Interaction of Ceramides, Sphingosine, and Sphingosine 1-Phosphate in Regulating DNA Synthesis and Phospholipase D Activity*

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—C2- and C6-ceramides (N-acetylphosphoglycerol and N-hexanoylphosphoglycerol, respectively) abolished the stimulation of DNA synthesis by sphingosine 1-phosphate in rat fibroblasts. This inhibition by ceramide was partially prevented by insulin. C2-ceramide did not alter the stimulation of DNA synthesis by insulin and decreased the sphingosine-induced stimulation by only 16%. The ceramides did not significantly modify the actions of sphingosine or sphingosine 1-phosphate in decreasing cAMP concentrations. C2- and C6-ceramides blocked the activation of phospholipase D by sphingosine 1-phosphate, and this inhibition was not affected by insulin. Okadaic acid decreased the activation of phospholipase D by sphingosine 1-phosphate and did not reverse the inhibitory effect of C2-ceramide on this activation. Therefore, this effect of C2-ceramide is unlikely to involve the stimulation of phosphoprotein phosphatase activity. Sphingosine did not activate phospholipase D activity significantly after 10 min. C2-ceramide stimulated the conversion of exogenous [3H]sphingosine 1-phosphate to sphingosine and ceramide in fibroblasts. Ceramides can inhibit some effects of sphingosine 1-phosphate by stimulating its degradation via a phosphohydrolase that also hydrolyzes phosphatidate. Furthermore, C2- and C6-ceramides stimulated ceramide production from endogenous lipids, and this could propagate the intracellular signal. This work demonstrates that controlling the production of ceramide versus sphingosine and sphingosine 1-phosphate after sphingomyelinase activation could have profound effects on signal transduction. —

The activation of the SM\textsuperscript{1} cycle plays an important role in signal transduction (1–3). Stimulation of cells with 1,2-dihydroxyvitamin D\textsubscript{3} (4), TNF-\alpha, or γ-interferon (1–3, 5) activates a neutral sphingomyelinase that hydrolyzes membrane SM to generate intracellular ceramides. The latter event is linked to the induction of cell differentiation and the inhibition of cell growth in HL-60 human leukemia cells (1–5). Other stimulators of SM hydrolysis in different cell types include interleukin-1 (1–3, 6, 7), dexamethasone (8), and complement proteins (9). The other product of SM hydrolysis, phosphorylcholine, was thought not to play a role in signal transduction (10). However, Lacal et al. (11) showed that phosphorylcholine is essential for late events in the induction of DNA synthesis by platelet-derived growth factor. The stimulation of a neutral sphingomyelinase by TNF-α may involve phospholipase A\textsubscript{2} and the release of arachidonic acid (12). TNF-α also stimulates an acidic sphingomyelinase through the production of 1,2-diacylglycerol via stimulation of a phosphatidylcholine-specific phospholipase C.

There is abundant evidence that ceramides are second messengers for cell regulation. The generation of intracellular ceramides or the addition of cell-permeable ceramides leads to the rapid induction of a nuclear transcription factor system, NF-κB (13, 14), although a recent report presented evidence that dissociates NF-κB activation from an increase in cell ceramide concentrations (15). Incubation of cells with exogenous bacterial sphingomyelinase to generate intracellular ceramides (a) inhibits the phorbol ester-induced differentiation in HL-60 cells (16), (b) inhibits hydroxyethylglutaryl-CoA reductase (17, 18), (c) increases the binding, internalization, and degradation of low density lipoproteins and the synthesis of cholesterol esters in cultured human fibroblasts (19), and (d) stimulates PA and lyso-PA metabolism in rat fibroblasts (20). Exogenous cell-permeable ceramides mimic the action of 1,25-dihydroxyvitamin D\textsubscript{3}, TNF-α, and γ-interferon (4). In addition, cell-permeable ceramides exhibit specific antiproliferative effects in HL-60 cells (2), Madin-Darby canine kidney cells (21), and rat fibroblasts stimulated with PA or lyso-PA (20). C2-ceramide can also induce internucleosomal DNA fragmentation, which leads to programmed cell death or apoptosis (22), and this process can be initiated by TNF-α (23). Ceramide levels are increased significantly in T lymphocytes that are infected with the human immunodeficiency virus and that undergo apoptosis (24). Ceramides also play a role in inflammatory processes, and they modulate the secretion of prostaglandin E\textsubscript{2} in response to interleukin-1 (7). Furthermore, ceramides stimulate the secretion of interleukin-2 in lymphocytes (6).

