Cell-permeable Ceramides Inhibit the Stimulation of DNA Synthesis and Phospholipase D Activity by Phosphatidate and Lysophosphatidate in Rat Fibroblasts*

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The interactions of ceramides with phosphatidate and lysophosphatidate in the regulation of signal transduction in rat fibroblasts were examined. C2 and C6-ceramides (N-acetylsphingosine and N-hexanoylsphingosine, respectively) at 10 μM inhibited the stimulation of DNA synthesis that was produced by 50-100 μM phosphatidate, or lysophosphatidate, or by exogenous phospholipase D. Sphingosine (10 μM) had the opposite effect to the ceramides on DNA synthesis. C2- or C6-ceramides failed to inhibit the stimulation of DNA synthesis by insulin or serum. The ceramides did not modify the actions of phosphatidate, or lysophosphatidate, in decreasing the forskolin-induced increase in cAMP. C2- and C6-ceramides inhibited the stimulation of phospholipase D activity by: (a) phosphatidate, lysophosphatidate, phosphorib ester, thrombin, or serum in intact fibroblasts and (b) phosphatidyl ester or guanosine 5’-3-O-(thio)triphosphate in permeabilized fibroblasts. The ceramides can therefore modify cell signaling via phosphatidate D, but this effect alone could not explain the decreased DNA synthesis. Incubation of fibroblasts with C2- or C6-ceramides or sphingomyelinase inhibited the interaction of exogenous phosphatidate or lysophosphatidate with the fibroblasts by 42 and 53%, respectively. Furthermore, a greater proportion of the phosphatidate, or lysophosphatidate, that was associated with the fibroblasts was metabolized further when the cells were pretreated with ceramides or sphingomyelinase. This effect was accompanied by an increased activity of N-ethylmaleimide-insensitive phosphatidate phosphohydrolase. Ceramides may therefore produce part of their growth inhibitory effects by blocking some of the signal transducing effects of phosphatidate and lysophosphatidate.

Sphingomyelin turnover is now recognized as an important pathway of signal transduction. Several reports have suggested that sphingomyelin may participate in a lipid second messenger system that mediates the action of various important biomodulators (1, 2). It has also been suggested that ceramide, the immediate product of sphingomyelin degradation, may play an important role in cellular differentiation (2, 3). Stimulation of cells with 1,2-dihydroxyvitamin D3 (4), tumor necrosis factor-α, or γ-interferon (5) activates a neutral sphingomyelinase that hydrolyzes membrane sphingomyelin to generate intracellular ceramides. Tumor necrosis factor-α also activates an acidic sphingomyelinase through the production of 1,2-diacylglycerol via stimulation of a PC-PLC. The subsequent generation of intracellular ceramides then triggers the rapid induction of a nuclear transcription factor system, NF-κB (6). An activation of the sphingomyelin signaling pathway by interleukin-1 has also been demonstrated in EL4 cells (7). The addition of exogenous sphingomyelinase to cells: (a) inhibits phorbol ester-induced differentiation of human promyelocytic leukemic (HL-60) cells (8); (b) inhibits HMGB-CoA reductase, a key regulatory enzyme in the biosynthesis of cholesterol, in rat intestinal epithelial cells (IEC-6); human skin fibroblasts (GM-45), human hepatoma (Hep G2) cells (9); and Caco-2 cells (10); (c) decreases the uptake of cholesterol from bile salt micelles and increases the rate of cholesterol esterification (10); and (d) increases the binding, internalization, and degradation of low density lipoproteins and the synthesis of cholesterol esters in cultured human fibroblasts (11).

Cell-permeable ceramides are valuable tools in studying the mechanisms of action of naturally occurring ceramides in signal transduction. C2-ceramide induces cell differentiation potently in HL-60 cells and inhibits cell growth (12). It also decreases the mRNA for the proto-oncogene, c-myc, an important step in the induction of cell differentiation (5). The potency of C2-ceramide, and probably other cell permeable ceramides, is modulated by the starting cell density rather than its molar concentration (13). The IC50 also depends upon the protein concentration of the medium (13). C2-ceramide has also been reported to induce internucleosomal DNA fragmentation which leads to programmed cell death (apoptosis) (14). In this respect, part of the effect of the human immunodeficiency virus, which is known to induce apoptosis, could be mediated by ceramides (15). C2-ceramide also activates a specific serine/threonine protein phosphatase in mammalian cell extracts (16), and in Saccharomyces cerevisiae (17).

