Sphingosine-1-Phosphate, a Novel Lipid, Involved in Cellular Proliferation

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Abstract. Sphingosine, a metabolite of membrane sphingolipids, regulates proliferation of quiescent Swiss 3T3 fibroblasts (Zhang, H., N. E. Buckley, K. Gibson, and S. Spiegel. 1990. *J. Biol. Chem.* 265:76–81). The present study provides new insights into the formation and function of a unique phospholipid, a metabolite of sphingosine, which was unequivocally identified as sphingosine-1-phosphate. The rapid increase in ^{32}P-labeled sphingosine-1-phosphate levels induced by sphingosine was concentration dependent and correlated with its effect on DNA synthesis. Similar to the mitogenic effects of sphingosine, low concentrations of sphingosine-1-phosphate stimulated DNA synthesis and induced pronounced morphological alterations. Both sphingosine and sphingosine-1-phosphate stimulated DNA synthesis in cells made protein kinase C deficient by prolonged treatment with phorbol ester and sphingosine still elicited similar increases in sphingosine-1-phosphate levels in these cells. Although both sphingosine and sphingosine-1-phosphate acted synergistically with a wide variety of growth factors, there was no additive or synergistic effect in response to a combination of sphingosine and sphingosine-1-phosphate. Using a digital imaging system for measurement of calcium changes, we observed that both sphingosine and sphingosine-1-phosphate are potent calcium-mobilizing agonists in viable 3T3 fibroblasts. The rapid rise in cytosolic free calcium was independent of the presence of calcium in the external medium, indicating that the response is due to the mobilization of calcium from internal stores. Our results suggest that sphingosine-1-phosphate may be a component of the intracellular second messenger system that is involved in calcium release and the regulation of cell growth induced by sphingosine.

THE biochemical mechanisms whereby eukaryotic cells regulate their proliferation are not well understood. One approach to this problem is to identify the second messengers responsible for the initiation of the progression of G_0-arrested cells into S phase. In Swiss 3T3 cells, which are sensitive to a wide range of mitogenic agents, some growth factors appear to function through conventional second messengers such as cAMP, whereas others use the signal pathways associated with increased degradation of polyphosphoinositides leading to the generation of lipid second messengers. Diacylglycerol (DAG)[1] is an endogenous activator of protein kinase C (Nishizuka, 1986) and inositol trisphosphate (IP_3) causes a release of Ca^{2+} from intracellular stores (Berridge, 1984; Berridge et al., 1984). Although the roles of these intracellular second messengers which appear to be important for the mitogenic response have been well characterized, it is evident that not all of the second messenger systems involved in cell growth regulation have been elucidated (Berridge, 1985; Besterman et al., 1986; Spiegel and Panagiotopoulos, 1988). In particular, it is clear that the early responses of quiescent cells to a variety of growth factors, such as changes in Ca^{2+} and pH_i, and activation of phospholipase C and protein kinase C, are insufficient by themselves to cause the cells to progress to DNA synthesis (Hesketh et al., 1988). Thus, it appears that there are still some undiscovered intracellular second-messenger pathways which are important for cell growth regulation.

Attention has recently been focused on other metabolites of membrane lipids, including phosphatidic acid (Exton, 1990; Moolenaar et al. 1986; Yu et al., 1988), arachidonic acid, and prostaglandins and related derivatives (Needleman et al., 1986) in signal transduction systems. A new addition to this growing family of biologically active lipids emerged from the discovery that sphingosine, a metabolite of membrane sphingolipids, inhibits protein kinase C, a pivotal regulatory enzyme in cell growth, opposing the action of DAG (Hannun and Bell, 1989; Merrill and Stevens, 1989). Thus, it has been suggested that sphingosine may function as an endogenous negative effector of protein kinase C (Hannun and Bell, 1989; Merrill and Stevens, 1989). We have shown previously that sphingosine stimulates the proliferation of quiescent Swiss 3T3 fibroblasts via a protein kinase

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1. *Abbreviations used in this paper*: DAG, diacylglycerol; EGF, epidermal growth factor; fura-2/AM, fura-2/acetoxy-methyl ester; IP_3, inositol trisphosphate; SPC, sphingosylphosphorylcholine; TPA, 12-*o*-tetradecanoylphorbol 13-acetate.

C-independent pathway (Zhang et al., 1990*a*). Recently, we have shown that the mitogenic effect of sphingosine is accompanied by an increase in phosphatidic acid levels (Zhang et al., 1990*b*), a potent mitogen for Swiss 3T3 cells (Yu et al., 1988; Moolenaar et al., 1986; Zhang et al., 1990*b*). This finding raises the possibility that sphingosine may play an important role as a positive regulator of cell growth acting through a novel pathway. A new action of sphingosine in mediating rapid and profound translocation of Ca^{2+} from intracellular stores has recently been described in permeabilized smooth muscle cells (Ghosh et al., 1990). Ghosh et al. have proposed that sphingosine must be converted intracellularly to sphingosine-1-phosphate which affects calcium release from an intracellular calcium pool that includes the IP_3-sensitive and -insensitive pools (Ghosh et al., 1990). In this paper, we present evidence that sphingosine-1-phosphate is produced in viable cells in response to mitogenic stimulation induced by sphingosine, is a potent mitogen for 3T3 fibroblasts, and mediates calcium release. These results suggest that sphingosine-1-phosphate may be a component of the intracellular second messenger system that is involved in calcium release and the regulation of cell growth induced by sphingosine.

Materials and Methods

Materials

[*methyl*-^{3}H]Thymidine (55 Ci/mmol), [^{32}P]orthophosphate (carrier-free), and [2-^{3}H]glycerol (1 Ci/mmol) were purchased from Amersham Chemical Corp. (Arlington Heights, IL). Epidermal growth factor (EGF), insulin, and transferrin were from Collaborative Research (Lexington, MA). Phospholipase D (from *Streptomyces chromofuscus*, type VI, 3,000 U/mg), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), D-sphingosine (from sphingomyelin), and sphingosylphosphorylcholine (free base) were from Sigma Chemical Co. (St. Louis, MO). The various standard phospholipids were from Avanti Polar Lipids (Birmingham, AL). Silica Gel 60 G plates were from Electron Microscopy Sciences (Cherry Hill, NJ). DMEM and PBS were from Advanced Biotechnologies (Silver Spring, MD) and Waymouth medium was from Gibco Laboratories (Grand Island, NY). Fura-2/acetoxy-methyl ester (fura-2/AM) was from Molecular Probes, Inc. (Eugene, OR).

Synthesis of Sphingosine-1-phosphate

Sphingosine-1-phosphate was prepared by enzymatic digestion of sphingosylphosphorylcholine (SPC) with phospholipase D as previously described, with minor modifications (Van Veldhoven et al., 1989). Briefly, SPC (10 μmol) was treated with 65 μg of phospholipase D in 2 ml of 50 mM ammonium acetate buffer, pH 8.0, at 30°C. As the reaction proceeds, sphingosine-1-phosphate, being insoluble in aqueous solution starts to precipitate. After 1 h, the suspension was centrifuged and the supernatant was allowed to react for a further 1 h and an additional amount of insoluble product isolated. The pellets were combined, dispersed in water by sonication, and cooled to 4°C. The precipitate was washed twice more with water to remove any traces of the substrate. Finally, the pellet was dissolved in 1 ml of methanol and purified by TLC using butanol/water/acetic acid (3:1:1 vol/vol, solvent system I, R_f = 0.47). The purity of sphingosine-1-phosphate was confirmed by analysis of the purified product in five different solvent systems. In each system, there was a single spot that was positive with ninhydrin and phosphate sprays. In these systems sphingosine-1-phosphate was well separated from SPC. The mobilities in the different solvent systems were identical to published values for sphingosine-1-phosphate (Van Veldhoven et al., 1989; Stoffel et al., 1970, 1973). Sphingosine-1-phosphate was quantified by the malachite green method (Hess and Derr, 1975). Sphingosine-1-phosphate was stored under N_2 in either methanol or water at −20°C.

Cell Culture

Swiss 3T3 cells were from the American Type Culture Collection (CCL 92; Rockville, MD). Stock cultures of cells were routinely cultured as previously described (Spiegel, 1989*a*). The cells were subcultured at a density of 1.5 × 10^4 cells/cm^2 in DMEM supplemented with 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FCS. For measurement of DNA synthesis, the cells were seeded and grown on multicluster plastic tissue culture dishes (24 × 16-mm wells, Costar, Cambridge, MA). For phospholipid analysis, the cells were cultured in six-well cluster plastic tissue culture dishes (6 × 34-mm wells, Costar Corp., Cambridge, MA). For measurements of cytoplasmic free Ca^{2+} concentration, cells were grown on glass cover slips in six-well cluster plastic tissue culture dishes. The cells were refed with the same medium after 2 d and were used 5 d later when the cells were confluent and quiescent (Spiegel and Panagiotopoulos, 1988).