Several intracellular targets for ceramide action have been described. C2-ceramide decreases the mRNA for c-myc, an important step in cell differentiation (5). C2-ceramide also stimulates a specific serine/threonine protein phosphatase in mammalian cell extracts (25) and in Saccharomyces cerevisiae (26). Ceramides stimulate specific kinases (2) and the phosphorylation of the epidermal growth factor receptor in A431 human epidermoid carcinoma cells (27) and act as competitive inhibi-
tors of diacylglycerol kinase in HL-60 cells (28). Sphingomyelinase and cell-permeable ceramides activate mitogen-activated protein kinase in HL-60 cells (29), and this effect has also been demonstrated for TNF-α in human fibroblasts (30). We have also demonstrated that cell-permeable ceramides inhibit the activation of PLD by several agonists (20). Further details of the SM cycle and the actions of ceramides have been described by Hannun (1).

The role of sphingolipids in regulating cell functions has been described by Merrill (31, 32) and Merrill and J ones (33). Sphingosine inhibits protein kinase C (34) and Mg\(^{2+}\)-dependent and -independent phosphatidylinositol phosphokinase (35–39). Sphingosine also activates PLD in NG108-15, rat pituitary, and NIH-3T3 cells (40), and it stimulates an 80-kDa diacylglycerol kinase (41). These combined actions increase the accumulation of PA relative to diacylglycerol (42), which could also decrease protein kinase C activation. Sphingosine also stimulates cell proliferation in fibroblasts (43) and induces intracellular Ca\(^{2+}\) mobilization (44, 45). Some of these effects of sphingosine have been attributed to the formation of SPP (46). SPP stimulates PLD activity in Swiss 3T3 fibroblasts (46). It is a mitogenic agent (47) and may be a second messenger that mediates the proliferative effects of platelet-derived growth factor and fetal calf serum (48). However, conversion of sphingosine to SPP is not necessarily essential for the stimulation of mitogenesis. Treatment of fibroblasts with fumonisin B1, a n inhibitor of sphingosine (sphinganine) N-acetyltransferase (49), enhanced \(^{[3H]}\)thymidine incorporation into DNA, but did not cause any accumulation of SPP, suggesting that sphingoid bases per se can also stimulate DNA synthesis (50). Sphingosine-induced Ca\(^{2+}\) mobilization may also be independent of conversion to SPP (45).

The agonist-stimulated breakdown of SM can potentially generate ceramides, sphingosine, and SPP. Therefore, we studied the interactions of these compounds on signal transduction and cell activation in cultured fibroblasts to define more precisely the effects and mechanisms of action of these putative second messengers. Cell-permeable ceramides increase the production of ceramide from endogenous lipids and stimulate the conversion of SPP to sphingosine and ceramide. This work emphasizes that regulating the balance in the production of ceramide versus sphingosine and SPP after sphingomyelinase activation could have profound effects on signal transduction and cell division.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of most of the materials have been described (20, 35, 51). Sphingosylphosphorylcholine (free base) was from Sigma, and acetic acid was from LC Laboratories (Warren, MA). p-Amydro-[\(^{3H}\)]sphingosine was from DuPont NEN, and this compound was converted to C\(_2\)-[\(^{3H}\)]ceramide by N-acetylation with p-nitrophenyl octanolate by Drs. H.-S. Byun and R. Bittman.

**Culture of Fibroblasts**—Rat 1 fibroblasts, and Rat 2 fibroblasts that lack nuclear thymidylate kinase (52), were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 10 units of penicillin/ml, and 10 \(\mu\)g of streptomycin/ml. Fibroblasts were seeded at 1.5 \(\times\) 10\(^7\) cells/35-mm culture dish and incubated at 37°C with 5% CO\(_2\) in air for 3 days for the studies on \(^{[3H]}\)thymidine incorporation or CAMP determinations. For determinations of PLD and sphingosine 1-phosphatase activities, 60- and 100-mm culture dishes were used, respectively, with equivalent cell densities. 60- and 100-mm cultured dishes were used, respectively, with equivalent cell densities.

**Determination of DNA Synthesis**—Fibroblasts were grown as described above and were maintained in medium containing 0.1% fetal bovine serum for 24 h to cause growth arrest. Cells were then incubated in the presence or absence of agonists as indicated for 22 h in serum-free medium, and \(^{[3H]}\)thymidine (0.5 \(\mu\)Ci/dish) was present for the last 6 h of the incubation (20). This time period was chosen because it gave the maximum incorporation of radiolabel (data not shown). The cells were washed, and the incorporation of \(^{[3H]}\)thymidine into DNA was measured as described previously (20).

