0026-895X/96/030451-07$3.00/0

MOLECULAR PHARMACOLOGY, 50:451–457 (1996).

Structural Requirements of Sphingosylphosphocholine and Sphingosine-1-phosphate for Stimulation of Activator Protein-1 Activity

ALVIN BERGER,[1] ROBERT BITTMAN, RICHARD R. SCHMIDT, and SARAH SPIEGEL

Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, DC 20007 (A.B., S.S.), Department of Chemistry and Biochemistry, Queens College, The City University of New York, Flushing, New York, 11367 (R.B.), and Universität Konstanz, Fakultät für Chemie, D-78434 Konstanz, Germany (R.R.S.)

Received January 23, 1996; Accepted May 14, 1996

SUMMARY

The sphingolipids sphingosine-1-phosphate (SPP) and sphingosylphosphocholine (SPC) stimulate mitogenesis in Swiss 3T3 fibroblasts and stimulate DNA binding activity of activator protein-1 (AP-1). We show that SPP and SPC were more potent agents than nonphosphorylated sphingosines and *N*-acylsphingolipids (ceramides) with respect to DNA synthesis, AP-1 DNA binding activity, and AP-1 *trans*-activation, illustrating the importance of the terminal phosphate group. The free 2-amino group and the 4*E* double bond of SPC and SPP were found to be important for these activities. Although the combination of decreasing the sphingoid backbone chain length of SPC by two carbons and hydrogenating the 4*E* bond only slightly reduced its effects, in contrast, the same modifications in SPP significantly decreased its mitogenic and AP-1 *trans*-activation effects. Furthermore, substitution of the 3-hydroxyl group in SPP with hydrogen decreased its ability to stimulate DNA synthesis and to stimulate AP-1 transcriptional activity. Thus, critical sphingolipid structural components for AP-1 activation and mitogenic stimulation include the free 2-amino group, the free 3-hydroxyl group, the 4,5-*trans* double bond, and terminal phosphorylation. These observations may be relevant for clinical uses of these compounds in applications such as wound healing and inhibition of metastasis.

In recent years, the sphingolipid metabolite ceramide (*N*-acylsphingosine) has been extensively studied for its antiproliferative properties and its role in cytokine signaling and apoptosis (1, 2). Less is known of two other sphingolipid metabolites, SPC {[(2*S*,3*R*,4*E*)-2-amino-3-hydroxyl-4-octadecen-1-yl][2′-(trimethyammonium)ethyl] hydrogen phosphate}, the *N*-deacyl derivative of sphingomyelin, and SPP {[(2*S*,3*R*,4*E*)-2-amino-3-hydroxyl-4-octadecen-1-yl]dihydrogen phosphate}.

SPC has been reported to be elevated (≤40-fold) in spleen, liver, and brain of patients with Niemann-Pick type A (1A) disease who have a deficiency of acid sphingomyelinase, hepatosplenomegaly, and brain dysfunction (3). SPC has been detected in minute amounts in normal mouse cerebrum (4), in A431 cells (5), and at higher levels (2 mol% of total phospholipid) in human meningiomas (4).

SPC is an extremely potent mitogen in various cell types (6). In fibroblasts, the mitogenic signaling pathways are partly protein kinase C dependent, and SPC did not increase levels of SPP, phosphatidate, inositol phosphate, or cAMP but did stimulate arachidonate release (6–8). SPC has also been reported to rapidly activate MAPK through protein kinase C-dependent and pertussis toxin-sensitive pathways (9), to stimulate tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$) and paxillin (9), and to stimulate a protein kinase related to casein kinase II (10). SPC has been found to mobilize calcium from internal sources, predominately through a previously uncharacterized, inositol-1,4,5-trisphosphate-independent pathway (reviewed in Refs. 6, 11, and 12). In rat basophilic leukemia cells, a novel intracellular SPC-gated calcium-permeable channel with unique pharmacological properties has been characterized (13).

SPP is a bioactive lipid with important second messenger properties: SPP levels increase rapidly and transiently in response to fetal calf serum, PDGF (14, 15), and TPA (16); SPP releases calcium from internal sources in an inositol-1,4,5-trisphosphate-independent manner (7, 17); and SPP

[1] Current affiliation: Nestle Research Center, Nestle, Ltd., Lausanne, Switzerland.

This work was supported by National Institutes of Health Research Grants RO1-CA61774 (S.S.) and HL16660 (R.B.) and National Cancer Institute Postdoctoral Fellowship F32-CA09249 (A.B.).

ABBREVIATIONS: SPC, sphingosylphosphocholine ; SPP, sphingosine-1-phosphate; AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay.

may link sphingolipids and Ras/Raf/Mek/MAPK signaling pathways by elevating phosphatidate levels (18). Like SPC, SPP is a mitogen in several cell types (19–23). In Swiss 3T3 fibroblasts, the induction of DNA synthesis by SPP corresponded with activation of MAPK (24). SPP was also found to decrease cAMP levels in Swiss 3T3 fibroblasts (22). In contrast, in human smooth muscle cells, SPP increased cAMP levels, decreased MAPK, and induced actin filament disassembly and calcium mobilization (15). SPP is not only a strong inhibitor of cell motility and phagokinesis but also an inhibitor of chemoinvasion of tumor cells (25). This effect may be a consequence of changes in assembly of actin filaments. SPP also induced platelet shape changes, aggregation, and intracellular calcium mobilization, indicating that it may play a role in thrombosis, hemostasis, and wound healing (26).

The nuclear events following these early events regulated by SPC and SPP are receiving attention. The transcription factor AP-1 is our focus of study because AP-1 proteins bind to regulatory AP-1 consensus sequences in numerous genes involved in cellular proliferation (27). Previously, we found that SPC (11) and SPP (28) stimulated DNA binding activity of AP-1. SPC also rapidly increased levels of c-*fos* transcripts and *trans*-activated a reporter gene containing AP-1 recognition sites, linking the action of SPC to gene expression (11). The mechanism by which SPC affects AP-1 DNA-binding activity is thought to involve a combination of increased AP-1 protein synthesis and post-translational changes (11).

In the current study, sphingosine, *N*-acylsphingosine, SPC, and SPP analogs were evaluated for effects on DNA synthesis, DNA binding activity, and *trans*-activation of a reporter gene containing AP-1 recognition sites. Structural modifications studied included *N*-acylation, replacement of the hydroxyl group with hydrogen, double bond hydrogenation, changes in sphingoid base chain length, and stereochemical changes (Table 1). This study may have importance for the clinical uses of these compounds. In fact, the multitude of potent biological properties of sphingolipids has sparked pharmacological interest in these compounds. For example, the mitogenic potential of SPC was recently exploited to enhance wound healing in healing-impaired diabetic mice (29). SPC enhanced proliferation of keratinocytes, fibroblasts, endothelial cells, and cells surrounding the sebaceous glands and hair follicles, resulting in complete reepithelialization and granulation tissue formation in excisional and incisional wound sites.