C2-ceramide inhibits protein and DNA synthesis in Madin-Darby canine kidney cells, and it is involved in the inhibition of a phosphocholine transferase that synthesizes sphingomyelin (18). C2-ceramide stimulates phosphorylation of the epidermal growth factor receptor in A431 human epidermoid carcinoma cells (19) and acts as a competitive inhibitor of diacylglycerol kinase in human leukemia (HL-60) cells (20).

* This work was supported by grants from the Medical Research Council of Canada and the Cancer Research Fund of the University of Alberta. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PC-PLC, phosphatidylcholine-specific phospholipase C; C2, acetyl; C6, hexanoyl; PAP-2, N-ethylmaleimide-insensitive phosphatidate phosphohydrolase (EC 3.1.3.4.); PLC, phospholipase C; PLD, phospholipase D; PMA, 4β-phorbol 12-myristate 13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; GTPγS, guanosine 5’3-O-(thio)triphosphate.
In the present work, we studied the interaction of cell-permeable ceramides with two mitogenic lipids, phosphatidate, and lysophosphatidate. Both of these lipids when added to cells can increase intracellular concentrations of arachidonic acid, and inhibit adenylate cyclase, thus decreasing cAMP through pertussis toxin-sensitive mechanisms (21, 22). Phosphatidate also causes: (a) superoxide generation in neutrophils (23, 24), (b) activation of protein kinase(s) (25), (c) stimulation of purified phosphatidylinositol-4-phosphate kinase (26), (d) inactivation of ras GTPase activating protein (27), and (e) inhibition of a cytosolic protein that inhibits the GTPase activity of ras in vitro (28). The latter two events could cause prolonged activation of p21ras. Lysophosphatidate also induces tyrosine phosphorylation of proteins via a pertussis toxin-sensitive mechanism, thereby activating p21ras (29). PLD activity is stimulated by exogenous lysophosphatidate, and this generates intracellular phosphatidate and diacylglycerol (30). Lysophosphatidate, but not phosphatidate, induces chemotaxis in amoeba (31), activates PLC-γ, and increases intracellular Ca2+ in several cell types. In addition, PLC-γ activity in a pertussis toxin-sensitive manner (32). Although phosphatidate is unable to stimulate PLC-γ, the two phospholipids are equally mitogenic (33).

In the present study, we demonstrate that cell-permeable ceramides block the mitogenic effect of phosphatidate and lysophosphatidate in fibroblasts. The mechanisms by which ceramides exert their effects might involve an inhibition of the interaction and uptake of phosphatidate and lysophosphatidate by the fibroblasts. This may be relevant to the interaction of albumin-bound lysophosphatidate that is found in the circulation and which may interact with the surface of various cells and stimulate cell division (34). We also showed that a greater proportion of the phosphatidate or lysophosphatidate, that was taken up by the fibroblasts, was metabolized further, and this was probably related to the increased PAP-2 activity of the plasma membranes (35). The ceramides also decreased the stimulation of PLD by phosphatidate and lysophosphatidate but were unable to reverse the decrease in cAMP concentrations.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of most of the materials have been described (35-36). DMEM (high glucose), fatty acid-poor bovine serum albumin, cAMP, GTPγS, insulin, isobutylmethylxanthine, sphingosine, phosphatidate, lysophosphatidate, Type VI PLD from Streptomyces chromofuscus, and thrombin were purchased from Sigma. The fetal bovine serum, penicillin, and streptomycin were from Gibco BRL, Burlington, Ontario. C2 and C6-ceramides were gifts from Drs. R. M. Bell and B. A. Borchardt or were purchased from Matreya, Inc. Pleasant Gap, PA, and [3H]-ceramide or 100 milliunits of sphingomyelinase/ml as indicated. Culture of Fibroblasts—Rat-1 fibroblasts, rat-2 fibroblasts that lack nuclear thymidine kinase (37), and the same cell line transfected by exogenous thymidine-kmDNA (R2TK+) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 10 units penicillin/ml, and 10 μg streptomycin/ml. Fibroblasts were seeded at 1.5 x 10^6 cells/35-mm culture dish and incubated at 37°C with 5% CO2 in air for 3 days for the studies of [3H]thymidine incorporation or cAMP determinations. For determinations of PLD and PAP-2 activities, 60-mm culture dishes were used with equivalent cell densities.