**Assay of cAMP**—For these measurements, the lysates from the cells were boiled for 5 min and centrifuged at 12,000 \(\times\) g for 10 min. Samples of the supernatant were then used to determine the cAMP concentrations using a radioimmunoassay procedure in which samples were acetylated prior to analysis (53).

**Assay of PLD and Ceramide Production**—The activity of PLD was determined by measuring the accumulation of \(^{[3H]}\)phosphatidylethanol, which is the product of its transphosphatidyltransferase reaction and is considered to be a definitive assay for PLD (54–56). The labeling of fibroblasts with \(^{[3H]}\)myristate and the details of the PLD assay have been described previously (51). The formation of \(^{[3H]}\)ceramide was determined by scraping the ceramides from the same thin-layer plate as the phosphatidylethanol. The identity of the ceramide was confirmed by cochromatography with authentic long-chain ceramides. Similar studies on ceramide formation were also performed after labeling fibroblasts with \(^{[3H]}\)palmitate.

**Measurement of Sphingosine 1-Phosphate and Phosphatidylethanol Activity**—Rat 2 fibroblasts were harvested in 0.5 ml of 100 mM Tris-HCl, pH 7.4, containing 1 mM benzamidine, 2 \(\mu\)g/ml leupeptin, 25 \(\mu\)g/ml pepstatin A, and 1 mM dithiothreitol. The cells were sonicated and then dialyzed against 100 mM Tris-HCl, pH 7.4, to decrease the P, content. The assays contained (in 100 \(\mu\)l) 100 mM Tris-HCl, pH 7.4, 0.4 mM SPP, and 30–90 \(\mu\)g of cell protein; and the incubations were for 50 min at 37°C. The reactions were stopped with 200 \(\mu\)l of 0.342 M HClO\(_4\), and the mixture was cooled to 4°C and then incubated for 10 min at 37°C in a bench. The supernatant (240 \(\mu\)l) was transferred to a tube, and 25 \(\mu\)l of 2.5 g of ammonium molybdate/100 ml and 25 \(\mu\)l of 10 g of ascorbic acid/100 ml were added. After incubation for 30 min at room temperature, 270 \(\mu\)l of the solution was transferred to a microfilter plate, and the concentration of P, was determined by measuring the absorbance at 700 nm. Reaction rates were constant with time and proportional to the amount of cell protein added. The amount of P, was calculated relative to a standard curve of P, prepared in HClO\(_4\). Phosphatidylethanol activity was determined using 20 \(\mu\)M \(^{[3H]}\)IPA and 8 mM Triton X-100 as a substrate and evaluating the formation of \(^{[3H]}\)diacylglycerol (51).

**Synthesis of Sphingosine 1-Phosphate**—This sphingolipid was synthesized by enzymatic digestion of sphingosylphosphorylcholine with phospholipase D essentially as described by Van Veldhoven et al. (57), but with modifications. The first incubation was for 2 h, followed by a second incubation that lasted for 3–4 h. After each of these incubations, the samples were frozen, instead of being maintained at 4°C. This helped to improve the precipitation of SPP. Tubes were centrifuged, and the pellets pooled and then dispersed in water by sonication. After freeze-drying and centrifuging, the final pellet was dispersed in 1 ml of acetone by sonication. The acetone sample was centrifuged, and the final pellet was dissolved in methanol. SPP was then purified by thin-layer chromatography according to Zhang et al. (47). SPP had an \(R_p\) of 0.47–0.49 when the plates were developed with 1-butanol/acetic acid/water (3:1:1, v/v/v). The purity of SPP was confirmed by analysis of the phospholipid product in three additional solvent systems, which include solvents I and V from Ref. 46 and a solvent system that consisted of chloroform/acetone/acidic acid/methanol/water (50:20:15:10.5:5, by volume). The \(R_p\) for SPP in the latter solvent was 0.39. In each system, there was a single spot that was positive with ninhydrin and phosphate sprays (58). SPP was eluted from the silica in three washes with methanol/chloroform/CH\(_3\)OH (9:1:0.05, v/v/v).