Experimental Procedures

Cell culture. Swiss 3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium/calf serum (9:1) (11).

Growth factors and lipids. Sphingomyelinase (*Streptomyces* sp.) and TPA were from Sigma Chemical (St. Louis, MO), calf serum was from Colorado Serum (Denver, CO), and EGF and insulin were from Collaborative Research (Bedford, MA). SPC (Matreya, Pleasant Gap, PA) was produced by acidic hydrolysis of sphingomyelin and has been reported to be a 72:28 D-*erythro*/L-*threo* mixture (30). *N*-Acetyl-SPC, *N*-hexanoyl-SPC, C_{16}-Dihydro-SPC[2] (90% *erythro*), C_{18}-dihydro-SPC (99% *erythro*), and various sphingosines and *N*-acylsphingosines were also from Matreya. The DL-*threo*- and DL-*erythro*-sphingosines and dihydrosphingosines were from Sigma. D-*erythro*-SPC, D-*erythro*-SPP, and *N*-palmitoyl-SPP were synthetically prepared (31). D-*erythro*-SPP was also obtained from BIOMOL Laboratories (Plymouth Meeting, PA) and produced similar results in all assays to the above synthetically prepared material. 3-Deoxy-D-SPP was prepared in a synthetic sequence[3] that entailed ring opening of (*R*)-benzyl-glycidol with lithium pentadecyne, followed by mesylation of the secondary hydroxy group, azide substitution, and Birch reduction to yield 3-deoxysphingosine, which was then phosphitylated and oxidized (scheme 2 in Ref. 32). C_{16}-dihydro-SPP and C_{18}-dihydro-SPP compounds were produced by phospholipase D transphosphatidylation of the respective SPC products by a method modified from Van Veldhoven *et al.* (30). Briefly, SPC analogs (10 mg) were evaporated to dryness, mixed with 100 μg (220 units) of phospholipase D (*Streptomyces chromofuscus* Type VI, EC 3.1.4.4; Sigma) in 500 μl of 75 mM ammonium acetate, incubated for 3 hr at 30°, and, after pH adjustment to 4, pelleted by centrifugation at 16,000 × *g* for 5 min at 4°. The pellets were redissolved in MeOH and purified by thin layer chromatography using a butanol/water/acetic acid (3:1:1 v/v/v) solvent system. Based on the migration of authentic SPP standard, the silica regions containing SPP analogs were scraped, extracted in 1 ml of MeOH, warmed to 37°, bath-sonicated for 5 min, and then vortexed vigorously for 2 min. After centrifugation at 3000 × *g* for 10 min, the supernatant was passed through a glass syringe with a Teflon filter (Millex-FG, 0.2 μM; Millipore, Bedford, MA) to remove silica particulates. The silica regions containing SPP analogs were reextracted with 5 ml of $CHCl_3$/MeOH/0.1 N HCl (2.7:1.3:1.0 v/v/v), and the $CHCl_3$ layer containing the lipids was pH neutralized. SPP analogs were quantified by phosphorus determination. Unless indicated, lipid solutions were stored in methanol, dried under N_2, and redissolved as 1–2 mM solutions in a 4 mg/ml BSA (Sigma) solution by sonication. Dihydrosphingosines, glucosylsphingosines, and galactosylsphingosines were dissolved in ethanol at 25–50 mM, and small volumes were delivered directly to the cell culture media (final ethanol concentrations were 0.02–0.05%). Cells were treated with appropriate vehicle or ≤20 μM of lipids, with 10 μM being the most common concentration used, because this is a widely used, mitogenic, noncytotoxic concentration for numerous sphingolipids that have been evaluated (6).

DNA synthesis. Cells were grown in 1.77-cm^2 clusters, and DNA synthesis measured as previously described (22). After 18 hr of incubation with 1–20 μM sphingolipids, 1 μCi of [*methyl*-^{3}H]thymidine (55 Ci/mmol; Amersham, Arlington Heights, IL) was added, the cells were incubated for an additional 6 hr, and the incorporation of radioactivity into trichloroacetic acid-insoluble material was measured.

EMSA. Swiss 3T3 cells were cultured in 150-cm^2 petri dishes, refed every 3 days until 90% confluent, and used 5 days later when quiescent. Before treatment, medium was aspirated and replaced with 10 ml of Dulbecco's modified Eagle's medium containing transferrin (5 μg/ml) and BSA (20 μg/ml) for 12 hr. Nuclear extracts (5 μg; prepared according to Ref. 11) were incubated with 1 μg of poly[d(I-C)] (Pharmacia LKB, Piscataway, NJ) and double-stranded [^{32}P]AP-1 consensus oligonucleotide probe (0.3 ng, 2 × 10^6 cpm; Stratagene, La Jolla, CA). The AP-1 oligonucleotide (AP-1 consensus sequence is underlined) was as follows: 5′-CTAGTGA<u>TGAGTCA</u>GCCGGATC-3′. Probes were 5′-end-labeled with [γ-^{32}P]ATP (Amersham) and T4-polynucleotide kinase (New England Biolabs, Beverly, MA) and purified with Sephadex G-50 spin columns (Pharmacia LKB, st. Quentin, France). DNA/protein complexes were resolved on 1.5-mm 6%

[2] C_n refers to the carbon length in the sphingoid base; if not otherwise indicated, the carbon chain length is C_{18}. SPC is also known as sphingosylphosphorylcholine and lysosphingomyelin. Sphingosine and dihydrosphingosine are also known as sphingenine and sphinganine, respectively; galactosylsphingosine is also known as gal psychosine; glucosylsphingosine is also known as glc psychosine; *N*-acylsphingolipids are also known as ceramides; D(+)-*erythro* is 2*S*,3*R*, the naturally occurring isomer; L(−)-*erythro* is 2*R*,3*S*; L(−)-*threo* is 2*S*,3*S*; D(+)-*threo* is 2*R*,3*R*; and DL-*threo* is 2*S*,3*S*/2*R*,3*R* and indicates a racemic mix.

[3] R. Bittman and K. C. Reddy, unpublished observations.

polyacrylamide gels (acrylamide/bisacrylamide 60:1) using 0.5× electrophoresis buffer (1× contains 90 mM Tris, 90 mM boric acid, 2 mM EDTA) at 200 V for 1.5 hr at 25°.