Assay of DNA Synthesis

Quiescent cultures were washed with DMEM to remove residual serum and 1 ml of DMEM/Waymouth medium (1:1) supplemented with 20 μg/ml BSA and 5 μg/ml transferrin were added (Spiegel and Panagiotopoulos, 1988; Spiegel, 1989*a*). When indicated, the cells were treated with various growth factors, sphingosine added as a complex with BSA (Zhang et al., 1990*a*), or sphingosine-1-phosphate, and after 18 h, the cells were pulsed with 0.5 μCi of [^{3}H]thymidine for 6 h. The incorporation of radioactivity into trichloroacetic acid-insoluble material was measured as described (Spiegel and Panagiotopoulos, 1988). Values are the means of triplicate determinations. Standard deviations were routinely <10% of the mean.

Labeling of Cells with $^{32}P_i$

Quiescent cultures of 3T3 cells were washed with DMEM without phosphate and incubated with 2 ml of this buffer containing $^{32}P_i$ (40 μCi/ml) for 24 h. The cells were treated with sphingosine, or vehicle, or sphingosine-1-phosphate alone for various times and the reactions were terminated by placing the dishes on ice, and rapidly removing the medium. The cells were scraped from the dish in 1 ml of 0.1 N HCl. In some experiments, confluent cultures were labeled with (2-^{3}H)glycerol (2 μCi/ml) for the last 3 d of culture and $^{32}P_i$ (40 μCi/ml) was added for the final 24 h. Cells were rinsed with serum-free medium, and treated with sphingosine or vehicle and the lipids were extracted as described below.

Extraction and Analysis of Lipids

Lipids were extracted with chloroform/methanol/concentrated HCl (100:200:1 vol/vol) and the phases were separated as described (Hasegawa-Sasaki, 1985). The lipids in the lower chloroform phase were separated by two dimensional-thin-layer chromatography on silica gel 60 G. The plates were developed in solvent system V in the first dimension (Rouser et al., 1970) and solvent system IV in the second dimension. The phospholipid standards were visualized with molybdenum blue spray (Supelco, Inc., Bellefonte, PA). Phospholipids were located by autoradiography and the silica gel areas containing the labeled phospholipids were scraped off and counted by liquid

Table I. Mobilities of Sphingosine-1-phosphate (SPP) and Sphingosylphosphorylcholine (SPC) on TLC

Solvent System (vol/vol)	R_f SPP	R_f SPC
I. Butanol/water/acetic acid (3:1:1)	0.47	0.12
II. Chloroform/methanol/water (60:35:8)	0.23	0.04
III. Chloroform/methanol/water (65:35:8)	0.31	0.08
IV. Chloroform/methanol/water/acetic acid (30:30:2:5)	0.35	0.04
V. Chloroform/methanol/ammonium hydroxide (13:7:1)	0.00	0.04

scintillation spectrometry. In some experiments, sphingosine-1-phosphate was analyzed by thin-layer chromatography using different solvent systems as described in Table I.

Hydrolysis and Periodate Treatment of Sphingosine-1-Phosphate

Sphingosine-1-phosphate and putative radiolabeled product were treated with methanolic KOH (0.1 M) at 37°C for 1 h. After phase separation, the radioactive lipids recovered in the organic phase were analyzed by TLC. Sphingosine-1-phosphate was also subjected to periodate oxidation followed by borohydride reduction exactly as described previously (Hirschberg et al., 1970). The product, ethylene glycol monophosphate, was identified by paper chromatography (Hirschberg et al., 1970).

Measurements of Cytoplasmic Free Ca^{2+} Concentration

Levels and variations of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) after application of mitogens were determined by the fluorescent calcium-sensitive dye fura-2/AM as described previously (Brooker et al., 1990). Briefly, cells grown on glass coverslips were loaded with 10 μM fura-2/AM for 60 min in DMEM at 37°C, washed three times with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 5 mM Hepes, pH 7.4) and then mounted in a 35-mm holder, which created a chamber with the coverslip on the bottom. Changes in fura-2 fluorescence in single cells were monitored by dual wavelength simultaneous detection imaging using an Attofluor Digital Fluorescence Microscopy System (Atto Instruments, Inc., Potomac, MD). Fura-2 was excited at the wavelength pair of 334 and 390 nm using 10-nm bandpass interference filters that were alternatively selected by the computer-controlled excitation and shutter control unit (Brooker et al., 1990). Emission was monitored with an intensified CCD camera whose sensitivity was set for each wavelength and switched to that sensitivity by the computer just before each wavelength of excitation was selected. A 510-nm long-pass emission filter was used to select fluorescence emission >500 nm. The $[Ca^{2+}]_i$ was determined from the ratio of fura-2 fluorescence emissions after excitation at the dual wavelengths calibrated with external standards (Brooker et al., 1990). For experiments done at 37°C, the mitogens were dissolved in Locke's solution maintained at 37°C and perfused over the cells at 1 ml/min with a peristaltic pump. It should be noted that in these experiments, the errors in estimating the lag time may be increased due to slow diffusion of the mitogens in the perfusion system.

Results

Sphingosine Induces Formation of Sphingosine-1-Phosphate in Swiss 3T3 Cells

The effect of sphingosine on phospholipid metabolism was determined in cells metabolically prelabeled with $^{32}P_i$ to isotopic equilibrium (Fig. 1, *A* and *B*). In agreement with our previous studies (Zhang et al., 1990*b*), mitogenic concentrations of sphingosine significantly stimulated ^{32}P incorporation into phosphatidic acid. In addition, two-dimensional thin-layer chromatography analysis revealed another unidentified phospholipid spot which did not comigrate with any of the major known phospholipids of Swiss 3T3 fibroblasts (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, or sphingomyelin). To further identify this unknown phospholipid, the cells were double-labeled with both ^{3}H-glycerol and $^{32}P_i$ to isotopic equilibrium (Table II). In untreated cells, there was some incorporation of ^{32}P but no labeling with ^{3}H into this lipid. Mitogenic concentrations of sphingosine induced significant increases in incorporation of ^{32}P without corresponding increases in labeling with ^{3}H (Table II). In agreement with our previous studies (Zhang et al., 1990*b*), identical stimulations of incorporation of [^{3}H]-glycerol and $^{32}P_i$ into phosphatidic acid were found in response to sphingosine. In contrast, the incorporation of ^{32}P and ^{3}H into the other phospholipids was not affected by treatment with sphingosine. The ratio of ^{32}P to ^{3}H incorporated into all of the known glycerophospholipids was constant for both the control and sphingosine-treated cells.

These results indicate that the new phospholipid does not contain the glycerol moiety and that sphingosine enhances

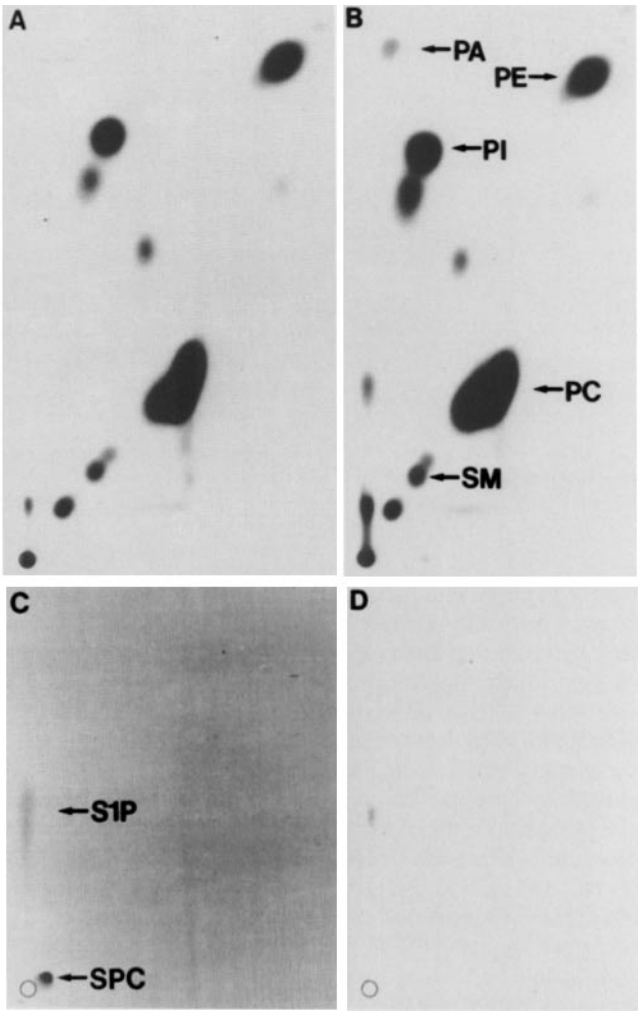

Figure 1. Sphingosine-induced changes in ^{32}P-labeled phospholipids. Confluent and quiescent cultures of Swiss 3T3 cells were prelabeled with $^{32}P_i$ for 24 h and stimulated with sphingosine-BSA complex (10 μM) or vehicle for 1 h. The lipids were extracted, separated by two dimensional TLC as described under Materials and Methods and autoradiograms developed (DuPont Cronex Lightning screen [DuPont Co., Wilmington, DE], 40 h of exposure). (*A*) Phospholipids extracted from untreated cells. (*B*) Phospholipids extracted from sphingosine-treated cells. (*C*) Sphingosylphosphorylcholine (SPC) after hydrolysis by phospholipase D (SPC and sphingosine-1-phosphate mixture) prepared as described in Materials and Methods. (*D*) Pure sphingosine-1-phosphate prepared as described in Materials and Methods. Unlabeled standard phospholipids were visualized by spraying with ninhydrin (*C*) or molybdenum blue (*D*). Arrows indicate location of standard phospholipids: phosphatidic acid (*PA*), phosphatidylethanolamine (*PE*), phosphatidylinositol (*PI*), phosphatidylcholine (*PC*), sphingomyelin (*SM*), sphingosylphosphorycholine (*SPC*), and sphingosine-1-phosphate (*SIP*).