\(^{[3H]}\)SPP was prepared by deacylation of \(^{[3H]}\)ceramide 1-phosphate essentially according to Desai et al. (46). \(^{[3H]}\)Ceramide 1-phosphate was synthesized from N-octanol/[\(^{3H}\)]phosphoglycerol essentially as described previously (59) by using Escherichia coli diacylglycerol kinase. \(^{[3H]}\)Ceramide 1-phosphate was treated with 6 \(\mu\)M HCl/I betaine (1.1, v/v) at 100°C to prepare \(^{[3H]}\)SPP. This compound was purified by thin-layer chromatography (47) using 1-butanol/acidic acid/water (3:1:1, v/v/v). The product also cochromatographed with nonradioactive SPP in the solvent systems described above.

**Metabolism of \(^{[3H]}\)Sphingosine 1-Phosphate in Rat Fibroblasts**—Rat 2 fibroblasts were preincubated for 2 h in DMEM containing 0.5% bovine serum albumin in the presence or presence of 1 mM 

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chose not to separate chloroform and methanol/water phases to avoid partitioning SPP into the aqueous phase.

For the analysis of the sphingolipid metabolites of SPP, we developed a thin-layer system that separated SPP, sphingosine, SM, ceramide, and ceramide 1-phosphate efficiently in one dimension. After lipids were extracted and dried down, they were spotted on glass-backed Silica Gel 60 plates, which were developed sequentially with three different solvent systems. Solvent A consisted of chloroform/methanol/10mM HEPES, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.5% Tween 20, and 0.25% gelatin. The blot was probed with an affinity-purified antibody raised against a homogeneous preparation of liver phosphohydrolase (61). Immunoreactive proteins were detected using ECL (Amersham Corp.).

RESULTS

Cell-permeable Ceramides Inhibit the Stimulation of [3H]Thymidine Incorporation into DNA Induced by Sphingosine 1-Phosphate—The proliferation of the fibroblasts was arrested after 3 days in culture by decreasing the concentration of serum from 10 to 0.1% in the incubation (62). At this stage, cells were ~80% confluent. This procedure was adopted in order to study the effects of mitogenic and antimitogenic agents before the cells became confluent (20). Fibroblasts were then treated for 22 h with cell-permeable ceramides in the presence or absence of 2 μM SPP. As expected (47), SPP stimulated DNA synthesis. This effect was overcome by 5–10 μM C2-ceramide (Fig. 1). Similar results were obtained in an additional experiment performed with C6-ceramide (results not shown). SPP produced optimum stimulations of DNA synthesis of 2–2.5-fold at 2 μM. This effect was completely inhibited by C2-ceramide (Figs. 1 and 2). SPP concentrations higher than 5 μM were less effective in stimulating DNA synthesis or caused inhibition (Fig. 3). This effect of high SPP concentrations was further potentiated by the ceramides, particularly at 10 μM concentrations of both SPP and C2-ceramide (Fig. 3). Relatively high insulin concentrations enhance the mitogenic effect of SPP (47). Fig. 2 shows that 1 μg/ml insulin partially protects the stimulation of DNA synthesis by SPP against the inhibition by C2-ceramide.

Sphingosine also stimulates the incorporation of [3H]thymidine into DNA (20, 43), and this effect has been suggested to be mediated by its conversion to SPP in Swiss 3T3 fibroblasts (44). However, in rat fibroblasts, sphingosine is more mitogenic than SPP, and 20 μM sphingosine caused a 4-fold stimulation of DNA synthesis. Concentrations greater than 25 μM sphingosine were toxic to the fibroblasts. Furthermore, C2-ceramide, which blocks the effect of SPP completely (Figs. 1 and 2), had only a marginal effect on the inhibition of DNA synthesis induced by sphingosine (Fig. 3). Sphingosine and SPP appear to stimulate DNA synthesis by different mechanisms, and therefore, they might be expected to increase this process in an additive manner. However, the combination of an optimum SPP concentration of 2 μM with 20 μM sphingosine stimulated DNA synthesis ~11% less than did sphingosine alone. This effect is compatible with the results of Spiegel et al. (44). It is difficult to rationalize these results except to postulate that the addition of extra SPP to cells already exposed to 20 μM sphingosine causes cell damage, as does extra sphingosine.