**Determination of DNA Synthesis—**Fibroblasts were grown as indicated above and were maintained in DMEM containing 0.1% fetal bovine serum for 24 h prior to the addition of agonists. 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 μCi/ml) was present for the last 6 h of the incubation (35). This time period was chosen because it gave the maximum incorporation of [3H]thymidine into DNA after stimulation with phosphatidate or lysophosphatidate (Ref. 33 and our own results, not shown). The medium was then removed, and the cells were washed once with phosphate-buffered saline and three times with 10% trichloroacetic acid. Cells were kept in contact with trichloroacetic acid for 10 min in each wash. The precipitated material was dissolved in 0.3 M NaOH containing 1% SDS, and radioactivity was determined by liquid scintillation counting.

**Assay of cAMP—**For these measurements, the lysates from the cells were boiled for 5 min and centrifuged at 12,000 x 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 (38).

**Assay of PLD—**The activity of PLD was determined by measuring the accumulation of [3H]phosphatidylethanol, since this is the product of its transphosphatidylation reaction which is considered to be a definitive assay for PLD (39-41). The treatment of intact fibroblast and the details of the assay have been described previously (36). The activity of PLD in permeabilized fibroblasts was determined in a similar manner to that in intact cells, but in this case, agonists were added in 1 ml of buffer containing: 20 mM Hepes, pH 7.2, 155 mM KCl, 5 mM NaHCO3, 4 mM MgCl2, 1.5 mM CaCl2, 5 mM EGTA, 5.6 mM glucose, 2 mM ATP, 15 μM bovine serum albumin, 85 mM ethanol, and 20 μg/ml digitonin (41). Incubations were then terminated by addition of 1 ml of ice-cold methanol. Cells were scraped into tubes containing 1.5 ml of methanol and 1.25 ml of chloroform. Phases were separated by the addition of 1.25 ml of chloroform and 1.25 ml of 2 M KC1 in 0.2 M H2PO4.

**Assay of PAP—**This enzyme was assayed within its natural membrane using liposomes of phosphatidate as described elsewhere (36).

**Preparation of [3H]Phosphatidate and [3H]Lysophosphatidate—**[3H]Phosphatidate was prepared enzymatically from [3H]glycero-3-phosphate and by using cleate plus palmitate as reported previously (42). Lysophosphatidate was obtained from phosphatidate by mild alkali hydrolysis. [3H]Phosphatidate was dissolved in 3 ml of chloroform/methanol (1:1, v/v). To this was added 0.5 ml of 0.35 M NaOH in 90% methanol. After 10 min at room temperature, the solution was adjusted to about pH 7 with 0.9 ml of 0.2 M HCL. Then 0.9 ml of 2 M KC1 and 0.5 ml of chloroform were added. The tube was inverted three times very gently and then centrifuged at 1,000 rpm for 10 min to separate the phases. The bottom phase was removed, and the top phase was washed twice with chloroform. All of the chloroform phases were then added and the lipid was then resuspended in chloroform and applied to a thin layer plate of silica gel. The plate was developed with chloroform/methanol/water/acetic acid (50:25:3:7, by volume). The positions of the standard lipids were identified after staining part of the plate with iodine vapor. The position of the [3H]lysophosphatidate was visualized by spraying the remainder of the plate lightly with distilled water, and the coincidence with the [3H]was confirmed with a Bioscan System 200 Imaging Scanner. The [3H]lysophosphatidate was scraped from the plates and was then eluted from the silica gel with chloroform/methanol/acetic acid/water (50:39:1:10, by volume). After centrifuging at 1,000 rpm for 10 min, the solvent containing [3H]lysophosphatidate was separated from the silica gel and was dried under rotary evaporator. The [3H]lysophosphatidate was then resuspended in chloroform and stored at -20°C until it was diluted with nonradioactive lysophosphatidate for use in experiments.