Table II. Effect of Sphingosine on Incorporation of $^{32}P_i$ and [^{3}H]Glycerol into Phospholipids in Quiescent Swiss 3T3 Fibroblasts

	Incorporation of radioactivity into phospholipids			
	Control		Sphingosine-treated	
Phospholipids	[^{32}P]P_i	[^{3}H]glycerol	[^{32}P]P_i	[^{3}H]glycerol
	cpm			
SM	680 ± 20	ND	670 ± 100	ND
PC	26,780 ± 460	10,600 ± 375	21,020 ± 870	9,670 ± 600
PE	4,180 ± 900	2,730 ± 470	3,740 ± 760	3,030 ± 630
PI	4,040 ± 310	1,490 ± 100	4,170 ± 380	1,845 ± 270
PA	560 ± 90	220 ± 50	1,340 ± 200	500 ± 100
SPP	355 ± 70	ND	4,930 ± 310	ND

Quiescent cultures of Swiss 3T3 cells prelabeled with [2-^{3}H]glycerol (2 μCi/ml) for the last 3 d of culture and $^{32}P_i$ (40 μCi/ml) was added for the final 24 h. Cells were treated with sphingosine (20 μM) or vehicle, harvested after 2 h and the lipids extracted and chromatographed as described in Materials and Methods. The indicated phospholipid bands were excised from the plates and the radioactivity determined by liquid scintillation counting. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphigomyelin; SP1P, sphingosine-1-phosphate.

the ^{32}P incorporation into this phospholipid. Thus, this unknown phospholipid might be derived directly from sphingosine. It is known that cells contain sphingosine kinase, an enzyme which catalyzes the phosphorylation of free sphingosine at the 1-OH position (Stoffel et al., 1970, 1973; Hirschberg et al., 1970; Louie et al., 1976). To further substantiate the identity of this compound, we prepared sphingosine-1-phosphate by a facile enzymatic synthesis from sphingosinephosphorylcholine using phospholipase D. This elegant procedure, described recently by Bell's group (Van Veldhoven, et al., 1989), enabled us to prepare milligram quantities of pure sphingosine-1-phosphate. Fig. 1 demonstrates the cochromatography of the putative ^{32}P-labeled sphingosine-1-phosphate and the authentic material by two-dimensional thin-layer chromatography (compare Fig. 1, *B* with *D*). To further identify the putative ^{32}P-labeled sphingosine-1-phosphate, this spot was eluted from the thin-layer chromatography plate and analyzed by thin-layer chromatography in another solvent system (Fig. 2). In this solvent system, sphingosine-1-phosphate was well resolved from SPC and the ^{32}P-labeled phospholipid comigrated with authentic sphingosine-1-phosphate. This product also cochromatographed with authentic sphingosine-1-phosphate in four other solvent systems. The R_f values of the putative sphingosine-1-phosphate were identical to the authentic material in solvent systems I-V (Table I). In addition, ^{32}P-labeled sphingosine-1-phosphate was unaffected by mild alkali treatment, whereas ^{32}P-labeled phosphatidic acid was degraded. Furthermore, ^{32}P-labeled sphingosine-1-phosphate, sphingosine-1-phosphate standard, and L-α-glycerophosphate were subjected to periodate oxidation followed by borohydride reduction in order to establish the position of phosphorylation (Hirschberg et al., 1970). This procedure converts sphingosine-1-phosphate to an aqueous-extractable material that comigrates with the expected product, ethylene glycol monophosphate, and which was also formed from glycerophosphate. Whereas sphingosine-3-phosphate would be converted to a chloroform-soluble long chain alcohol, >85% of the ^{32}P-labeled sphingosine-1-phosphate was converted to ethylene glycol monophosphate by this procedure and there was no significant amount of chloroform-soluble material remaining.

Together, these results establish unequivocally the identification of this compound as a sphingosine-based phospholipid which does not contain an ester-linked acyl group (glycerophospholipid).

Sphingosine-1-Phosphate Is Rapidly Induced in Response to Sphingosine

The time course of ^{32}P-labeled sphingosine-1-phosphate accumulation in response to an optimal mitogenic concentration of sphingosine is shown in Fig. 3 *A*. A significant increase in [^{32}P]sphingosine-1-phosphate was detected within 5 min after exposure of the cells to sphingosine. Thus, the response to sphingosine was very rapid, reaching nearly maximal levels within 60 min, when four to sixfold stimulation over the basal value was observed. After 1–2 h, sphingosine-1-phosphate levels decrease but are still elevated up to 8 h (data not shown). The rapid response to mitogenic concentrations of sphingosine suggests that the formation of sphingosine-1-phosphate should be considered to be an early event that precedes the entry into the S phase of the cell cycle. The accumulation of ^{32}P-labeled-sphingosine-1-phosphate in response to sphingosine was also concentration dependent, with a maximal accumulation at 20 μM (Fig. 3 *B*). This dose response correlated closely with the dose response for sphingosine-induced stimulation of DNA synthesis (Zhang et al., 1990*b*).

Sphingosine-1-Phosphate Stimulates Proliferation of Quiescent Swiss 3T3 Fibroblasts

The effect of sphingosine-1-phosphate on DNA synthesis was examined in quiescent Swiss 3T3 cells grown in chemically defined medium. We found that sphingosine-1-phosphate stimulated proliferation of quiescent Swiss 3T3 fibroblasts as measured by [^{3}H]thymidine incorporation (Fig. 4). A mitogenic effect was observed at a concentration of sphingosine-1-phosphate as low as 0.5 μM and maximum stimulation was achieved at 2–5 μM (Fig. 4 *A*). Up to this concentration, there was no loss of cell viability and >95% of the cells were viable, as measured by trypan blue exclusion. Sphingosine-1-phosphate not only stimulated DNA synthesis, but also caused an increase in cell division (Fig. 4 *B*). A maximal increase in cell number was observed after 48 h exposure

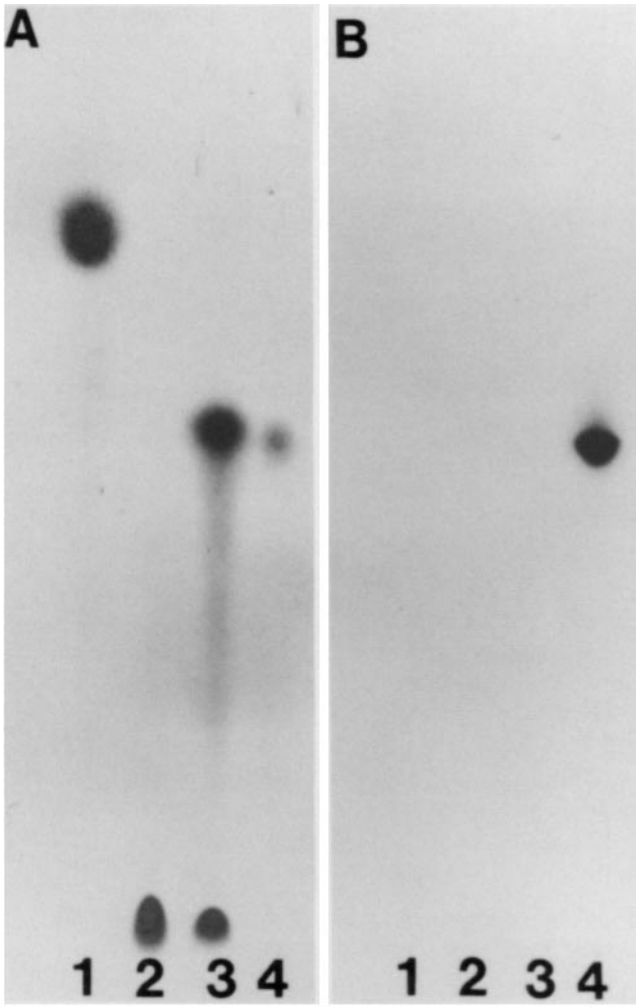

Figure 2. Thin layer chromatography of the putative ^{32}P-labeled sphingosine-1-phosphate induced by sphingosine. (*A*) Confluent and quiescent cultures of Swiss 3T3 cells were prelabeled with ^{32}Pi for 24 h and stimulated with sphingosine-BSA complex (10 μM). After extraction, lipids were separated by TLC and the putative ^{32}P-labeled sphingosine-1-phosphate spot was eluted from the TLC plate, analyzed by TLC using solvent system I and the chromatograms either visualized by spraying with ninhydrin (*A*) or autoradiographed (*B*). Lane *1*, authentic sphingosine. Lane *2*, authentic SPC. Lane *3*, SPC after hydrolysis by phospholipase D. Lane *4*, ^{32}P-labeled sphingosine-1-phosphate spot extracted from sphingosine-treated cells was mixed with standard sphingosine-1-phosphate prepared as described in Materials and Methods.