Cell-permeable Ceramides Do Not Reverse the Effects of Sphingosine and Sphingosine 1-Phosphate in Decreasing the Production of cAMP in Fibroblasts—A decrease in cAMP concentrations has been linked to the mitogenic effect of lysophosphatidate in rat fibroblasts (63). Sphingosine also decreases cAMP levels dramatically in Swiss 3T3 cells (44). We investigated whether C2-ceramide might modify the possible effects of sphingosine and SPP in lowering cAMP in rat fibroblasts. Sphingosine, SPP, and lysophosphatidate decreased cAMP concentrations in both the absence and presence of forskolin (Table

![Fig. 1. Effect of ceramide concentration on the stimulation of DNA synthesis by sphingosine 1-phosphate.](image)

**Fig. 1.** Effect of ceramide concentration on the stimulation of DNA synthesis by sphingosine 1-phosphate. Rat1 fibroblasts were incubated for 22 h in serum-free DMEM in the absence (□) or presence (●) of 2 μM SPP and the indicated concentrations of C2-ceramide. [3H]Thymidine was present during the last 6 h of incubation. Basal incorporation of [3H]thymidine corresponded to 23,102 ± 2,616 dpm/95% ethanol in 95% ethanol and viewing the lipid spots under light at 366 nm. Phospholipids were identified by staining with 0.2% 9-[9-dichlorofluorescein in 95% ethanol and viewing the lipid spots under light at 366 nm. Phospholipids were identified by spraying the plates with molybdenum blue reagent (58). In all cases, authentic standards were used to establish Rf values. Under the conditions used, the final Rf values for the sphingolipids were 0.15 for SM, 0.33 for SPP, 0.50 for ceramide 1-phosphate, 0.61 for sphingosine, and 0.83 for ceramide. Radioactive sphingolipids were detected with a Bioscan System 200 Imaging Scanner, and they were quantitated by liquid scintillation counting after scraping from the plate.

Electrophoresis and Western Blot Analysis—The proliferation of the fibroblasts was arrested after 3 days in culture by decreasing the concentration of serum from 10 to 0.1% in the incubation (62). At this stage, cells were ~80% confluent. This procedure was adopted in order to study the effects of mitogenic and antimitogenic agents before the cells became confluent (20). Fibroblasts were then treated for 22 h with cell-permeable ceramides in the presence or absence of 2 μM SPP. As expected (47), SPP stimulated DNA synthesis. This effect was overcome by 5–10 μM C2-ceramide (Fig. 1). Similar results were obtained in an additional experiment performed with C6-ceramide (results not shown). SPP produced optimum stimulations of DNA synthesis of 2–2.5-fold at 2 μM. This effect was completely inhibited by C2-ceramide (Figs. 1 and 2). SPP concentrations higher than 5 μM were less effective in stimulating DNA synthesis or caused inhibition (Fig. 3). This effect of high SPP concentrations was further potentiated by the ceramides, particularly at 10 μM concentrations of both SPP and C2-ceramide (Fig. 3). Relatively high insulin concentrations enhance the mitogenic effect of SPP (47). Fig. 2 shows that 1 μg/ml insulin partially protects the stimulation of DNA synthesis by SPP against the inhibition by C2-ceramide.

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Cell-permeable Ceramides Do Not Reverse the Effects of Sphingosine and Sphingosine 1-Phosphate in Decreasing the Production of cAMP in Fibroblasts—A decrease in cAMP concentrations has been linked to the mitogenic effect of lysophosphatidate in rat fibroblasts (63). Sphingosine also decreases cAMP levels dramatically in Swiss 3T3 cells (44). We investigated whether C2-ceramide might modify the possible effects of sphingosine and SPP in lowering cAMP in rat fibroblasts. Sphingosine, SPP, and lysophosphatidate decreased cAMP concentrations in both the absence and presence of forskolin (Table

![Fig. 2. Effect of C2-ceramide on the synthesis of DNA induced by increasing concentrations of sphingosine 1-phosphate.](image)
C₂⁻ceramide did not reverse the decrease in cAMP concentrations in the presence of forskolin. In the absence of this reagent, C₂⁻ceramide alone increased the concentration of cAMP by ~18% in these experiments. This effect accounts for the differences obtained with C₂⁻ceramide in the presence of SPP and sphingosine. However, there was no significant effect of C₂⁻ceramide on the percentage decrease in cAMP that was produced by SPP, sphingosine, and lysophosphatidate (Table I).