**Measurement of the Interaction and Metabolism of [3H]Phosphatidate and [3H]Lysophosphatidate—**Fibroblasts were preincubated in DMEM containing 0.5% bovine serum albumin with 50 μM C6-ceramide or 100 milliunits of sphingomyelinase/ml as indicated. After 2 h, 50 μM [3H]lysophosphatidate (7.2 Ci/mmol), or [3H]lysophosphatidate (2.75 Ci/mmol), were then added, and the incubation was continued for a further 5 min. The medium was removed, and the cells were washed immediately with three batches of ice-cold DMEM containing 0.1% albumin. The cells were removed from the plates with 1 ml of ice-cold methanol, and lipids were extracted and analyzed as described for the PLD activities (36).

**RESULTS**

**Effects of Ceramides on the Stimulation of Thymidine Incorporation into DNA—**The growth of the fibroblasts was inhibited after 3 days in culture by decreasing the serum concentration from 10 to 0.1% in the incubation. At this stage the fibroblasts were 70-80% confluent. We adopted this procedure because we wished to study the effects of mitogenic and antimitogenic agents before the cells had become contact inhibited for growth. Cells were then treated with phosphatidate, or lysophosphatidate to produce the expected mitogenic effect, and to characterize the rat-1 and R2TK+ fibroblast systems. Phosphatidate and lysophosphatidate were almost equally mi-
were incubated for 22 h in serum-free DMEM in the absence or presence of 50 μM phosphatidate (●, ■) and the indicated concentrations of C2-ceramide (▲) or C6-ceramides (△). [3H]Thymidine was present during the last 6 h of incubation. Basal [3H]thymidine incorporation corresponds to 26,492 ± 1,281 dpm/dish in 10 independent determinations. Results are expressed relative to the control value with no additions as described in the legend to Fig. 1. The results from one experiment performed in duplicate and they were confirmed in a second independent experiment. Results are expressed relative to the control value.

**FIG. 2.** Effect of cell-permeable ceramides on the synthesis of DNA induced by increasing concentrations of phosphatidate.

Fibroblasts (R2TK) were incubated for 22 h in serum-free DMEM with increasing concentrations of phosphatidate in the absence (●, ■) or in the presence of 10 μM C2-ceramide (▲) or 10 μM of C6-ceramide (△). [3H]Thymidine was present during the last 6 h of incubation. Results are from three to five independent experiments. The other values are means from two independent experiments and where large enough the range is shown.

**FIG. 3.** Effects of sphingosine or cell-permeable ceramides on the stimulation of DNA synthesis by phosphatidate or lysophosphatidate. Fibroblasts (R2TK) were incubated as in Fig. 1 with 10 μM sphingosine, 10 μM C2-ceramide, or 10 μM C6-ceramide in the presence or absence of 50 μM phosphatidate or 50 μM lysophosphatidate. Results are expressed relative to the control value and they are means ± S.E. of three to five independent experiments.

**TABLE I**

Effects of C2-ceramide on the synthesis of DNA induced by incubation with exogenous PLD

| PLD added | Relative incorporation of [3H]thymidine into DNA |
|-----------|-----------------------------------------------|
| No ceramide | +10 μM C2-ceramide |
| units/ml | 0 | 1 | 10 |
| 0 | 1.00 | 0.68, 0.74 |
| 1 | 2.83, 3.74 | 1.52, 0.61 |
| 10 | 3.58, 3.88 | 1.77, 1.31 |

As an alternative to adding exogenous phosphatidate to the cells, we also generated phosphatidate within the plasma membrane by treating the fibroblasts with 1 or 10 units of PLD/ml. This procedure stimulated DNA synthesis (Table I) to a similar extent to that obtained with phosphatidate (Fig. 1). Addition of C2-ceramide with both concentrations of PLD also diminished the mitogenic stimulus.