to sphingosine-1-phosphate and was comparable to the increase mediated by mitogenic concentrations of sphingosine or EGF. Similar to sphingosine, sphingosine-1-phosphate also induced a large morphological transformation, especially in cultures treated with 2 μM sphingosine-1-phosphate. The flattened appearance of untreated cells is contrasted with the elongated, refractile appearance of cells treated with sphingosine-1-phosphate, sphingosine, or a potent mitogen for these cells, such as TPA (Fig. 5). Furthermore, we found that the time course of the increase in DNA synthesis in quiescent cultures of Swiss 3T3 cells induced by sphingosine-1-phosphate treatment was essentially the same as that induced in response to sphingosine or EGF. The lag period preceding the onset of DNA synthesis was identical (15 h) and the rate of entry into S phase was the same regardless of whether the cultures were exposed to sphingosine-1-phosphate, sphingosine, or EGF (data not shown). Thus, the kinetics of the response of quiescent 3T3 cells to sphingosine-1-phosphate appear to be similar to other known mitogens.

Effect of Sphingosine-1-phosphate after Downregulation of Protein Kinase C

Previously, we have shown that sphingosine stimulates cellular proliferation in protein kinase C-deficient cells (Zhang et al., 1990*a*). Prolonged exposure of quiescent cultures of Swiss 3T3 cells to TPA leads to downregulation of protein kinase C and the enzymatic activity as well as [^{3}H]PDBu binding disappears completely (Spiegel, 1989*b*). Similar to our previous studies (Zhang et al., 1990*a*), TPA pretreatment decreased [^{3}H]PDBu binding by >92% and abolished the mitogenic effect of TPA. In contrast, downregulation of protein kinase C had no effect on the ability of sphingosine-1-phosphate to stimulate DNA synthesis (Table III). Thus, indicating that protein kinase C is apparently not required for the mitogenic effect of either sphingosine or sphingosine-1-phosphate. Not surprisingly, sphingosine still elevated levels of sphingosine-1-phosphate in protein kinase C-deficient cells to the same extent as in untreated cells (Table III).

Effects of Sphingosine-1-Phosphate on Reinitiation of DNA Synthesis Induced by Various Growth-promoting Agents

Sphingosine-1-phosphate is a modest mitogen by itself. It is noteworthy that sphingosine-1-phosphate alone at optimal concentrations is more mitogenic than insulin, and it is almost as potent as EGF, which is an exceptionally potent growth stimulator for Swiss 3T3 cells (Table IV). Similar to the synergistic effect between insulin and other growth factors, the mitogenic response to sphingosine-1-phosphate was potentiated by insulin. Sphingosine-1-phosphate also potentiated the growth promoting effects of optimal stimulatory concentrations of EGF, TPA, and even unfractionated FCS. This synergistic interaction between sphingosine-1-phosphate and growth factors was observed even in combinations with two growth factors, such as EGF plus insulin or TPA plus insulin. While any two of the growth factors synergized with each other, addition of sphingosine-1-phosphate caused a further potentiation of [^{3}H]thymidine incorporation (Table IV).

No Additive or Synergistic Stimulation of DNA Synthesis in Response to Combination of Sphingosine and Sphingosine-1-Phosphate

The striking similarity in the mitogenic properties of sphingosine-1-phosphate and sphingosine, combined with the demonstration that sphingosine induces an early rise in the levels of sphingosine-1-phosphate, raises the possibility that sphingosine-1-phosphate may be involved in the mitogenic effect of sphingosine. If these two lipid mitogens stimulate DNA synthesis by an identical pathway, they should not interact, either additively or synergistically, in stimulating DNA synthesis when tested at optimal mitogenic concentrations. As expected, in contrast to the potentiating effect on

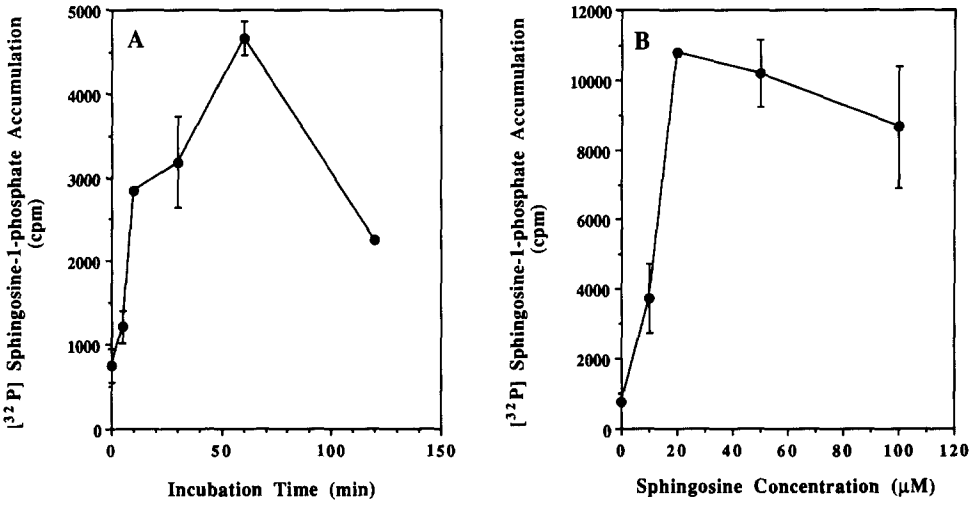

Figure 3. Time course and dose response for sphingosine-induced accumulation of ^{32}P-labeled sphingosine-1-phosphate. Quiescent cultures of 3T3 cells were washed with phosphate-free DMEM and incubated with this buffer containing $^{32}P_i$ (40 μCi/ml) for 24 h. The cells were treated with sphingosine-BSA complex (10 μM) for the indicated time periods (*A*) or with increasing concentrations of sphingosine-BSA complex for 1 h (*B*)). Lipids were extracted, separated by TLC and phospholipids were located by autoradiography. The silica gel areas containing the labeled sphingosine-1-phosphate were scraped off and counted by liquid scintillation spectrometry. The incorporation of ^{32}P into sphingosine-1-phosphate in untreated cells was 770 ± 25 cpm determined from an aliquot of the phospholipid extract containing 150,000 cpm.

DNA synthesis induced by insulin, TPA, EGF and even unfractionated FCS (Table IV), sphingosine-1-phosphate did not potentiate DNA synthesis induced by an optimal stimulatory concentration of sphingosine (Fig. 6). Even in the presence of insulin or EGF, agents which greatly potentiate the mitogenic response to sphingosine-1-phosphate or sphingosine, there were no further stimulatory effects when both sphingosine-1-phosphate and sphingosine were added together. The response of the cells to sphingosine plus sphingosine-1-phosphate was almost identical or even less than the responses to each of the effectors alone. This nonadditive response was observed even when both EGF and insulin were tested together, while the two growth factors synergized with each other and with the lipid mitogens, addition of these lipids together caused no further potentiation of [^{3}H]thymidine incorporation. Thus, sphingosine and sphingosine-1-phosphate do not interact additively or synergistically either in the presence or absence of other growth factors.