**Table I**

| Forskolin | Lipids added | Relative cAMP concentrations | Relative CAMP concentrations |
|-----------|--------------|------------------------------|-----------------------------|
| Absent    | None         | 1.0±0.02                     | 1.0±0.02                    |
|           | Sphingosine 1-phosphate (10 μM) | 0.51±0.05 (8) | 0.67±0.04 (8) |
|           | Sphingosine (20 μM) | 0.55±0.04 (8) | 0.83±0.10 (8) |
|           | Lysophosphatidate (50 μM) | 0.70±0.06 (7) | 0.73±0.08 (7) |
| Present   | None         | 35.6±4.9 (6)                  | 31.3±5.5 (6)                |
|           | Sphingosine 1-phosphate (10 μM) | 18.4±5.0 (6) | 21.9±5.9 (6) |
|           | Sphingosine (20 μM) | 24.4±5.1 (6) | 28.0±6.0 (6) |
|           | Lysophosphatidate (50 μM) | 17.5±5.5 (5) | 20.7±6.8 (5) |

# p < 0.01.

During the course of these experiments on PLD, we detected that the addition of cell-permeable ceramides to the cells that were labeled with [³H]myristate increased the production of [³H]ceramide. This increase was ~2.5- and 8.7-fold for C₂⁻ and C₆⁻ceramides, respectively, and the presence of SPP had no significant effect on these values (Table II). Furthermore, we established that the presence of ethanol in the incubations did not significantly modify the production of [³H]ceramides. Ceramide-induced production of endogenous ceramides was also observed when the cells were prelabeled with [³H]palmitate rather than [³H]myristate (data not shown).

Some of the effects of ceramides on cell signaling are caused by the stimulation of phosphoprotein phosphatase activity, and these effects can be blocked by okadaic acid (25, 26). Okadaic acid prevented the stimulation of PLD activity by optimum concentrations of C₂⁻ceramide. C₂⁻ceramide alone increased the concentration of cAMP by 6.3±0.04-fold to 1.2±0.11-fold in three different experiments. This concentration of ceramides is higher than that needed to inhibit thymidine incorporation into DNA (Figs. 1-3). However, this difference is explained by a decreased potency of C₂⁻ and C₆⁻ceramides in the presence of high concentrations of albumin (64). Treatment of fibroblasts with sphingosine concentrations of up to 20 μM did not cause any significant stimulation of PLD after 10 min in three independent experiments (data not shown).

**Fig. 4. Effect of C₂⁻-ceramide on the stimulation of PLD by sphingosine 1-phosphate.** In A, Rat2 fibroblasts were labeled with [³H]myristate for 2 h and then incubated with 0.5% albumin in the absence (○) or presence (●) of 50 μM C₂⁻-ceramide for an additional 2 h. The fibroblasts were then treated for 10 min with increasing concentrations of SPP, as indicated, in the presence of 200 mM ethanol. PLD activities were determined through the formation of [³H]phosphatidylethanol. The results were calculated as a percentage of radioactivity in phosphatidylcholine (20) and then expressed as the fold stimulation relative to the incubations that contained neither ceramide nor SPP. In B, incubations contained 1 μM SPP, and the concentration of C₂⁻-ceramide was varied as shown. Values are the means ± range of two independent experiments performed in duplicate, except for the 50 μM C₂⁻-ceramide point, which is the mean ± S.E. of five independent experiments performed in duplicate.
These characteristics are similar to those of plasma membrane-associated since it was recovered in the particulate fraction of cell sonicates after ultracentrifugation (data not shown). This effect was also seen to a smaller extent when C2-ceramide was present with SPP (Fig. 5A). As already shown in Table II, the presence of C2-ceramides in the incubations stimulated the production of endogenous [3H]ceramide (Fig. 5B). Neither okadaic acid nor SPP significantly changed the relative concentration of [3H]ceramide either in the presence or absence of C2-ceramide. We concluded that the inhibition by C2-ceramide of the stimulation of PLD activity by SPP does not appear to involve the stimulation of a phosphoprotein phosphatase since this effect is not reversed by okadaic acid.