It is possible that the effect of the ceramides might have been caused by their conversion to sphingosine. Therefore, the effect of this latter compound on DNA synthesis was also examined. Sphingosine (10 μM) increased DNA synthesis by 2.5-fold (Fig. 3) and 20 μM sphingosine caused a 4.3-fold increase (results not shown). This effect of sphingosine was expected from the work of Zhang et al. (43). Sphingosine concentrations above 25 μM in our studies caused the fibroblasts to detach from the culture dishes. The stimulation caused by 10 μM sphingosine was also seen in the presence of 50 μM of phosphatidate or lysophosphatidate, although the effects were not strictly additive. These results therefore demonstrate that sphingosine and ceramides have completely different effects on DNA synthesis either in the absence or presence of phosphatidate or lysophosphatidate.

We also tested to see whether the mitogenic effects of 10% serum or 340 nm insulin could be reversed by the C2- or C6-
TABLE II
Effect of C2-ceramide on the cAMP concentration in rat fibroblasts

| Forskolin | Lipids added | Relative cAMP concentration |
|------------|--------------|-----------------------------|
|            |              | No cAMP | 10 µM C2-ceramide |
| Absent     | None | 1 | 0.89 ± 0.03 |
| Phosphatidate (50 µM) | 0.65 ± 0.09 | 0.59 ± 0.11 |
| Lysocephosphatidate (50 µM) | 0.62 ± 0.06 | 0.61 ± 0.09 |
| Present    | None | 23.6 ± 5.2 | 19.56 ± 7.6 |
| Phosphatidate (50 µM) | 11.7 ± 3.2 | 10.0 ± 3.5 |
| Lysocephosphatidate (50 µM) | 13.0 ± 4.1 | 11.9 ± 6.8 |

C2- and C6-ceramides Antagonize the Effects of Phosphatidate and Lysocephosphatidate in Decreasing the Production of cAMP in Fibroblasts—Lysocephosphatidate decreases cAMP concentrations in fibroblasts, and this has been linked to its mitogenic effect (22). We therefore investigated whether C2- and C6-ceramides might modify the effects of phosphatidate or lysocephosphatidate in lowering cAMP.

Incubation of the fibroblasts with C2-ceramide alone had no significant effect on the actions of phosphatidate or lysocephosphatidate in decreasing cAMP concentrations (results not shown).

C2- and C6-ceramides Do Not Reverse the Effects of Phosphatidate or Lysocephosphatidate in Decreasing the Production of cAMP in Fibroblasts—Lysocephosphatidate decreases cAMP concentrations in fibroblasts, and this has been linked to its mitogenic effect (22). We therefore investigated whether C2- and C6-ceramides might modify the effects of phosphatidate or lysocephosphatidate in lowering cAMP.

The two ceramides also inhibited the stimulating effects of PMA, thrombin, and serum on PLD activity (Table III). The maximum inhibition of the PMA-induced stimulation of PLD activity was obtained after about 30 min of incubation with either C2- or C6-ceramide (Fig. 4A). The maximum inhibition was observed with about 50 µM of either ceramide in the presence of 0.5% bovine serum albumin (Fig. 4B).

Experiments were also performed with permeabilized fibroblasts to investigate the mechanisms whereby ceramides inhibit the activation of PLD. Both C2- and C6-ceramides antagonized the effects of either PMA or GTPγS in stimulating PLD activity (Table IV).