Transfection. The 2×AP-1 LUC plasmid contained two copies of the AP-1 binding site (TCGAGTTGAGTCAGGGTAACGATTGAGTCAGGAG) located 5′ of a 72-bp region of the rat prolactin promoter linked to the luciferase gene and was kindly provided by K. Chien (University of California, San Diego, CA). NIH-3T3 cells (1×10^6 cells; American Type Culture Collection) were grown in 75-cm^2 petri dishes in 15 ml of media and stably transfected by treatment with 1.5 ml of 1× solution (25 mM N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 140 mM NaCl, 0.75 mM Na_2HPO_4, pH 6.96; Sigma) (33) containing 125 mM $CaCl_2$, 5 μg of 2×AP-1 LUC plasmid, 1 μg of pSV2neo plasmid (conferring G-418 resistance), and 30 μg of sonicated salmon sperm carrier DNA (Stratagene, La Jolla, CA). The cells were incubated in 3% CO_2/97% air overnight and then washed twice with phosphate-buffered saline; at 72 hr after transfection, selection was initiated with 1 mg/ml G-418 (GIBCO BRL, Baltimore, MD). Pooled clones were used for experimentation after 1 month of continuous culture. To examine the effect of sphingolipids on luciferase activity, transfectants were seeded onto 10-cm^2 dishes, cultured for 48 hr until 90% confluent, incubated in media containing 0.5% calf serum for 12 hr, and then treated with lipids or vehicle for 6 hr. After two washings with phosphate-buffered saline, cells were incubated with 150 μl of E397A lysis buffer (Promega, Madison, WI) supplemented with phenylmethylsulfonyl fluoride and dithiothreitol (0.5 mM each; Sigma) for 15 min at 25°, scraped to microfuge tubes, vortexed for 15 sec, and centrifuged at 16,000 × *g* for 2 min at 25°, and protein concentration was determined (DC Protein Assay Kit; BioRad Laboratories, Hercules, CA). For luciferase determination, 10 μl (3–6 μg) of lysate was transferred into luminometer cuvettes (Analytical Luminescence Laboratories, Ann Arbor, MI) and thawed to 25°, and 100 μl of assay buffer was autoinjected into each tube using a Monolight 2001 Luminometer (Analytical Luminescence Laboratories). The assay buffer consisted of 20 mM tricine, pH 7.8, 33.3 mM dithiothreitol, 2.67 mM $MgSO_4$, 1.07 mM $MgCO_3$, 0.53 mM ATP (Sigma), 0.47 mM beetle luciferin (potassium salt; Analytical Luminescence), 0.27 mM Coenzyme A (lithium salt; Sigma), and 0.1 mM EDTA. This assay buffer system produced a constant light intensity for ≤1 min.

Results

Mitogenicity. As previously reported (6), SPC and SPP greatly stimulated DNA synthesis and cell division of quiescent cultures of Swiss 3T3 fibroblasts, with 10 μM producing the maximal increase in [^{3}H]thymidine incorporation into DNA (Table 2). SPC and SPP were equally effective at stimulating [^{3}H]thymidine incorporation and were approximately half as effective as PDGF. SPC and SPP were more potent mitogenic agents than any of the nonphosphorylated sphingosine bases tested such as sphingosine, *N*-hexanoyl-sphingosine, and sphingosines with glucose and galactose residues coupled to the 1-hydroxyl group (Table 2).

Hydrogenation of the 4*E* bond of SPC and acetylation of the free amino group greatly diminished mitogenicity. Increasing the hydrophobicity of the *N*-acyl group (as in *N*-hexanoyl-SPC) further reduced activity of SPC.

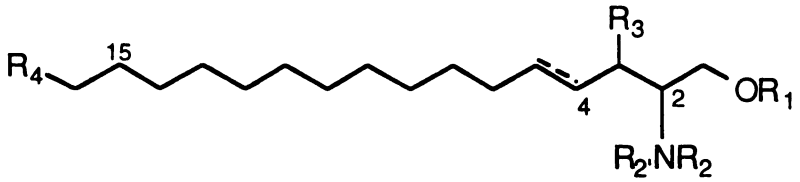
Similarly, hydrogenation of the 4*E* bond of SPP reduced mitogenicity. However, activity was still evident when the free amino group was palmitoylated. The 3-hydroxyl group of SPP was also found to be an important structural component for mitogenicity because 3-deoxy-SPP (lacking the hydroxyl group) had lower activity than SPP.

Interestingly, the combination of shortening the sphingoid

TABLE 1

Structures of various sphingolipids

All of the sphingolipids contained a long-chain hydrophobic sphingoid backbone (saturated or with a 4*E* bond), with either 18 carbons or 16 carbons, a 2-amino- or 2-amide group, and a 3-hydroxyl group. The polar head group (position 1) was either hydroxyl for sphingosines and *N*-acylsphingolipids, phosphate for SPP, phosphocholine for SPC, or a carbohydrate residue.



Compound	R_1	R_2/R_2	R3	4-5-*trans* bond	R_4
Sphingosine	H	H/H	OH	4*E*	C_2H_5
Dihydrosphingosine	H	H/H	OH	Saturated	C_2H_5
N-Acetylsphingosine	H	H/$COCH_3$	OH	4*E*	C_2H_5
N-Acetyldihydrosphingosine	H	H/$COCH_3$	OH	Saturated	C_2H_5
N-Hexanoylsphingosine	H	H/$CO(CH_2)_4CH_3$	OH	4*E*	C_2H_5
N-Hexanoyldihydrosphingosine	H	H/$CO(CH_2)_4CH_3$	OH	Saturated	C_2H_5
1-Glucosylsphingosine	glc	H/H	OH	4*E*	C_2H_5
1-Galactosylsphingosine	gal	H/H	OH	4*E*	C_2H_5
1-Galactosyl-(3′-sulfonyl)-sphingosine	gal (3′ SO_3^-)	H/H		4*E*	C_2H_5
N-Acylsphingosinetrihexoside	trihexose	H/$CO(CH_2)_nCH_3$	OH	4*E*	C_2H_5
SPP	PO_3H^-	$(H/H_2)^+$	OH	4*E*	C_2H_5
Dihydro-SPP	PO_3H^-	$(H/H_2)^+$	OH	Saturated	C_2H_5
C_{16}-Dihydro-SPP	PO_3H^-	$(H/H_2)^+$	OH	Saturated	H
3-Deoxy-SPP	PO_3H^-	$(H/H_2)^+$	H	4*E*	C_2H_5
N-Palmitoyl-SPP	PO_3H_2	H/$CO(CH_2)_{14}CH_3$	OH	4*E*	C_2H_5
SPC	$PO_2^-O(CH_2)_2N^+(CH_3)_3$	H/H	OH	4*E*	C_2H_5
Dihydro-SPC	$PO_2^-O(CH_2)_2N^+(CH_3)_3$	H/H	OH	Saturated	C_2H_5
C_{16}-Dihydro-SPC	$PO_2^-O(CH_2)_2N^+(CH_3)_3$	H/H	OH	Saturated	H
N-Acetyl-SPC	$PO_2^-O(CH_2)_2N^+(CH_3)_3$	H/$COCH_3$	OH	4*E*	C_2H_5
N-Hexanoyl-SPC	$PO_2^-O(CH_2)_2N^+(CH_3)_3$	H/$CO(CH_2)_4CH_3$	OH	4*E*	C_2H_5

TABLE 2

Stimulation of DNA synthesis in quiescent Swiss 3T3 fibroblasts treated with SPC, SPP analogs, and PDGF

Quiescent cultures of Swiss 3T3 cells were treated with 10 μM of the indicated sphingoid bases or 10 ng/ml PDGF-AB (Upstate Biotechnology, Lake Placid, NY). [^{3}H]Thymidine incorporation was measured as described in Experimental Procedures and is expressed as fold stimulation relative to vehicle-treated controls. Each value is the mean ± standard deviation of triplicate determinations from a representative experiment from a minimum of three experiments. If known, the stereochemistry of the compound is given in brackets.