C2-ceramide Stimulates the Metabolism of Sphingosine 1-Phosphate by Fibroblasts—Recently, we demonstrated that at least part of the mechanism by which ceramides block the effects of PA and lyso-PA on the stimulation of DNA synthesis and PLD activity is by interfering with the interaction of the latter two mitogens with the cell, and by increasing the rates of metabolism (20). By contrast, the total amount of [3H]labeled sphingolipids, which were derived from exogenous [3H]SPP, was not significantly affected by C2-ceramides (Fig. 6E). [3H]SPP was rapidly converted in the fibroblasts to [3H]sphingosine (Fig. 6B), which is the precursor for the synthesis of ceramides and SM (33). C2-ceramide accelerated this process by increasing the rate of metabolism of SPP to sphingosine after 30 min and to ceramide at 5 and 12 h (Fig. 6, B and C). However, C2-ceramides decreased the subsequent conversion of ceramides to SM (Fig. 6D). The latter finding is in agreement with previous work (21) that suggested that C2-ceramide inhibits a phosphocholine transferase that synthesizes SM in Madin-Darby canine kidney cells. This inhibition of SM synthesis might cause a higher accumulation of ceramides within the cell. However, the magnitude of the decreased synthesis of SM in this experimental system is small by comparison with the increased accumulation of [3H]ceramide (Fig. 6, C and D). Also in this experimental system, we did not detect any significant production of ceramide 1-phosphate.

The mechanism of the ceramide-induced increase in SPP degradation was studied further in cell-free preparations. Sonicates of the Rat2 fibroblasts exhibited an SPP-dependent formation of P, that was optimum at 0.4–0.5 mM SPP (Fig. 7A). This activity was inhibited by ~90% with 5–10 mM NaF (Fig. 7B). The reaction was not dependent upon the presence of Mg2+ or Ca2+, and preincubation with N-ethylmaleimide did not alter the activity significantly. The phosphohydrolase is membrane-associated since it was recovered in the particulate fraction of cell sonicates after ultracentrifugation (data not shown). These characteristics are similar to those of plasma membrane phosphatidate phosphohydrolase (35, 42). Phosphatidate phosphohydrolase activity in fibroblasts is increased by cell-permeable ceramides or by treatment with sphingomyelinase (20). The presence of a protein in rat fibroblasts similar to rat liver phosphatidate phosphohydrolase was confirmed by Western blot analysis (Fig. 8) using affinity-purified antibody generated against the homogeneous liver phosphatidate phosphohydrolase (61). The predominant immunoreactive protein in both rat fibroblasts and liver homogenates has an apparent molecular mass of 51–53 kDa. If the same enzyme were hydrolyzing both PA and SPP in Rat2 fibroblasts, then SPP should inhibit the activity of phosphatidate phosphohydrolase. This prediction was confirmed since increasing concentrations of SPP decreased PA hydrolysis by >80% (Fig. 8). The ceramide-induced hydrolysis of SPP (Fig. 6) is therefore explained by the stimulation of a phosphatidate phosphohydrolase (20) that also degrades SPP.

**DISCUSSION**

We recently demonstrated that cell-permeable ceramides (C2- and C6-ceramides) specifically inhibit the mitogenic effects of PA, lyso-PA, and ceramide 1-phosphate in rat fibroblasts (20, 66). We have now shown a ceramide-dependent inhibition of the stimulation of DNA synthesis by SPP (Figs. 1 and 2). Maximum effects on DNA synthesis were obtained at 2 µM SPP, which was ~25 times less than for PA or lyso-PA (Fig. 2) (20). Higher concentrations (~5 µM) of SPP failed to stimulate [3H]thymidine incorporation into DNA or were inhibitory (Fig. 3). Microscopic examination of cells incubated with 10 and 20 µM SPP did not reveal any obvious toxic effects. These high concentrations of SPP could generate sufficient quantities of

**TABLE II**

| Additions      | Relative production of [3H]ceramide |
|---------------|-----------------------------------|
|               | No SPP                             |
|               | +2 µM SPP                          |
| None          | 0.22 ± 0.02 (12)                   | 0.21 ± 0.01 (4)           |
| C2-ceramide   | 0.55 ± 0.03 (12)                   | 0.53 ± 0.05 (4)           |
| C6-ceramide   | 1.92 ± 0.19 (7)                    | 2.04 ± 0.22 (2)           |
ceramides to block the stimulation of DNA synthesis by SPP (Fig. 6). Moreover, C2-ceramide enhanced the inhibition of DNA synthesis at 10 μM SPP (Fig. 3), and this could have resulted from a more rapid conversion of SPP to ceramide (Fig. 6) together with ceramide production from endogenous lipids (Table II). Cell-permeable ceramides do not inhibit DNA synthesis induced by insulin alone (20), and insulin also reverses the inhibitory effect of C2-ceramide on the SPP-induced synthesis of DNA.