C2-ceramide and Sphingomyelinase Decrease the Interaction but Increase the Metabolism of Phosphatidate and Lysocephosphatidate by Fibroblasts—In order to study the mechanism of ceramide action, we determined whether they or sphingomyelinase might interfere with the interaction of phosphatidate or

FIG. 4. Effect of cell-permeable ceramides on the PMA-stimulated PLD in rat fibroblasts. Rat-2 fibroblasts were labeled with 1H[3]myristate for 2 h and then incubated in the presence or absence of 50 µM phosphatidate with 0.5% albumin for a further 2 h. The fibroblasts were then treated for 10 min in the presence of 200 µM ethanol, and agonists were added as indicated. PLD activities were determined through the formation of [3H]phosphatidylethanol. The results were calculated as a percentage of radioactivity in phosphatidylcholine (36) and are expressed relative to the incubations that contained neither ceramide nor agonist. The values are given as means ± S.E. for the number of experiments indicated in parentheses or as means ± range for two experiments.

TABLE III
Effects of C2- and C6-ceramides on the activity of PLD in intact rat fibroblasts

| Lipid | No addition | C2-ceramide | C6-ceramide |
|-------|-------------|-------------|-------------|
| None  | 1.00 ± 0.13 (3) | 0.66 ± 0.04 (2) |
| Phosphatidate (50 µM) | 2.12 ± 0.09 (3) | 1.12 ± 0.25 (3) |
| Lysocephosphatidate (50 µM) | 2.00 ± 0.25 (2) | 1.43 ± 0.41 (2) |

The maximum inhibition of the PMA-induced stimulation of PLD activity was obtained after about 30 min of incubation with either C2- or C6-ceramide (Fig. 4A). The maximum inhibition was observed with about 50 µM of either ceramide in the presence of 0.5% bovine serum albumin (Fig. 4B).

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TABLE IV
Effects of C2- and C6-ceramides on the activity of PLD in permeabilized rat fibroblasts

| Treatment with ceramides | No addition | C2-ceramide | C6-ceramide |
|-----------------|-------------|-------------|-------------|
| None            | 1.00 ± 0.13 (3) | 0.66 ± 0.04 (2) |
| Phosphatidate (50 µM) | 2.12 ± 0.09 (3) | 1.12 ± 0.25 (3) |
| Lysocephosphatidate (50 µM) | 2.00 ± 0.25 (2) | 1.43 ± 0.41 (2) |

The maximum inhibition of the PMA-induced stimulation of PLD activity was obtained after about 30 min of incubation with either C2- or C6-ceramide (Fig. 4A). The maximum inhibition was observed with about 50 µM of either ceramide in the presence of 0.5% bovine serum albumin (Fig. 4B).

Experiments were also performed with permeabilized fibroblasts to investigate the mechanisms whereby ceramides inhibit the activation of PLD. Both C2- and C6-ceramides antagonized the effects of either PMA or GTPγS in stimulating PLD activity (Table IV).
lysophosphatidate with the fibroblasts. The cells were preincubated with 50 μg C6-ceramide or with 100 milliunits of sphingomyelinase/ml in 2 h in the presence of 0.5% bovine serum albumin. The fibroblasts were then treated with 50 μg [3H]phosphatidate or [3H]lysophosphatidate, and after 5 min the association of the radioactive lipids with the cells was determined. Incubation of the fibroblasts with C6-ceramide decreased the association of phosphatidate and lysophosphatidate with the fibroblasts by 42 and 45%, respectively. Sphingomyelinase treatment also produced a 53% decrease in the phosphatidate that was associated with the cells (Table V).

We also determined the relative metabolism of the phosphatidate and lysophosphatidate in the presence or absence of C6-ceramide or sphingomyelinase. The relative proportion of the phosphatidate that was metabolized, as calculated by conversion to diacylglycerol, phosphatidylcholine, and triacylglycerol, was increased from 4.1 to 7.7 and 6.6%, respectively, in the presence of C6-ceramide or sphingomyelinase (Table V). Lysophosphatidate was metabolized more rapidly than was phosphatidate. In this case, preincubation of the cells with C6-ceramide increased the relative conversion of lysophosphatidate to monoacylglycerol from 30 to 38%. Sphingosine is an inhibitor of phosphatidate and lysophosphatidate with the cells relative to that in the nontreated cells. This value represented 0.9 ± 0.05% of the total phosphatidate and lysophosphatidate, respectively, that were added to the cells. The values for relative metabolism give the percentages of the phosphatidate, and lysophosphatidate that interacted with the cells which were metabolized further. For phosphatidate this was calculated by the summation of [3H] in diacylglycerol, triacylglycerol, and phosphatidylcholine compared with the total [3H] associated with the fibroblasts. For lysophosphatidate the only product detected after 5 min was monoacylglycerol. Results are means ± S.E. for three independent experiments for phosphatidate and means ± range of two experiments for lysophosphatidate.