Effect of Sphingosine and Sphingosine-1-Phosphate on the Release of Cytosolic Free Calcium

Recently, a new action of sphingosine derivatives in mediating rapid and profound release of calcium from intracellular stores within permeabilized cells was described (Ghosh et al., 1990). It was proposed that sphingosine must be converted to sphingosine-1-phosphate which then mediates the

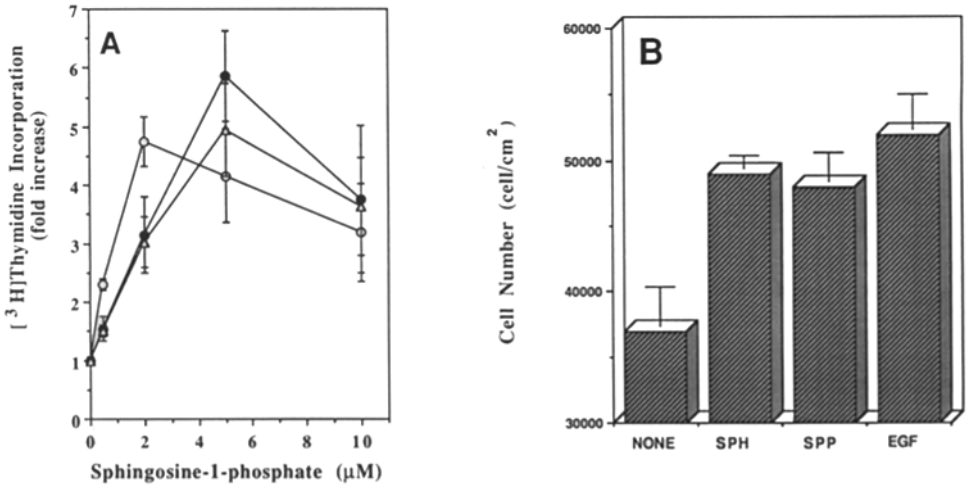

Figure 4. Stimulation of cell growth by sphingosine-1-phosphate. (*A*) Increase in DNA synthesis. Confluent and quiescent cultures of Swiss 3T3 cells were incubated with various concentrations of sphingosine-1-phosphate in the absence (○) or in the presence of (●) insulin (2 μg/ml) or (△) TPA (100 nM). [^{3}H]Thymidine incorporation was measured as described in Materials and Methods. The results are expressed as percent of the control incorporation measured in the absence of sphingosine-1-phosphate. Similar results were obtained in at least five additional experiments. All concentrations of sphingosine-1-phosphate >0.5 μM were statistically significant (*t* test, $P \leq 0.01$) compared with the control cells. (*B*) Stimulation of cell division. Confluent and quiescent cultures were incubated in DMEM/Waymouth (1:1) supplemented with 20 μg/ml BSA, 5 μg/ml transferrin, and 4 μg/ml insulin in the absence or the presence of the indicated mitogens: sphingosine (*SPH*, 20 μM); sphingosine-1-phosphate (*SPP*, 5 μM); or EGF (10 ng/ml). After 48 h, the cells were removed from the dishes and counted with a cell counter (model ZBI; Coulter Electronics, Hialeah, FL). Data represent the mean ± SD of three independent cultures treated identically.

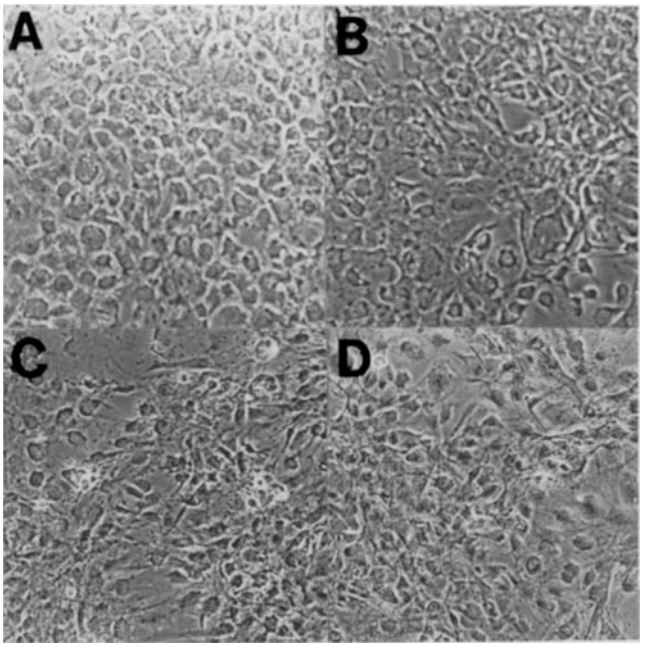

Figure 5. Morphologic alterations of Swiss 3T3 cells induced by sphingosine-1-phosphate. Confluent and quiescent cultures of Swiss 3T3 cells were washed with DMEM and incubated at 37°C in DMEM/Waymouth (1:1) supplemented with BSA (20 μg/ml), transferrin (5 μg/ml), and insulin (2 μg/ml) in the absence (*A*) or in the presence of 10 μM sphingosine (*B*) or in the presence of 2 μM sphingosine-1-phosphate (*C*) or in the presence of 100 nM TPA (*D*). Photomicrographs of the cultures were taken with the aid of a phase-contrast microscope 24 h after addition of the mitogens (×450). The flattened appearance of untreated cells (*A*) is contrasted with the elongated, refractile appearance of treated cells (*B–D*).

release of calcium (Ghosh et al., 1990). However, there has not yet been any direct experimental evidence presented that sphingosine-1-phosphate can cause the release of calcium from internal sources. We used fluorescence imaging to determine the effect of sphingosine and sphingosine-1-phosphate on cytosolic free calcium.

In quiescent Swiss 3T3 fibroblasts, basal $[Ca^{2+}]_i$ was 30–90 nM (n = 30). These values are similar to those obtained previously in these cells by a different method (Spiegel and Panagiotopoulos, 1988; Hesketh et al., 1988). Upon addition of mitogenic concentrations of sphingosine-1-phosphate at room temperature, there was an immediate and transitory rise in cytosolic free calcium concentration (Fig. 7).

Table III. Effect of Prolonged Phorbol Ester Pretreatment on ^{32}P Incorporation into Sphingosine-1-Phosphate and DNA Synthesis Initiated by Various Mitogens

Treatment	Stimulants	[^{3}H]Thymidine incorporation	^{32}P incorporated into SPP
		fold increase	
Untreated	TPA	13.8	1
	Sphingosine	1.82	6
	Sphingosine-1-phosphate	3.01	ND
TPA pretreatment	TPA	0.8	1
	Sphingosine	1.95	6
	Sphingosine-1-phosphate	3.06	ND

Control cultures or cultures pretreated for 24 h with 1 μM TPA were washed thoroughly and then incubated in DMEM/Waymouth (1:1) supplemented with 20 μg/ml BSA, 5 μg/ml transferrin, and 4 μg/ml insulin in the presence of the indicated mitogens: TPA (100 nM); sphingosine (20 μM); sphingosine-1-phosphate (5 μM). [^{3}H]Thymidine incorporation into DNA was measured. Incorporation of ^{32}P into sphingosine-1-phosphate was measured in duplicate cultures as described in Materials and Methods. The data are expressed relative to the none value obtained in the absence of additional mitogens.

Table IV. Effects of Sphingosine-1-Phosphate on DNA Synthesis in Quiescent Cultures of Swiss 3T3 Fibroblasts

Stimulants	[^{3}H]Thymidine incorporation	
	Absence of SPP	Presence of SPP
	cpm × 10^{-3}/well	
None	1.6 ± 0.2	12.7 ± 0.7
Insulin	3.8 ± 0.4	39.5 ± 3.7
EGF	12.1 ± 1.2	49.9 ± 0.5
TPA	11.5 ± 2.4	40.9 ± 0.4
EGF plus insulin	109.0 ± 13	155.6 ± 0.8
TPA plus insulin	47.1 ± 8.4	112.3 ± 12
FCS	56.5 ± 6.7	112.9 ± 5.5

Quiescent cultures of Swiss 3T3 cells were exposed to the indicated mitogens in the absence or presence of sphingosine-1-phosphate (2 μM) and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. Each value is the mean ± SD of triplicate determinations from a representative experiment. Similar results were obtained in at least five additional experiments. The concentrations of the mitogenic agents were as follows: insulin (2 μg/ml); EGF (10 ng/ml); TPA (25 nM); FCS, 5% (vol/vol). SPP, sphingosine-1-phosphate.

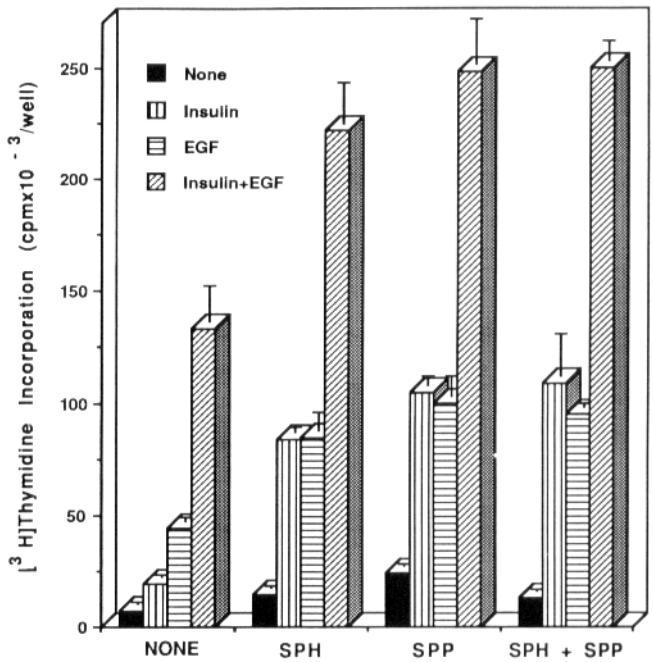

Figure 6. Lack of synergistic effect between sphingosine and sphingosine-1-phosphate on DNA synthesis. Quiescent cultures of Swiss 3T3 cells were exposed to the indicated mitogens in the absence (none) or in the presence of sphingosine (*SPH*) or sphingosine-1-phosphate (*SPP*) or both and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. The concentrations of the mitogenic agents were as follows: insulin (4 μg/ml); EGF (10 ng/ml); sphingosine (20 μM); sphingosine-1-phosphate (5 μM).