Treatment	[^{3}H]Thymidine
	fold increase
SPC (D-*erythro*)	17.9 ± 2.3
C_{16}-Dihydro-SPC (90% *erythro*)	10.4 ± 1.4
Dihydro-SPC (99% *erythro*)	6.9 ± 1.9
N-Acetyl-SPC	3.1 ± 0.3
N-Hexanoyl-SPC	1.2 ± 0.1
SPP (D-*erythro*)	17.5 ± 2.1
N-Palmitoyl-SPP (D-*erythro*)	7.9 ± 0.04
3-Deoxy-SPP (D)	2.6 ± 0.5
Dihydro-SPP	1.6 ± 0.2
C_{16}-Dihydro-SPP	1.5 ± 0.6
Sphingosine	2.9 ± 0.2
N-Hexanoylsphingosine (D-*erythro*)	2.5 ± 0.2
1-Galactosyl-(3′-sulfonyl)-sphingosine	1.5 ± 0.1
N-Acylsphingosinetrihexoside	1.5 ± 0.2
1-Glucosylsphingosine	1.3 ± 0.1
1-Galactosylsphingosine	1.2 ± 0.1
PDGF	34.1 ± 1.3

backbone chain length by two carbons and hydrogenating the 4*E* bond more profoundly diminished activity in SPP compared with SPC. Cells treated with C_{16}-dihydro-SPC (but not C_{16}-dihydro-SPP) adapted a stellate-like appearance, indicating that this compound induces fibroblast morphological alterations (data not shown).

EMSA. As previously reported, SPC (11) and SPP (28) stimulated AP-1 DNA binding activity as demonstrated by the appearance of a distinct and specific complex in EMSA that could be inhibited by a 10-fold excess of cold AP-1 probe (data not shown). Structural modifications of SPC, such as hydrogenation of the 4*E* bond and *N*-acetylation, decreased binding activity (Fig. 1A). However, the combination of chain shortening of the sphingoid backbone (C_{18} to C_{16}) of SPC and reducing the 4*E* bond did not diminish DNA binding activity (Fig. 1A). In contrast to SPC, the same modifications in the SPP structure resulted in a profound reduction in DNA binding activity (Fig. 1B). Removal of the 3-hydroxyl group in SPP (i.e., 3-deoxy-SPP) or 1-phosphate group (i.e., sphingosine) also diminished DNA binding activity (Fig. 1, B and C). Increasing cellular levels of ceramide by treatment with exogenous sphingomyelinase or by the addition of the cell-permeable ceramide analog *N*-hexanoylsphingosine, conditions that have previously been shown to stimulate DNA synthesis in Swiss fibroblasts (34), also increased DNA binding activity of AP-1 (Fig. 1D).

Transactivation through AP-1 sites. To more quantitatively compare the effects of subtle structural changes of sphingolipids on AP-1 activation, a more sensitive method was used. To this end, the effects of different stimuli on AP-1 transcriptional activity were examined in NIH-3T3 cells stably expressing a truncated AP-1 responsive promoter fused to the luciferase reporter gene (2×AP-1 LUC). Calf serum, PDGF, and TPA (an activator of protein kinase C) were more potent *trans*-activators than were SPC or SPP, whereas EGF, even in the presence of insulin, was less effective (Table 3). As expected, the effect of TPA was abolished in cells made protein kinase C deficient by prolonged TPA pretreatment (Table 3). A similar extent of stimulation of luciferase reporter activity was obtained when cells were maintained in chemically defined media (Opti-MEM, Collaborative Research) supplemented with 2–5% calf serum, when cells were grown on fibronectin- or poly-lysine-coated surfaces and then serum-starved for ≤24 hr before administration of the sphingolipids (data not shown), and when cells were incubated in media containing 0.5% calf serum for 18 hr and then treated with sphingolipids for 6 hr (see below). After 6-hr treatment with 10 μM SPC, luciferase reporter activity was increased 2.4-fold (Table 3). As previously reported (11), a similar induction of luciferase activity was obtained with 20 μM SPC, and the fold increase was either the same or slightly higher in cells treated with D-*erythro*-SPC compared with cells treated with racemic SPC (data not shown).

