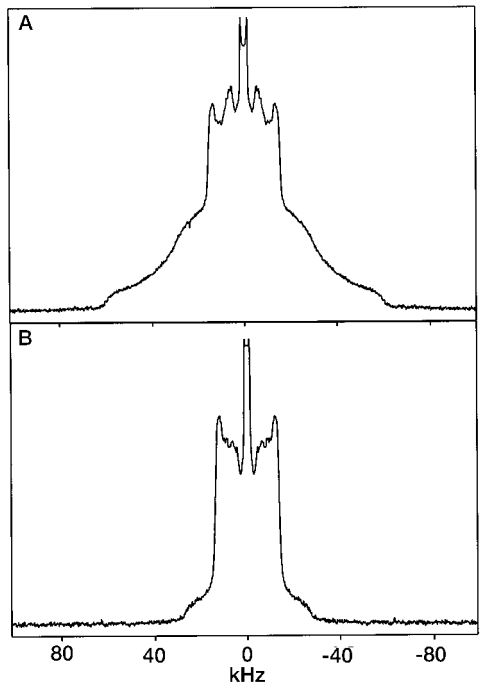

FIGURE 6 ^{2}H-NMR spectra of DPPC-d_{62} in BLPC/DPPC-d_{62}/DPPS mixtures with 15 mol % DO and 20 mol % natural ceramide. (*A*) 30°C; (*B*) 60°C.

Holopainen et al., (1997) have recently reported formation of microdomains in dimyristoylPC/natural ceramide mixtures. Addition of ceramide to dimyristoylPC resulted in increased temperature of gel-to-liquid crystalline phase transition, as followed by differential scanning calorimetry, and increased polarization of a fluorescent probe in the membranes. Poor mixing of natural ceramide with DPPC was also recently reported by Veiga et al. (1999). These results are consistent with those obtained by us in the present study with PC/PS, and in the previous publications with PC/ceramide (Huang et al., 1996, 1998) systems. Moreover, the use of ^{2}H-NMR allowed us to obtain the superposition of spectra corresponding to the lipids in gel-like and fluid liquid crystalline domains, directly demonstrating the coexistence of the corresponding laterally separated phases. Noting that the average chain length of the natural ceramide greatly exceeds that of dimyristoylPC, Holopainen et al. (1997) suggested that the hydrophobic mismatch is the main driving force of the ceramide-enriched domain formation. We, however, obtained similar results with natural ceramide or much shorter C_{16}-ceramide using either PC/PS system or DPPC system. We therefore conclude that the main contribution to the observed lateral phase separation is, similarly to the effects of DAGs, the lack of a bulky headgroup in the ceramide molecule, which allows for closer chain interactions and complex-like formation between ceramide and PC or PS molecules. These phospholipid-ceramide complexes then laterally phase-separate from the bulk lipids. Such a process was described in detail for DAGs (De Boeck and Zidovetzki, 1989, 1992; Heimburg et al., 1992; Goldberg et al., 1994; López-García et al., 1994; Quinn et al., 1995; Schorn and Marsh, 1996).

Ruiz-Argüello et al. (1996), using a different lipid system, have reported that, although both natural DAG and ceramide induce fusion of sphingomyelin/PE/cholesterol or PC/PE/cholesterol vesicles, the mechanisms and efficiency of this process are not the same for the two molecules; it was suggested that the difference may be due to the different effects of DAG and ceramide in inducing nonbilayer lipid structures. Indeed, our current and previous results are consistent with this interpretation and suggest that the different effects observed by Ruiz-Argüello et al. (1996) are due to the difference in the acyl chain compositions of natural ceramide and DAG. As described above, we have found that the membrane effect of C_{16}-ceramide is similar to that of dipalmitin. However, the fatty acid compositions of the natural ceramides and DAGs are different: 85% of natural ceramide have long saturated acyl chains at the second position (manufacturer information), whereas the predominant acyl chain at the second position of natural DAG is polyunsaturated, commonly arachidonic. Our previous studies showed that 25 mol % 1-stearoyl, 2-arachidonoyl-*sn*-glycerol efficiently induces nonbilayer lipid phases in PC/PS (Goldberg et al., 1995) and erythrocyte lipid (Zidovetzki et al., 1993) systems, whereas natural ceramide at 25 mol % induces only lateral phase separation in DPPC (Huang et al., 1996) or PC/PS (this study). Natural ceramide, however, can induce nonbilayer lipid phases in pure PE bilayers (Veiga et al., 1999).

CONCLUSIONS

The present work demonstrated that synthetic short-chain ceramides, widely used in research to investigate sphingomyelin-dependent signal transduction pathway, also activate PKC, the effect not exhibited by natural ceramide. Caution, therefore, should be exercised when interpreting the results of the effects of the short-chain ceramides on cells. It has been pointed out that in the cells exposed to 1–10 μM C_2- or C_6-cer the membrane concentration of these ceramides reaches 1–10 mol % to the phospholipids (Hannun, 1996), which is within the range of ceramide concentrations where both membrane perturbation and PKC activation were observed in this study. Moreover, comparable concentration of natural ceramide is achieved after prolonged response to tumor necrosis factor-α or serum deprivation (Hannun, 1996). Similar levels of DAGs were observed in some cellular systems, exceeding 5 mol % in PC12 cells (Altin and Bradshaw, 1990), human epidermal A431 cells (van Veldhoven and Bell, 1988), human endothelial cells (Whatley et al., 1993), and transformed 3T3 cells (Wolfman and Macara, 1987). We have found that the distinct effects of natural ceramide and short-chain ceramides are even more pronounced in the presence of DAG. Natural ceramide has little effect on the enzyme activity, whereas C_2-, C_6-, and C_8-ceramides strongly activate PKC synergistically with DO.

The present results, combined with our previous studies with various DAGs and unesterified fatty acids, provide strong support to the hypothesis that the physicochemical parameters of the lipid membranes play an important role in the mechanism of PKC activation. The specific membrane parameter associated with PKC activation is the increased tendency to form nonbilayer lipid structures, promoted by the addition of the lipids with high intrinsic curvature (Gruner, 1989). Many cellular membranes exist under conditions of high curvature stress, being close to the bilayer-to-hexagonal phase transition (Rilfors et al., 1994; Rietveld et al., 1994), and the physicochemical properties of these membranes are expected to be quite sensitive to changes in DAG and ceramide concentration, resulting in modulation of the activity of PKC and other membrane-associated enzymes.

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