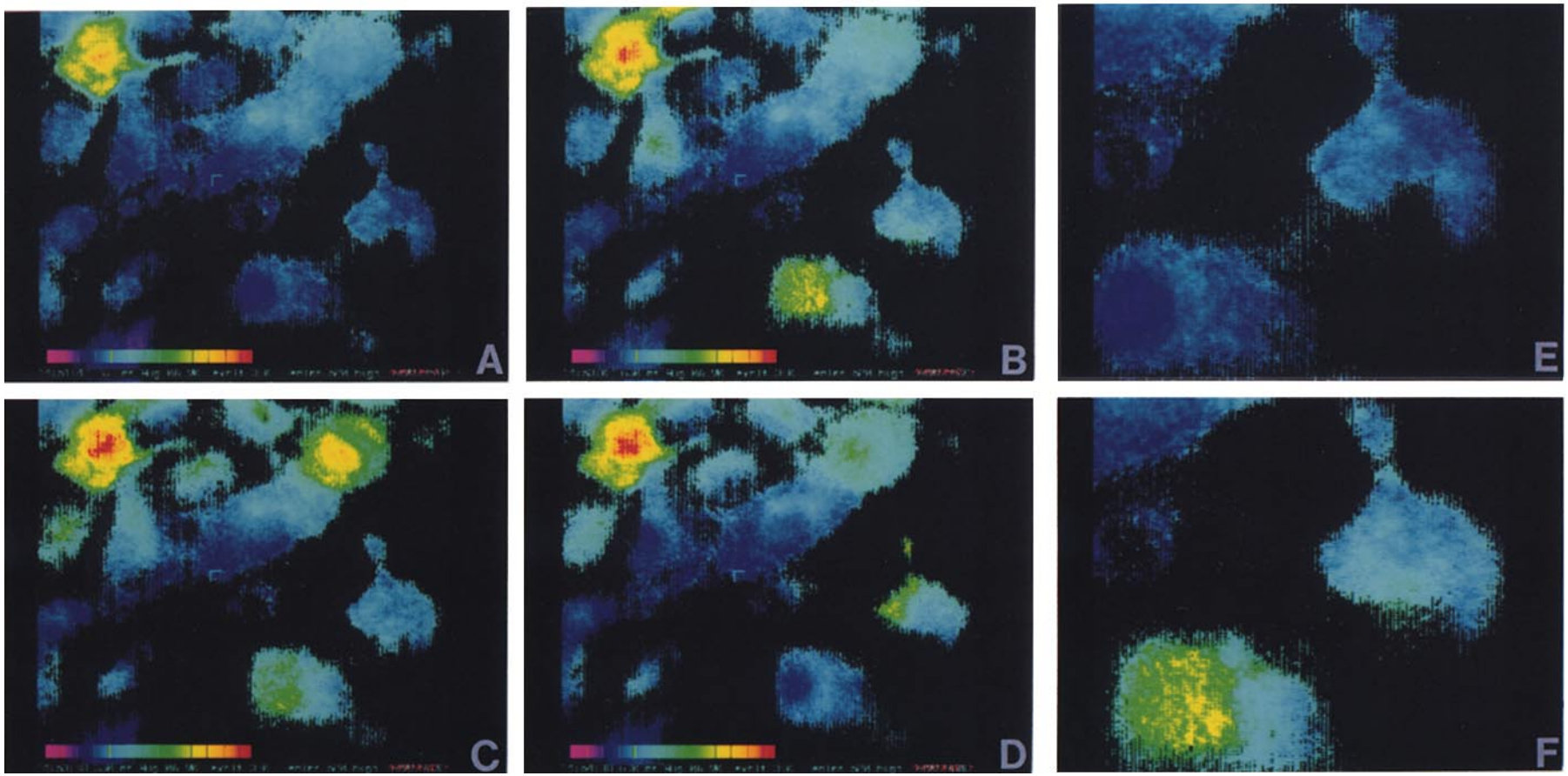

Figure 7. Pseudo color images of fura-2 fluorescence intensity ratio imaging in Swiss 3T3 cells stimulated with 2 μM sphingosine-1-phosphate (room temperature, 340 nm image/380 nm image). (*A*) 5 s before addition of sphingosine-1-phosphate. (*B*) 15 s; (*C*) 65 s; (*D*) 125 s after addition of sphingosine-1-phosphate (2 μM). (*E* and *F*) Enlargement of the same areas of *A* and *B*. Color scale at bottom depicts pixel intensity of $[Ca^{2+}]_i$ from 0 nM (*pink*) to 800 nM (*red*).

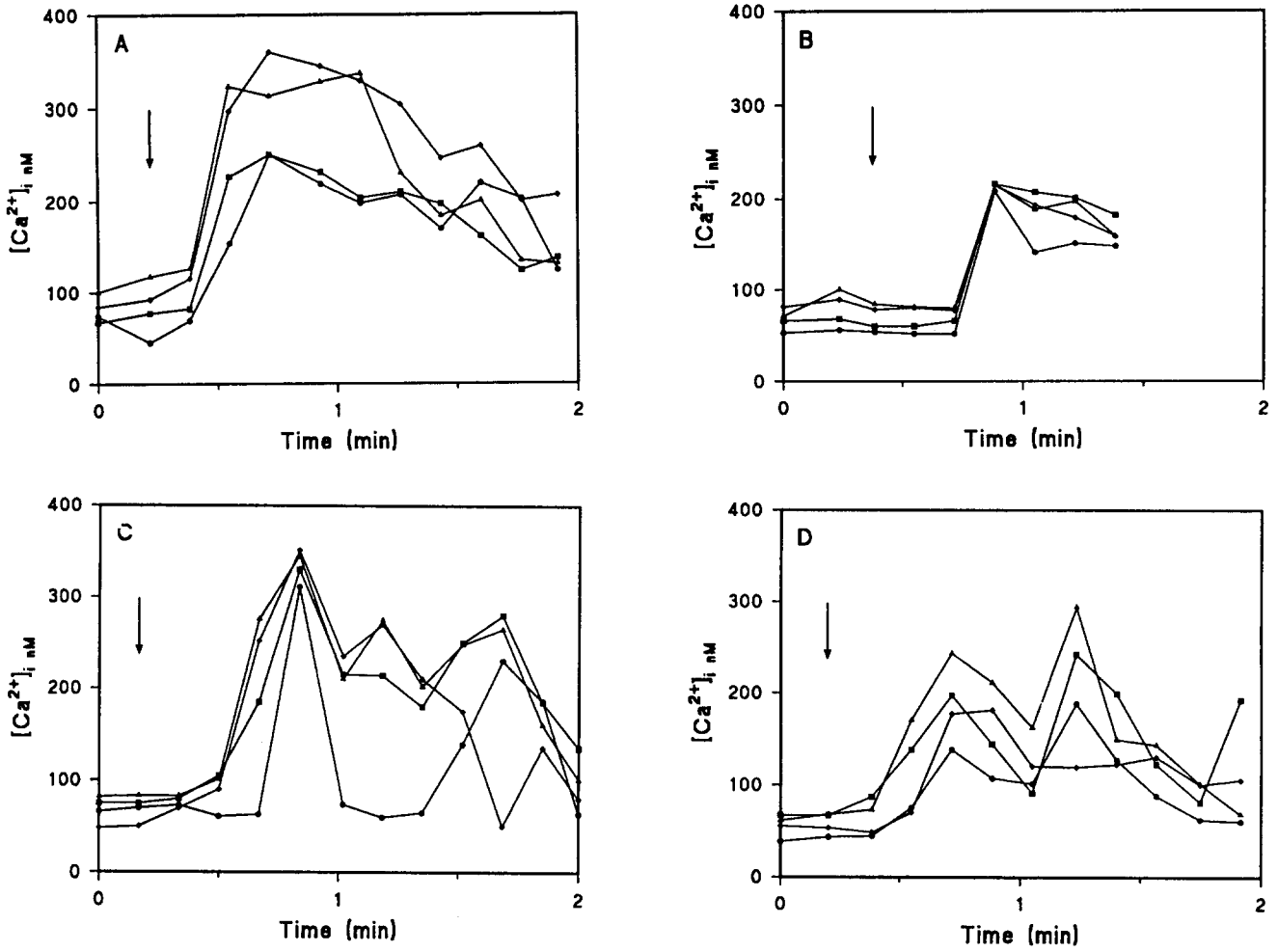

Figure 8. Changes in cytoplasmic free Ca^{2+} after addition of sphingosine-1-phosphate and sphingosine. Quiescent cultures of Swiss 3T3 cells were loaded with fura-2/AM, washed and incubated at 37°C in Locke's buffer containing Ca^{2+} (*A* and *C*) or in Ca^{2+}-free buffer containing 2 mM EGTA (*B* and *D*). At the indicated times, 2 μM sphingosine-1-phosphate (*A* and *B*) or 50 μM sphingosine (*C* and *D*) were added. $[Ca^{+2}]i$ was determined by fura-2 imaging as described in Materials and Methods. In each case, the calcium changes of four responsive cells are shown.