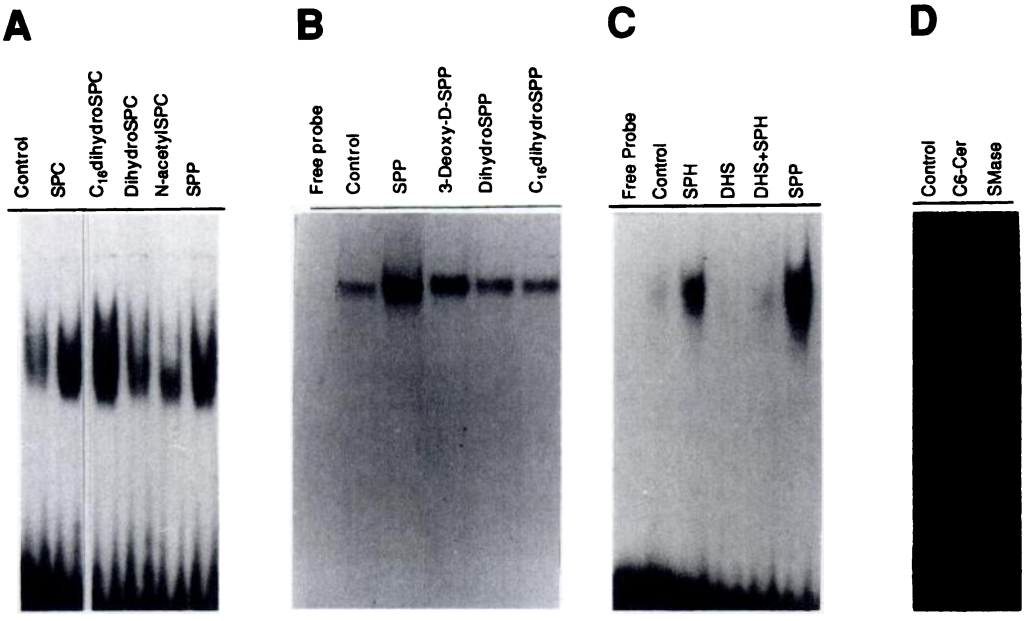


Fig. 1. Activation of AP-1 DNA binding activity by SPC, SPP, sphingosine, and analogs. EMSA were performed using 0.3 ng of [^{32}P]AP-1 probe and nuclear extract (5 μg) prepared from quiescent Swiss 3T3 fibroblasts treated with BSA (*Control*), 10 μM concentration of the indicated sphingolipids, or 20 μM C_6-ceramide for 3 hr. Vehicle (BSA)-treated controls did not show any stimulation in AP-1 DNA binding activity relative to non-vehicle-treated controls, and basal activities did not change with time. *Top band*, specific AP-1 probe/protein complex; *bottom band*, free probe. Autoradiographic results are representative of two or three separate experiments. Refer to Tables 1–2 for the stereochemistry of the various sphingolipids. *DHS*, DL-*threo*-dihydrosphingosine; *SMase*, sphingomyelinase (1 unit/ml); *SPH*, sphingosine; *C6-Cer*, *N*-hexanoyl sphingosine.

TABLE 3

Regulation of AP-1 transcriptional activity by SPC and SPP analogs

NIH-3T3 cells were stably transfected with the 2×AP-1 LUC reporter plasmid as described in Experimental Procedures. Luciferase activity, expressed as light units/10 sec/μg of protein, was measured 6 hr after treatment with 10 μM of the indicated sphingoid bases or growth factors and is expressed as fold stimulation relative to vehicle-treated controls. Fold-stimulations of ≤1.1 are denoted by NE (no effect). Results are mean ± standard deviation from two to seven combined experiments (3–4 samples). If known, the stereochemistry of the compound is given in brackets.

Treatment	Luciferase activity
	fold increase
SPC (D-*erythro*)	2.4 ± 0.3
C_{16}-Dihydro-SPC (90% *erythro*)	2.0 ± 0.1
Dihydro-SPC (99% *erythro*)	1.2 ± 0.2
N-Acetyl-SPC	1.3 ± 0.2
N-Hexanoyl-SPC	NE
SPP (D-*erythro*)	2.5 ± 0.2
Dihydro-SPP	1.2 ± 0.3
3-Deoxy-SPP (D)	1.2 ± 0.1
C_{16}-Dihydro-SPP	NE
N-Hexanoylsphingosine (D-*erythro*)	1.6 ± 0.1
N-Hexanoyldihydrosphingosine (99% *erythro*)	NE
N-Acetylsphingosine (D-*erythro*)	1.3 ± 0.1
N-Acetyldihydrosphingosine (99% *erythro*)	NE
Sphingomyelinase (2 units)	2.1 ± 0.1
Sphingomyelinase (1 unit)	1.4 ± 0.2
Sphingosine (DL-*erythro*)	1.2 ± 0.03
1-Galactosylsphingosine	NE
1-Galactosyl-(3′-sulfonyl)-sphingosine	NE
N-Acylsphingosinetrihexoside	NE
1-Glucosylsphingosine	NE
Calf serum (10%)	15.1 ± 1.9
TPA (100 nM)	10.5 ± 1.5
PDGF (10 ng/ml)	7.9 ± 1.3
EGF (20 ng/ml) plus insulin (4 μg/ml)	1.2 ± 0.2
TPA (1 μM, 24 hr) plus TPA (100 nM, 6 hr)	NE

SPC and SPP were found to be more potent than any nonphosphorylated sphingoid bases evaluated. 1-Glucosylsphingosine and 1-galactosylsphingosine bases had no effects on reporter activity. Hydrogenation of the 4*E* double bond of SPC and attachment of an *N*-acetyl group reduced activity, whereas attachment of an *N*-hexanoyl group completely eliminated SPC-stimulated AP-1 transcriptional activity (Table 3). Hydrogenation of the 4*E* double bond of SPP and replacement of the free 3-hydroxyl group with a hydrogen atom markedly reduced activity (Table 3). Similar to results of the mitogenicity assay and the AP-1 DNA binding assay, the combination of shortening the sphingosine carbon backbone chain length by two carbons and hydrogenating the 4*E* double bond (C_{16}-dihydro-SPP) substantially reduced activity in the SPP structure but not the SPC structure.

Because the double bond in ceramide is critical for its biological effects (1), it was of interest to compare the effects of ceramides with those of dihydroceramides. The dihydro derivatives of *N*-acetylsphingosine and *N*-hexanoylsphingosine, which lack the 4,5-*trans* double bond, were less active than the respective 4*E* monounsaturated parent compounds (Table 3).

Discussion

Structural modifications of SPC and SPP had profound effects on DNA synthesis, AP-1 binding activity, and AP-1 *trans*-activation. It is likely that SPC and SPP mediate their effects by binding to specific targets. These modifications may thus have affected the ability of SPC and SPP to interact with their respective cell surface or internal targets. Another possibility is that structural modifications may affect the uptake and subsequent metabolism of these sphingolipids.

In general, there is better agreement between the *trans*-activation assay of AP-1 activity and DNA synthesis because they are both *in vivo* assays, whereas EMSA only measures *in vitro* DNA binding activity of AP-1. Consistency between the results of the [^{3}H]thymidine assay and AP-1 transcriptional activity is not surprising because AP-1 is a critical transcription factor for cellular proliferation (27). However, it is now established that enhanced Myc synthesis is also required for progression of quiescent fibroblasts from the G_0 to the G_1 phase of the cell cycle (35), and inconsistencies could be due to the relative importance of this pathway. In our study, there was an example of a lack of agreement between the various assays: C_{16}-dihydro-SPC stimulated DNA binding activity similarly to SPC but was less active in the transcriptional and mitogenesis assays. There are several possible explanations for the apparent discrepancy, including temperature differences (25°, EMSA; 37°, *trans*-activation assay); kinetic differences (3 hr, EMSA; 6 hr, *trans*-activation assay); AP-1 binding site differences, which can possibly affect binding of AP-1 dimers (one AP-1 binding site, EMSA; two AP-1 binding sites with a different surrounding sequence, *trans*-activation assay); and the fact that the EMSA is a qualitative assay designed to detect large differences in binding activity, whereas the *trans*-activation assay is a sensitive quantitative assay.

The role of sphingoid base phosphorylation. Consistent with previous results in Swiss 3T3 fibroblasts (6), SPC was much more mitogenic than other lysophospholipids and structurally related sphingolipids that were examined (including *N*-acylsphingosine and sphingosine) and more potent than some growth factors (insulin and EGF). The fact that SPP was more potent than sphingosine in all three assays exemplifies the importance of terminal phosphorylation with respect to bioactivity. Interestingly, in Rat-1 fibroblasts, sphingosine was found to be more mitogenic than SPP (21), demonstrating differences in sphingolipid metabolism and signaling pathways in different cell lines.