### Table V

| Additions       | Phosphatidate | Lysophosphatidate |
|-----------------|---------------|-------------------|
|                 | Relative interaction | Relative metabolism | Relative interaction | Relative metabolism |
| None            | 100           | 4.1 ± 1.0         | 100                  | 30 ± 2.1           |
| C6-ceramide     | 58 ± 4        | 7.7 ± 1.8         | 55 ± 10              | 38 ± 0.9          |
| Sphingomyelinase| 47 ± 3        | 6.6 ± 2.0         | Not determined       |                    |

**DISCUSSION**

Previous work showed that ceramides can induce cell differentiation and inhibit cell growth (4, 5, 12–16). It has been suggested that these effects may involve the stimulation of a serine/threonine protein phosphatase in mammalian cells (16). Ceramide-mediated growth inhibition and ceramide-activated protein phosphatase are conserved in yeast (17). We have now demonstrated that cell-permeable ceramides (C2- and C6-ceramides) specifically inhibit the mitogenic effects of phosphatidate or lysophosphatidate in rat fibroblasts. Both phosphatidate and lysophosphatidate are equally mitogenic in this system, and they often appear to act through the same signaling pathways (32, 33). The mitogenic effect of lysophosphatidate has been associated with the early inhibition of adenylate cyclase (22). Our results (Table II) show that the inhibitory effect of phosphatidate or lysophosphatidate on the generation of cAMP cannot be reversed by the ceramides. Furthermore, the inhibitory effect of the two phospholipids on the forskolin-induced generation of cAMP still occurred in the presence of C2 or C6-ceramides. This implies that the mechanism by which ceramides inhibit the mitogenic effects of phosphatidate or lysophosphatidate is independent of the interaction with adenylate cyclase.

Phosphatidate and lysophosphatidate (Table III) stimulate PLD rapidly, and these effects are strongly inhibited by the cell-permeable ceramides (Table III). Furthermore, the cell-permeable ceramides also blocked the stimulation of PLD by thrombin and serum, showing that this effect is not specific to phosphatidate or lysophosphatidate. PLD activity is also inhibited by the ceramides regardless of whether the agonist activates via protein kinase C or a G-protein mediated event (Table

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2 A. Gomez-Muñoz and D. N. Brindley, unpublished work.
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IV. These results indicate that the PLD pathway could be an important target for the modulation of signal transduction by ceramides. However, the exact mechanism of this effect is unclear at present.

The rapid activation of PLD by different agonists need not be related directly to the mitogenic effect. In fact, endothelin, which is more efficient than lysophosphatidate in generating phosphatidate from phosphatidylcholine via PLD, is a poor mitogen (46). Furthermore, higher concentrations of phosphatidate or lysophosphatidate are required for DNA synthesis than for stimulating early responses (32).

The mechanism of action by which cell-permeable ceramides inhibit thymidine incorporation into DNA is unclear. However, the inhibition of the phosphatidate- or lysophosphatidate-stimulated synthesis of DNA by ceramides is probably related to a decreased interaction of phosphatidate and lysophosphatidate with the fibroblasts and to an enhanced metabolism of these phospholipids (Table V). PAP-2 is responsible for the degradation of phosphatidate in the plasma membrane (35), and this enzyme helps to regulate the concentration of phosphatidate relative to diacylglycerol during agonist stimulation (36). The specific activity of PAP-2 was increased when ceramides were generated by exogenous sphingomyelinase (Fig. 5) or when they were added directly. Therefore, the inhibition of the phospholipid-cell membrane interactions, by ceramides, and the increased rate of degradation of phosphatidate and lysophosphatidate could be important steps in the termination of the mitogen signal of these phospholipids, thus inhibiting cell proliferation. In this respect monacoglycerol, the major metabolic breakdown product of lysophosphatidate failed to induce mitogenesis in rat fibroblasts (47).