The onset in the rise in calcium was almost instantaneous, reaching a peak response within a short time (<60 s) after stimulation and decaying over the next 2 min. Fig. 8 illustrates the variation in the basal levels of calcium between cells and also the response to sphingosine-1-phosphate. It is clear that there are genuine variations in the level of $[Ca^{2+}]_i$ from cell to cell, even among quiescent cells, that were not distinguishable on the basis of morphology. Application of sphingosine-1-phosphate induced a wave of increased free Ca^{2+} that spread to involve almost the entire fibroblast. The rate of this spread varied slightly among cells. A significant observation from the single cell experiment is that there exists a population of cells whose calcium levels are unresponsive to sphingosine-1-phosphate. There were no obvious morphological features by which it was possible to distinguish responsive from unresponsive cells. The same results were found when the experiments were performed at either room temperature or at 37°C (Fig. 8).

Changes in $[Ca^{2+}]_i$ can result from an influx of extracellular Ca^{2+} or a release from intracellular stores (Berridge, 1984; Berridge et al., 1984). To distinguish between these sources, experiments were performed in calcium-free Locke's solution containing 2 mM EGTA. Nearly identical calcium responses were observed in the absence of external calcium (Table V, Fig. 8), thus indicating that the rapid rise in cytosolic free calcium induced by sphingosine-1-phosphate is due to the release of calcium from internal stores and not due to influx from extracellular sources.

In contrast to the action of sphingosine-1-phosphate, sphin-

Table V. Effects of Sphingosine and Sphingosine-1-Phosphate on Cytosolic Free Calcium

Stimulus	Medium calcium	$[Ca^{+2}]i$ Basal	$[Ca^{+2}]i$ Maximum
Sphingosine	−	61 ± 27	278 ± 65
	+	60 ± 19	261 ± 88
Sphingosine-1-phosphate	−	68 ± 11	206 ± 17
	+	75 ± 21	298 ± 49

Cytoplasmic free calcium concentration was measured by dual imaging using fura-2/AM as described in Materials and Methods. Basal values were determined 10 s before the addition of mitogens. Experiments were carried out in the absence (−) or presence (+) of 2 mM extracellular calcium. The concentrations of the mitogens were 50 μM sphingosine and 2 μM sphingosine-1-phosphate.

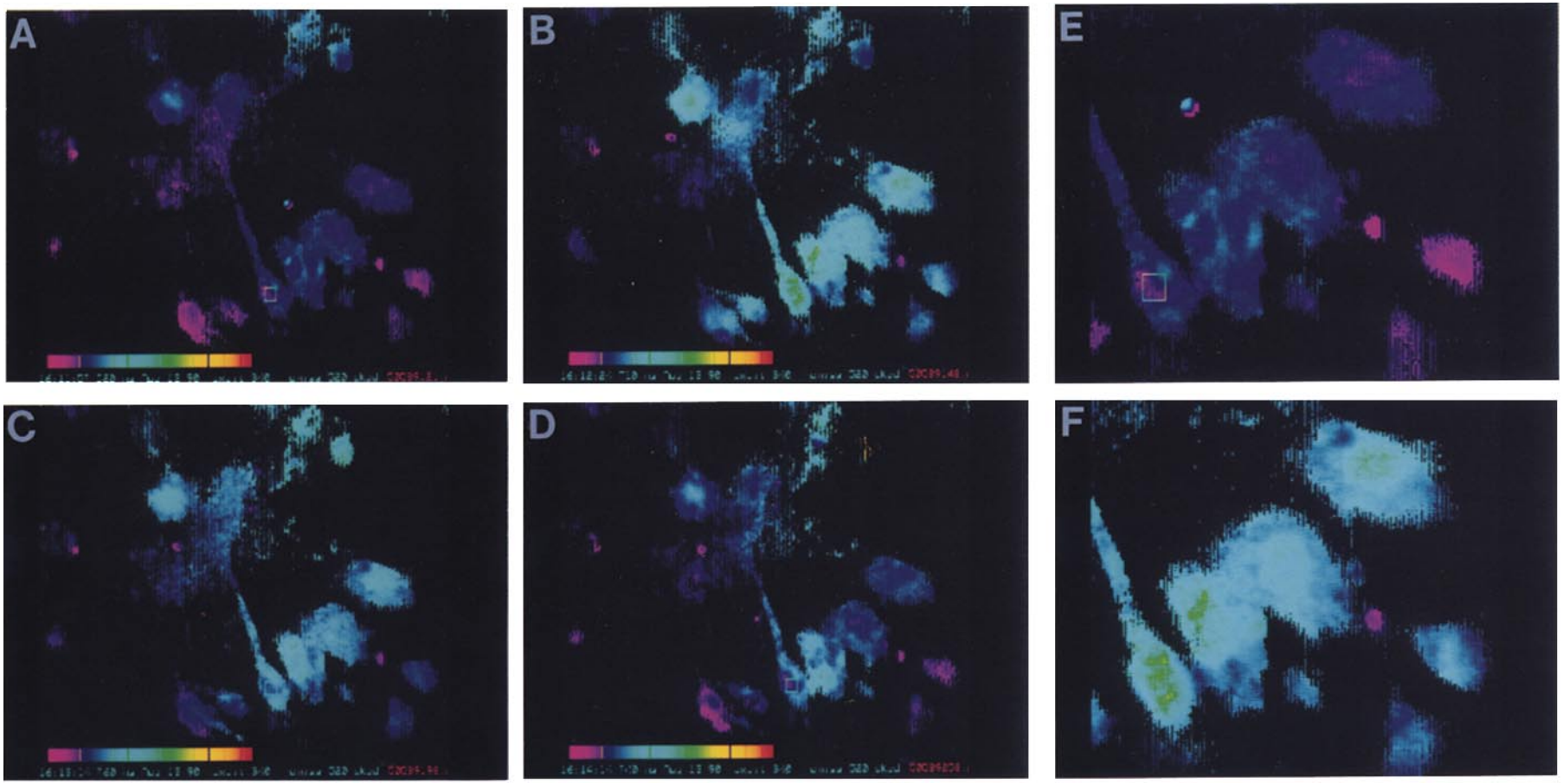

Figure 9. Pseudo color images of fura-2 fluorescence intensity ratio imaging in Swiss 3T3 cells stimulated with 50 μM sphingosine (37°C, 340 nm image/380 nm image). (*A*) 5 s before addition of sphingosine. (*B*) 75 s; (*C*) 120 s; (*D*) 170 s after addition of sphingosine (50 μM). (*E* and *F*) Enlargement of the same areas of *A* and *B*. Color scale at bottom depicts pixel intensity of $[Ca^{2+}]_i$ from 0 nM (*pink*) to 900 nM (*red*).

gosine itself had almost no detectable effects on cytosolic free calcium during the 5 min observation period when the cells were studied at room temperature. However, at 37°C, sphingosine increased cytosolic free calcium by three- to six-fold over the basal value after a lag of 30 s (Fig. 9 *C*). In most of the single cells that were examined, this peak was followed by another wave of increasing cytosolic free calcium (Fig. 8). The increase in $[Ca^{2+}]_i$ was also independent of the presence of calcium in the external medium (Table V). Similar to the effects of sphingosine-1-phosphate, the response of cytosolic free calcium to sphingosine was heterogeneous and also some of the cells were unresponsive (Fig. 9). The lag in the action of sphingosine combined with the temperature effect on release of calcium, lend support to the suggestion that sphingosine might require a temperature-sensitive enzymatic reaction for its function (Ghosh et al., 1990).