The role of sphingoid base glycosylation. Glycosylated sphingosine bases are naturally occurring structural components of gangliosides. *N*-Deacyl-glycosylated bases, such as those tested for bioactivity in the current study, occur in high levels in some sphingolipidoses and are reported to have cytotoxic effects (36). In both mitogenicity assays and AP-1 reporter assays, the glycosylated bases were ineffective (Tables 2 and 3). The uptake of these compounds was not examined in this study. However, in other cell systems they seem to be taken up and to have specific biochemical effects. For example, in thrombin-stimulated intact human platelets, 40 kDa protein phosphorylation (an index of protein kinase C activity) was inhibited by 25 μM concentrations of galactosylsphingosines, glucosylsphingosines, and sulfogalactosylsphingosines, whereas *N*-acetylgalactosylsphingosine was ineffective (36).

The role of the free amino group and *N*-acyl chain length. The importance of the free amino group for bioactivity of sphingolipids was readily apparent in the present studies (e.g., SPC versus *N*-acetyl-SPC; Tables 2 and 3). In agreement, *N*-acetyl-SPC was previously found to be less effective

than SPC in increasing intracellular calcium concentrations in rat pancreatic acinar cells, and longer-chain *N*-acyl-SPC (sphingomyelin) was found to be completely ineffective (37). Intracellular calcium concentrations are known to affect DNA synthesis and to affect AP-1 activation via calcium-induced transcriptional enhancement (38). Thus, the lesser effect of *N*-acetyl-SPC relative to SPC with respect to mitogenic potential (Table 2), AP-1 binding activity (Fig. 1A), and transcriptional activation (Table 3) could be linked to differences in abilities to mobilize calcium. SPC is also considerably more active at stimulating mitogenesis than sphingomyelin (6), which also suggests that the free amino group is important for biopotency. *N*-Palmitoyl-SPP was only half as effective as SPP in stimulating DNA synthesis, which further illustrates the importance of the free amino group. In contrast, in Rat-1 fibroblasts, *N*-acetyl-SPP and *N*-octanoyl-SPP were as effective as SPP in stimulating DNA synthesis at optimally mitogenic concentrations (39), suggesting that the free amino group may not be important for mitogenicity in this cell type. The uptake and metabolism of long-chain *N*-acyl-SPP, which is naturally occurring in some cell types (40), have not been well studied, but short-chain *N*-acyl-SPPs were not deacylated to SPP *in vivo* (39, 41). However, in other studies, the requirement of a free amino group in the sphingosine backbone for bioactivity is not apparent. For example, in the fusion of Semliki Forest virus with target membranes, sphingosine was ineffective in promoting fusion, whereas N-acylsphingosine or sphingomyelin was required (42, 43).

Differences observed in promoter activities between cells treated with sphingomyelinase and cells treated with short-chain *N*-acylsphingosines (Table 3) may be related to the chain length of the *N*-acyl group because treatment of fibroblasts with sphingomyelinase is known to produce long-chain endogenous *N*-acylsphingosines derived from sphingomyelin (34). In agreement, in other cell types, differences in bioactivity in response to treatment with short-chain *N*-acylsphingosines and sphingomyelinase have also been reported. Treatment of HL-60 cells with sphingomyelinase stimulated MAPK activity, whereas *N*-acetylsphingosine had no effect (44).

The role of the 3-hydroxyl group. Naturally occurring sphingolipids with modifications of the 3-hydroxyl group, such as ganglioside GM3 with a 3-*O*-acetyl sphingosine base, have been discovered (45), and 3-ketodihydrosphingosine is the natural precursor of dihydrosphingosine. To our knowledge, the bioactivity of 3-deoxy-SPP has not been previously examined. Compared with SPP, this compound was a less potent mitogen and produced lower AP-1 transcriptional activation. Replacing the hydroxyl group with hydrogen would reduce polarity and result in weaker intermolecular and intramolecular hydrogen bonding (46), which could ultimately affect uptake, stability, and target interactions. The 3-hydroxyl group has been shown to be of biological importance in other sphingolipids. In sphingomyelinase assays, 3-deoxy-sphingomyelin was not an effective substrate compared with sphingomyelin (47), and the 3-hydroxyl group of the sphingosine backbone was found to play a key role in supporting fusion of Semliki Forest virus with liposomes (43, 48). However, the 3-hydroxyl group was not essential for the interaction of sphingomyelin with cholesterol in vesicles and monolayers (49, 50).

The role of the 4*E* double bond. Sphingoid bases with saturated alkyl chains are naturally occurring precursors in the synthesis of sphingomyelin. Hydrogenation of the 4*E* bond of both SPC and SPP dramatically reduced activity in all three assays. Dihydro-SPP has been reported to be 10-fold less effective at inhibiting F1 melanoma cell haptotactic motility, which also illustrates the importance of the 4*E* bond with respect to bioactivity (51). Hydrogenation of the 4*E* bond of *N*-acylsphingosines has also been found to be critical for bioactivity in several cell systems (48, 52, 53). D-*Erythro*-*N*-acetylsphingosine, but not D-*erythro*-*N*-acetyldihydrosphingosine, was able to inhibit cell growth and c-*myc* expression and induce apoptosis and activation of a ceramide-activated protein phosphatase in HL-60 leukemia cells (52).

Differences in bioactivity between *trans*-monounsaturated and -saturated sphingosine bases may be related to differences in rates of cellular catabolism to inactive breakdown products. In this regard, it was recently shown that dihydro-SPP was more rapidly degraded than SPP in cultured skin fibroblasts (54).

The role of sphingoid base length. The predominant chain length for sphingolipids is C_{18}, although C_{20}-sphingosine has been detected in high amounts in some tissues, such as the central nervous system (45), and C_{16}-sphingosine backbones have been reported in some invertebrates (47). Chain length may be important for membrane intercalation and uptake of sphingosine because in human neutrophils incubated with 10 μM sphingosine, less C_{16}-sphingosine than C_{18}-sphingosine was associated with the cells (55).

The fact that hydrogenation and chain-shortening reduced activity in SPP but not SPC could be due to differences in stability, target recognition, and signaling pathways. In this regard, it is well established that SPC and SPP do not share common signaling pathways (7, 20).

In this study, we examined structure-function relationships of SPC, SPP, and analogs with respect to mitogenesis, AP-1 binding activity, and AP-1 *trans*-activation. Our results may have implications for the potential clinical use of these sphingolipids. SPC was recently found to directly accelerate dermal wound healing in healing-impaired diabetic mice (29), and SPP might be useful for inhibition of tumor cell invasiveness (25).

Acknowledgments

We express thanks to Yuan Su and Kim Goodemote for technical assistance, to K. C. Reddy for the synthesis of 3-deoxy-SPP, and to Mukesh Verma for reviewing the manuscript.