It is unlikely that the effect of ceramides on the stimulation of DNA synthesis by phosphatidate or lysophosphatidate is caused solely by a physical interaction of the ceramides with the phospholipids in the incubation medium since 5 μM C2-ceramide almost completely blocked the effect of 50 μM phosphatidate or lysophosphatidate. The interaction of phosphatidate with the fibroblasts was also decreased by sphingomyelinase which should have produced long chain ceramides in the plasma membrane (35). Furthermore, C2-ceramide inhibited the mitogenic action of exogenous PLD, which would have generated phosphatidate in the plasma membrane of the fibroblasts. Exogenous lysophosphatidate is thought to be a physiological mitogen, since it is released by activated platelets and becomes bound to albumin. The lysophosphatidate could instigate local tissue repair in response to vascular damage (34). The existence of a putative cell surface receptor for lysophosphatidate has recently been proposed (46). Therefore, it is possible that the ceramides could play a physiological role in modifying this agonist-receptor interaction in the plasma membrane.

There are likely to be many different mechanisms for the stimulation of DNA synthesis by different agonists, and these may not all be regulated by ceramides. In this regard, the incorporation of thymidine into DNA that was induced by insulin or serum was not inhibited by ceramides (data not shown). By contrast, C2- and C6-ceramides did inhibit the stimulation of PLD by phosphatidate, lysophosphatidate, serum, thrombin, PMA, or GTP-S (Tables III and IV). The activity of PLD alone is not sufficient to stimulate thymidine incorporation into DNA (46). Therefore, the inhibitory effect of ceramides on DNA synthesis must also be mediated by actions other than the inhibition of PLD. It may be significant that an inhibition of diacylglycerol kinase by C2-ceramide has been reported recently (19).

The effects of cell-permeable ceramides on DNA synthesis, or PLD activation, seem to be caused by the ceramides themselves, rather than by their conversion to sphingosine, since the latter compound stimulated these processes. Okazaki et al. (12) also reported the lack of conversion of cell-permeable ceramides to sphingosine in HL-60 cells. Several other reports have shown substantial differences between ceramides and sphingosine in signal transduction. For instance, sphingosine can inhibit protein kinase C, whereas cell-permeable ceramides cannot (48), or cause itsactivation (20). Sphingosine also inhibits phospholipase A2 activity, when C2-ceramide was unable to do so (49). An inhibition of HMG-CoA reductase by sphingosine and the lack of effect of ceramides has also been reported (9), although in this case the authors used long chain ceramides instead of cell-permeable molecules. More recently, ceramides have been shown to activate a protein phosphatase in mammalian cells (16) and yeast (17). The activity of this phosphatase was not affected by sphingosine. Also long term studies with retinoic acid on GH4C1 pituitary cells showed that the concentration of ceramides was elevated, whereas the sphingosine level remained unchanged (50). The concentration of ceramides was also elevated after human immunodeficiency virus infection, whereas the level of sphingosine was hardly changed (15).

In conclusion, this report shows for the first time that cell-permeable ceramides inhibit the effects of phosphatidate and lysophosphatidate in stimulating: (a) 3H-thymidine incorporation into DNA and (b) PLD activation. The antimitogenic effects of C2- and C6-ceramides were not mediated by increasing cAMP concentrations, and they are unlikely to depend solely on the inhibition of PLD activity. The ceramides decrease the interaction of phosphatidate and lysophosphatidate with the fibroblasts and stimulate the degradation of these lipids. These actions specifically decrease the amounts of phosphatidate or lysophosphatidate that can stimulate mitogenesis in the fibroblasts.

Acknowledgments—We thank Drs. R. M. Bell and R. A. Borchardt for their gifts of C2- and C6-ceramides and Dr. A. K. Ho for his help with the assay of cAMP.

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