C2-ceramide was much less effective in inhibiting DNA synthesis induced by sphingosine than that by SPP even when compared at equivalent stimulations of ~2-fold (Figs. 1 and 2 versus 3). Although some of the mitogenic effects of sphingosine may involve its conversion to SPP (44), our results indicate that sphingosine per se may also affect DNA synthesis by a different mechanism than that of SPP. Furthermore, although both sphingosine and SPP increase cellular Ca2+ concentrations (45, 46, 67), the effect of sphingosine depends on phosphoinositide breakdown, whereas that of SPP does not (45). Ca2+ ions have also been involved in the induction of early events of mitogenesis (44). However, cell-permeable ceramides have no effect on either phosphoinositide turnover or intracellular Ca2+ concentration (45), suggesting that the inhibition of sphingosine- or SPP-induced DNA synthesis by ceramides is independent of Ca2+ mobilization. Our results also imply that the ceramide inhibition of DNA synthesis is independent of effects on adenylate cyclase.

Stimulation of fibroblasts with as little as 10 nM SPP causes a significant increase in PLD activity (Fig. 4). It seemed possible that cell-permeable ceramides might inhibit PLD by stimulating the activity of a phosphoprotein phosphatase (25, 26). However, okadaic acid alone stimulated PLD activity slightly and did not reverse the effects of C2-ceramide in inhibiting PLD activation by SPP. Therefore, the effect of C2-ceramide in this case cannot be explained by stimulation of an okadaic acid-sensitive phosphoprotein phosphatase. Furthermore, the strong inhibition by okadaic acid of the activation of PLD by SPP indicates that this process may be dependent upon the stimulation of a phosphatase activity. The need for a relatively high concentration of okadaic acid (0.5–1.0 μM) indicates that phosphatase 1 as well as phosphatase 2A may be involved.

By contrast to the effects of SPP, sphingosine did not activate PLD significantly after 10 min. However, treating the fibroblasts for 2 h with 20 μM sphingosine increased PLD activity by 85 ± 28% in three independent experiments. This longer time course for PLD activation by sphingosine was also reported by Lavie and Liscovitch (40) and could involve the formation of SPP (44, 46).

Although externally added PA, lyso-PA (20, 62), or SPP is a potent mitogen for fibroblasts, the activation of PLD (and presumably the generation of PA at the inner surface of the plasma membrane) is not sufficient in itself to stimulate DNA synthesis (20). For example, endothelin is also a potent stimulator of PA generation through PLD, but it is a poor mitogen (68). Therefore, an inhibition of PLD activation by ceramides may not in itself be sufficient to account for their antimitogenic effects.

Exogenous SPP is rapidly dephosphorylated and metabolized by rat (Fig. 6) and human (65) fibroblasts. We have determined that the dephosphorylation of SPP is catalyzed by a F−-sensitive, membrane-associated phosphohydrolase that does not require bivalent cations and is not inhibited by N-
Insulin could be an important factor in controlling ceramide action since it can attenuate the inhibition of the SPP-induced DNA synthesis by ceramides (Fig. 2). However, insulin did not have any significant effect on the inhibition of the SPP-induced PLD activation by ceramide (data not shown). The stimulation of DNA synthesis is regulated by several independent mechanisms, and not all of these are controlled by ceramides. For example, the incorporation of thymidine into DNA that was induced by insulin (Fig. 2), platelet-derived growth factor, or epidermal growth factor (data not shown) was not inhibited by ceramide. Furthermore, the sphingosine-induced synthesis of DNA is decreased by only −16% by C2-ceramide (Fig. 3).

In conclusion, the effects of ceramides in decreasing PLD activity were not blocked by okadaic acid, and the latter inhibited the activation of PLD by SPP. The antiangiogenic effects of cell-permeable ceramides were not caused by reversing the SPP-induced decrease in CAMP. Insulin could play an important role in ceramide action since it counteracts the inhibition of the SPP-induced DNA synthesis by ceramides. SPP and sphingosine may function through different mechanisms to stimulate DNA synthesis since C2-ceramide inhibits the stimulation of DNA synthesis by SPP completely, but has little effect on DNA synthesis induced by sphingosine. Ceramides stimulate the degradation of SPP to sphingosine and ceramide and stimulate the production of ceramides from endogenous lipids. The increased degradation of SPP can now be attributed to the stimulation of a phosphohydrolase that also degrades PA. Our results emphasize that the balance in the production of ceramide versus sphingosine and SPP after sphingomyelinase activation could determine the patterns of cell signaling that control the extent of DNA synthesis, cell division, and cell death.

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