References

1. Obeid, L. M., and Y. A. Hannun. Ceramide: a stress signal and mediator of growth suppression and apoptosis. *J. Cell. Biochem.* **58:**191–198 (1995).
2. Kolesnick, R., and Z. Fuks. Ceramide: a signal for apoptosis or mitogenesis? *J. Exp. Med.* **181:**1949–1952 (1995).
3. Rodriguez-Lafrasse, C., and M. T. Vanier. Sphingosyl-phosphorylcholine in Niemann-Pick disease brain: accumulation in type A but not in type B. *J. Neurochem.* **61**(suppl)**:**101 (1993).
4. Seijo, L., T. E. Merchant, L. T. van der Ven, B. D. Minsky, and T. Glonek. Meningioma phospholipid profiles measured by ^{31}P nuclear magnetic resonance spectroscopy. *Lipids* **29:**359–364 (1994).
5. Lavie, Y., J. K. Blusztajn, and M. Liscovitch. Formation of endogenous free sphingoid bases in cells induced by changing medium conditions. *Biochim. Biophys. Acta* **1220:**323–328 (1994).
6. Desai, N. N., R. O. Carlson, M. E. Mattie, A. Olivera, N. E. Buckley, T. Seki, G. Brooker, and S. Spiegel. Signaling pathways for sphingosylphosphorylcholine-mediated mitogenesis in Swiss 3T3 fibroblasts. *J. Cell Biol.* **121:**1385–1395 (1993).
7. Mattie, M. E., G. Brooker, and S. Spiegel. Sphingosine-1-phosphate, a