Discussion

As our knowledge of the mechanism of cell proliferation increases, it has become apparent that there are many controlling elements. In some cases, activation of phospholipase C that leads to increases in cytosolic free calcium, increases in DAG, and activation of protein kinase C, correlates with activation of cellular proliferation (Rozengurt, 1986; Nishizuka, 1988). However, numerous exceptions have been reported and it has become clear that not all of the second messenger systems involved in cell growth regulation have been described (Berridge, 1985). Sphingosine, a metabolite of membrane glycosphingolipids, has been suggested by Bell and Merrill and their colleagues to be a possible new intracellular second messenger. Sphingosine was found to inhibit protein kinase C and has been implicated as a negative modulator of transmembrane signaling, opposing the action of DAG which stimulates protein kinase C (Hannun and Bell, 1987, 1989; Merrill et al., 1989). It has been shown that sufficient levels of free sphingosine are generated within cells to account for protein kinase C inhibition (Wilson et al., 1988). Recently, a metabolite of sphingosine, dimethylsphingosine, was found to be a much more potent inhibitor of protein kinase C and to enhance the tyrosine kinase activity of src-kinase and EGF receptor (Igarashi et al., 1989, 1990; Felding-Haberman et al., 1990).

We have previously reported that low concentrations of sphingosine stimulate proliferation of quiescent Swiss 3T3 fibroblasts acting in a fundamentally different, protein kinase C-independent pathway (Zhang et al., 1990*a*). Recently, we have shown that the mitogenic effect of sphingosine is accompanied by an increase in phosphatidic acid levels (Zhang et al., 1990*b*), which is a potent mitogen for Swiss 3T3 cells (Yu et al., 1988; Moolenaar et al., 1986; Zhang et al., 1990*b*).

The present study investigates in detail the effect of sphingosine on the metabolism of other phospholipids in Swiss 3T3 fibroblasts and provides new insights into the formation and function of a unique phospholipid, a metabolite of sphingosine, sphingosine-1-phosphate. This compound was identified unequivocally as a sphingosine-based phospholipid which does not contain an ester-linked acyl group (glycerophospholipid). In contrast to other phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid, this phospholipid, which was resistant to mild alkali treatment, did not contain the glycerol moiety. The compound was further identified by comparison to a standard prepared by a facile enzymatic synthesis from SPC using phospholipase D. The material obtained from Swiss 3T3 cells comigrated with sphingosine-1-phosphate standard in a variety of thin-layer chromatography systems and in addition, was converted to ethyleneglycol monophosphate by periodate/borohydride treatment. We demonstrated that mitogenic concentrations of sphingosine induce a rapid rise in the levels of ^{32}P-labeled sphingosine-1-phosphate. The dose response for the accumulation of sphingosine-1-phosphate correlated closely with the dose response of sphingosine-induced stimulation of DNA synthesis.

Similar to sphingosine, sphingosine-1-phosphate also stimulated DNA synthesis and cell division in quiescent cultures of Swiss 3T3 cells. It is noteworthy that sphingosine-1-phosphate alone at optimal concentrations is more mitogenic than insulin, and it is as effective as EGF, a potent growth stimulator for Swiss 3T3 cells. Both sphingosine and sphingosine-1-phosphate acted synergistically with a variety of growth factors, such as insulin, EGF, TPA, fibroblast growth factor, and unfractionated FCS. In sharp contrast, sphingosine and sphingosine-1-phosphate did not have additive or synergistic effects in the absence or presence of other growth factors.

Both sphingosine and sphingosine-1-phosphate stimulate DNA synthesis in cells made protein kinase C deficient by prolonged treatment with phorbol ester and sphingosine still elicited similar increases in sphingosine-1-phosphate levels in these cells. Furthermore, not only did sphingosine-1-phosphate stimulate [^3H]thymidine incorporation with similar efficiency and kinetics as sphingosine, it also induced similar morphological transformation. It should be noted that sphingosine-1-phosphate stimulated DNA synthesis to a greater extent than sphingosine and required a lower concentration for the maximum response. Therefore, sphingosine-1-phosphate is more potent in stimulating [^3H]thymidine incorporation than sphingosine, suggesting that it may mediate the mitogenic activity of sphingosine.

Recently, sphingosine derivatives were shown to cause the release of internal calcium from permeabilized cells and it has been suggested that their effects were mediated via the conversion of sphingosine to sphingosine-1-phosphate (Ghosh et al., 1990). However, direct experimental evidence that sphingosine-1-phosphate itself caused the release of calcium from internal sources was not presented. The experiments reported here show that sphingosine-1-phosphate and sphingosine are potent calcium-mobilizing agonists in viable 3T3 fibroblasts. The rapid rise in cytosolic free calcium was independent of the presence of calcium in the external medium, thus indicating that the response is due to the release of calcium from internal stores and not due to influx from extracellular sources. The advantage of the digital imaging system for measurement of free Ca^{2+} changes is that local concentrations of cytoplasmic free Ca^{2+} can be continuously imaged with great accuracy and resolution in single living cells within a large population of cells. This system makes it possible to detect small fluctuations in the $[Ca^{2+}]_i$ concentration within individual cells that would have been averaged out in the population of cells since the changes do not occur in exact synchrony. With this method, it is also possible to follow short term changes in the spatial distribution of Ca^{2+} with great accuracy, sensitivity, and resolution.

Both sphingosine and sphingosine-1-phosphate induced a wave of increased free Ca^{2+} which spread to involve almost the entire fibroblast. The rate of this spread varied slightly among cells. However, there was a population of cells unresponsive to sphingosine or sphingosine-1-phosphate which did not have any obvious characteristic morphological features. These results are similar to recent observations on heterogeneous responses to other growth factors, such as bombesin, EGF, and vasopressin (Hesketh et al., 1988), where 40% of cells were unresponsive to any mitogens.

In preliminary experiments, we found that ^{32}P-labeled sphingosine-1-phosphate (Dresler and Kolesnick, 1990) was rapidly taken up by cells. After 5 and 60 min of labeling with 1 μM sphingosine-1-phosphate, 13 and 48 pmol/10^6 cells were incorporated, respectively. During this period there was no appreciable degradation of the cellular sphingosine-1-phosphate. Furthermore, when the lipids were extracted from the labeled cells and analyzed by thin-layer chromatography, ^{32}P-sphingosine-1-phosphate was the only labeled lipid detected (Olivera, A., H. Zhang, and S. Spiegel, unpublished observations). These results suggest that the active species was sphingosine-1-phosphate itself and was not due to rapid reconversion to sphingosine by the action of a membrane phosphatase. In further support of this conclusion, only sphingosine-1-phosphate releases calcium from internal sources at room temperature and the response is almost instantaneous. Furthermore, the concentration of sphingosine required to release calcium at 37°C is much higher than the effective concentration of sphingosine-1-phosphate.

The temperature dependency of the effect of sphingosine on intracellular calcium, together with the increased lag in its action, support the suggestion that sphingosine must require a temperature-dependent enzymatic conversion to sphingosine-1-phosphate for its function (Ghosh et al., 1990). Sphingosine-1-phosphate has long been known to be produced from sphingosine in a variety of cell types (Stoffel et al., 1970, 1973) and it is known that cells contain sphingosine kinase, an enzyme which catalyzes the phosphorylation of free sphingosine at the 1-OH position (Stoffel et al., 1973). Sphingosine-1-phosphate is thought to be rapidly degraded by the action of a microsomal lyase which cleaves sphingosine-1-phosphate to trans-2-hexadecanal and phosphorylethanolamine (Stoffel, 1970). Thus, sphingosine-1-phosphate could be a suitable candidate for an intracellular second messenger as it can be rapidly induced and degraded. The results presented here are consistent with such an hypothesis. Sphingosine-1-phosphate is rapidly produced in response to mitogenic concentrations of sphingosine, is mitogenic by itself for 3T3 fibroblasts, and mediates calcium release in viable cells. Thus, sphingosine-1-phosphate may be an important component of the intracellular second messenger system that is involved in calcium release and the regulation of cell growth induced by sphingosine. However, there are some obvious caveats to this hypothesis. Sphingosine-1-phosphate was added exogenously to Swiss 3T3 cells and it is difficult to compare its effects to those of the endogenously generated compound. In this regard, we would like to point out that the amount of sphingosine-1-phosphate formed intracellularly in response to a mitogenic concentration of sphingosine was approximately the same order of magnitude as that taken up by the cells after treatment with sphingosine-1-phosphate (Olivera, A., H. Zhang, and S. Spiegel, unpublished observations). Unfortunately, the subcellular localizations of sphingosine and sphingosine-1-phosphate are still unknown, which makes the comparison of extracellular to intracellular concentrations ambiguous.

Our results raise the important question of whether other mitogenic agents will enhance degradation of glycosphingolipids (Stults et al., 1989) or sphingomyelin (Merrill and Jones, 1990) to regulate endogenous sphingosine levels that, in turn, will modulate sphingosine-1-phosphate levels. If so, sphingosine-1-phosphate could function as a novel intracellular second messenger that releases calcium from internal stores and also modulates cellular proliferation. Conclusive evidence is still lacking that sphingolipids, similar to phospholipids, can generate active metabolites in response to cell agonists. However, recently it has been shown that sphingomyelin turnover may be an important signaling mechanism transducing the actions of tumor necrosis factor α and gamma interferon on cell differentiation (Kim et al., 1991).

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Dedicated to Lea and Baruch Arazi on the occasion of their seventieth birthdays.

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