putative second messenger, mobilizes calcium from internal stores via an inositol trisphosphate-independent pathway. *J. Biol. Chem.* **269:**3181–3188 (1994).
8. Törnquist, K., and E. Ekokoski. Effect of sphingosine derivatives on calcium fluxes in thyroid FRTL-5 cells. *Biochem. J.* **299:**213–218 (1994).
9. Seufferlein, T., and E. Rozengurt. Sphingosylphosphorylcholine activation of mitogen-activated protein kinase in Swiss 3T3 cells requires protein kinase C and a pertussis toxin-sensitive G protein. *J. Biol. Chem.* **270:**24334–24342 (1995).
10. Pushkareva, M., and Y. A. Hannun. Modulation of cytosolic protein phosphorylation by sphingosylphosphorylcholine. *Biochim. Biophys. Acta* **1221:**54–60 (1994).
11. Berger, A., D. Rosenthal, and S. Spiegel. Sphingosylphosphorylcholine, a novel signaling molecule which accumulates in Niemann-Pick disease type A, stimulates DNA-binding activity of AP-1. *Proc. Natl. Acad. Sci. USA* **92:**5885–5889 (1995).
12. Dettbarn, C., R. Betto, G. Salviati, R. Sabbadini, and P. Palade. Involvement of ryanodine receptors in sphingosylphosphorylcholine-induced calcium release from brain microsomes. *Brain Res.* **669:**79–85 (1995).
13. Kim, S., V. Lakhani, D. J. Costa, A. I. Sharara, J. G. Fitz, L.-W. Huang, and L. A. Kindman. Sphingolipid-gated Ca^{2+} release from intracellular stores of endothelial cells is mediated by a novel Ca^{2+}-permeable channel. *J. Biol. Chem.* **270:**5266–5269 (1995).
14. Olivera, A., and S. Spiegel. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature (Lond.)* **365:**557–560 (1993).
15. Bornfeldt, K. E., L. M. Graves, E. W. Raines, Y. Igarashi, G. Wayman, S. Yamamura, Y. Yatomi, J. S. Sidhu, E. G. Krebs, S. Hakomori, and R. Ross. Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis of human arterial smooth muscle cells: spatial and temporal modulation of PDGF chemotactic signal transduction. *J. Cell Biol.* **130:**193–206 (1995).
16. Mazurek, N., T. Megidish, S.-I. Hakomori, and Y. Igarashi. Regulatory effect of phorbol esters on sphingosine kinase in Balb/C 3T3 fibroblasts (variant A31): demonstration of cell type-specific response: a preliminary note. *Biochem. Biophys. Res. Commun.* **198:**1–9 (1994).
17. Ghosh, T. K., J. Bian, and D. L. Gill. Sphingosine 1-phosphate generated in the endoplasmic reticulum membrane activates release of stored calcium. *J. Biol. Chem.* **269:**22628–22635 (1994).
18. Spiegel, S., and S. Milstien. Sphingolipid metabolites: members of a new class of lipid second messengers. *J. Membr. Biol.* **146:**225–237 (1995).
19. Zhang, H., N. N. Desai, A. Olivera, T. Seki, G. Brooker, and S. Spiegel. Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J. Cell Biol.* **114:**155–167 (1991).
20. Desai, N. N., H. Zhang, A. Olivera, M. E. Mattie, and S. Spiegel. Sphingosine-1-phosphate, a metabolite of sphingosine, increases phosphatidic acid levels by phospholipase D activation. *J. Biol. Chem.* **267:**23122–23128 (1992).
21. Gomez-Munoz, A., D. W. Waggoner, L. O'Brien, and D. N. Brindley. Interaction of ceramides, sphingosine, and sphingosine-1-phosphate in regulating DNA synthesis and phospholipase D activity. *J. Biol. Chem.* **270:**26318–26325 (1995).
22. Goodemote, K. A., M. A. Mattie, A. Berger, and S. Spiegel. Involvement of a pertussis toxin sensitive G protein in the mitogenic signaling pathways of sphingosine-1-phosphate. *J. Biol. Chem.* **270:**10272–10277 (1995).
23. Miyake, Y., Y. Kozutsumi, S. Nakamura, T. Fujita, and T. Kawasaki. Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. *Biochem. Biophys. Res. Commun.* **211:**396–403 (1995).
24. Wu, J., S. Spiegel, and T. W. Sturgill. Sphingosine 1-phosphate rapidly activates the mitogen-activated protein kinase pathway by a G protein-dependent mechanism. *J. Biol. Chem.* **270:**11484–11488 (1995).
25. Sadahira, Y., F. Ruan, S. Hakomori, and Y. Igarashi. Sphingosine 1-phosphate, a specific endogenous signaling molecule controlling motility and tumor cell invasiveness. *Proc. Natl. Acad. Sci. USA* **89:**9686–9690 (1992).
26. Yatomi, Y., F. Ruan, S. Hakomori, and Y. Igarashi. Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood* **86:**193–202 (1995).
27. Karin, M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **270:**16483–164866 (1995).
28. Su, Y., D. Rosenthal, M. Smulson, and S. Spiegel. Sphingosine-1-phosphate, a novel signaling molecule, stimulates DNA-binding activity of AP-1 in quiescent Swiss 3T3 fibroblasts. *J. Biol. Chem.* **269:**16512–16517 (1994).
29. Sun, L., L. Xu, F. A. Henry, S. Spiegel, and T. B. Nielsen. A new wound healing agent-sphingosylphosphorylcholine: its effects *in vitro* and vivo. *J. Invest. Derm.* **106:**232–237 (1996).
30. Van Veldhoven, P. P., R. J. Foglesong, and R. M. Bell. A facile enzymatic synthesis of sphingosine-1-phosphate and dihydrosphingosine-1-phosphate. *J. Lipid Res.* **30:**611–616 (1989).
31. Kratzer, B., and R. R. Schmidt. Efficient synthesis of sphingosine-1-phosphate, ceramide-1-phosphate, lysosphingomyelin, and sphingomyelin. *Liebigs Ann. Chem.* 957–963(1995).
32. Byun, H. S., R. K. Erukulla, and R. Bittman. Synthesis of sphingomyelin and ceramide-1-phosphate from ceramide without protection of the allylic hydroxyl group. *J. Org. Chem.* **59:**6495–6498 (1994).
33. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7:**2745–2752 (1987).
34. Olivera, A., N. E. Buckley, and S. Spiegel. Sphingomyelinase and cell-permeable ceramide analogs stimulate cellular proliferation in quiescent Swiss 3T3 fibroblasts. *J. Biol. Chem.* **267:**26121–26127 (1992).
35. Eisenman, R. N., and J. A. Cooper. Beating a path to Myc. *Nature (Lond.)* **378:**438–439 (1995).
36. Hannun, Y. A., and R. M. Bell. Lysosphingolipids inhibit protein kinase C: implications for the sphingolipidoses. *Science (Washington D. C.)* **235:**670–674 (1987).
37. Yule, D. I., D. Wu, T. E. Essington, J. A. Shayman, and J. A. Williams. Sphingosine metabolism induces Ca^{2+} oscillations in rat pancreatic acinar cells. *J. Biol. Chem.* **268:**12353–12358 (1993).
38. Lee, G., and M. Gilman. Dual modes of *c-fos* mRNA induction by intracellular calcium in T cells. *Mol. Cell. Biol.* **14:**4579–4587 (1994).
39. Gomez-Munoz, A., P. A. Duffy, A. Martin, L. O'Brien, H. S. Byun, R. Bittman, and D. N. Brindley. Short-chain ceramide-1-phosphates are novel stimulators of DNA synthesis and cell division: antagonism by cell-permeable ceramides. *Mol. Pharmacol.* **47:**833–839 (1995).
40. Dressler, K. A., and R. N. Kolesnick. Ceramide 1-phosphate, a novel phospholipid in human leukemia (HL-60) cells: synthesis via ceramide from sphingomyelin. *J. Biol. Chem.* **265:**14917–14921 (1990).
41. Shinghal, R., R. H. Scheller, and S. M. Bajjalieh. Ceramide 1-phosphate phosphatase activity in brain. *J. Neurochem.* **61:**2279–2285 (1993).
42. Nieva, J. L., R. Bron, J. Corver, and J. Wilschut. Membrane fusion of Semliki Forest virus requires sphingolipids in the target membrane. *EMBO J.* **13:**2797–2804 (1994).
43. Wilschut, J., J. Correr, J. L. Niera, R. Bron, L. Moesby, K. C. Reddy, and R. Bittman. Fusion of Semliki Forest virus with cholesterol-containing liposomes at low pH: a specific requirement for sphingolipids. *Mol. Membr. Biol.* **12:**143–149 (1995).
44. Westwick, J. K., A. E. Bielawska, G. Dbaido, Y. A. Hannun, and D. A. Brenner. Ceramide activates the stress-associated protein kinases. *J. Biol. Chem.* **270:**22689–22692 (1995).
45. Suetake, K., K. Tsuchihashi, K. Inaba, M. Chiba, Y. Ibayashi, K. Hashi, and S. Gasa. Novel modification of ceramide: rat glioma ganglioside GM3 having 3-*O*-acetylated sphingenine. *FEBS Lett.* **361:**201–205 (1995).
46. Koynova, R., and M. Caffrey. Phases and phase transitions of the sphingolipids. *Biochim. Biophys. Acta* **1255:**213–236 (1995).
47. Lister, M. D., Z. S. Ruan, and R. Bittman. Interaction of sphingomyelinase with sphingomyelin analogs modified at the C-1 and C-3 positions of the sphingosine backbone. *Biochim. Biophys. Acta* **1256:**25–30 (1995).
48. Corver, J., L. Moesby, R. K. Erukulla, K. C. Reddy, R. Bittman, and J. Wilschut. Sphingolipid-dependent fusion of Semliki Forest virus with cholesterol-containing liposomes requires both the 3-hydroxyl group and the double bond of the sphingolipid backbone. *J. Virol.* **69:**3220–3223 (1995).
49. Grönberg, L., Z. S. Ruan, R. Bittman, and J. P. Slotte. Interaction of cholesterol with synthetic sphingomyelin derivatives in mixed monolayers. *Biochemistry* **30:**10746–10754 (1991).
50. Kan, C. C., Z. S. Ruan, and R. Bittman. Interaction of cholesterol with sphingomyelin in bilayer membranes: evidence that the hydroxy group of sphingomyelin does not modulate the rate of cholesterol exchange between vesicles. *Biochemistry* **30:**7759–7766 (1991).
51. Sadahira, Y., M. Zheng, F. Ruan, S. Hakomori, and Y. Igarashi. Sphingosine-1-phosphate inhibits extracellular matrix protein-induced haptotactic motility but not adhesion of B16 mouse melanoma cells. *FEBS Lett.* **340:**99–103 (1994).
52. Wolff, R. A., R. T. Dobrowsky, A. Bielawska, L. M. Obeid, and Y. A. Hannun. Role of ceramide-activated protein phosphatase in ceramide-mediated signal transduction. *J. Biol. Chem.* **269:**19605–19609 (1994).
53. Venable, M. E., J. Y. Lee, M. J. Smyth, A. Bielawska, and L. M. Obeid. Role of ceramide in cellular senescence. *J. Biol. Chem.* **270:**30701–30708 (1995).
54. Van Veldhoven, P. P., and G. P. Mannaerts. Sphinganine 1-phosphate metabolism in cultured skin fibroblasts: evidence for the existence of a sphingosine phosphatase. *Biochem. J.* **299:**597–601 (1994).
55. Merrill, A. H., S. Nimkar, D. Menaldino, Y. A. Hannun, C. Loomis, R. M. Bell, S. R. Tyagi, D. Lambrth, V. L. Stevens, H. Hunter, and D. C. Liotta. Structural requirements for long-chain (sphingoid) base inhibition of protein kinase C *in vitro* and for the cellular effects of these compounds. *Biochemistry* **28:**3138–3145 (1989).

Send reprint requests to: Dr. Sarah Spiegel, Department of Biochemistry and Molecular Biology, 3900 Reservoir Road NW, 353 Basic Science Bldg., Washington, DC 20007.