Signaling Pathways for Sphingosylphosphorylcholine-mediated Mitogenesis in Swiss 3T3 Fibroblasts

Naishadh N. Desai, Robert O. Carlson, Mark E. Mattie, Ana Olivera, Nancy E. Buckley, Takashi Seki, Gary Brooker, and Sarah Spiegel

Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, DC 20007

Abstract. Sphingosylphosphorylcholine (SPC), or lysophingomyelin, a wide-spectrum growth promoting agent for a variety of cell types (Desai, N. N., and S. Spiegel. 1991. *Biochem. Biophys. Res. Comm.* 181: 361–366), stimulates cellular proliferation of quiescent Swiss 3T3 fibroblasts to a greater extent than other known growth factors or than the structurally related molecules, sphingosine and sphingosine-1-phosphate. SPC potentiated the mitogenic effect of an activator of protein kinase C, 12-*O*-tetradecanoylphorbol 13-acetate, and did not compete with phorbol esters for binding to protein kinase C in intact Swiss 3T3 fibroblasts. However, downregulation of protein kinase C, by prolonged treatment with phorbol ester, reduced, but did not eliminate, the ability of SPC to stimulate DNA synthesis, indicating that SPC may act via both protein kinase C-dependent and -independent signaling pathways. SPC induced a rapid rise in intracellular free calcium ($[Ca^{2+}]_i$) in viable 3T3 fibroblasts determined with a digital imaging system. Although the increases in $[Ca^{2+}]_i$ were observed even in the absence of calcium in the external medium, no increase in the levels of inositol phosphates could be detected in response to mitogenic concentrations of SPC. Furthermore, in contrast to sphingosine or sphingosine-1-phosphate, the mitogenic effect of SPC was not accompanied by increases in phosphatidic acid levels or changes in cAMP levels. SPC, but not sphingosine or sphingosine-1-phosphate, stimulates the release of arachidonic acid. Therefore, the ability of SPC to act as an extremely potent mitogen may be due to activation of signaling pathway(s) distinct from those used by sphingosine or sphingosine-1-phosphate.

SPHINGOSINE and lysophingolipids have been implicated in signal transduction in various cellular processes (Hannun and Bell, 1987, 1989; Merrill and Stevens, 1989; Merrill, 1991). It is well established that sphingosine and other lysosphingolipids have diverse biological functions that are dependent on protein kinase C (PKC),[1] a pivotal regulatory enzyme in signal transduction (Hannun and Bell, 1987, 1989; Merrill and Stevens, 1989). Nevertheless, other studies indicate a more versatile role for sphingosine which does not seem to be restricted to its effects on PKC (Davis et al., 1988; Faucher et al., 1988; Zhang et al., 1990*a*; and for review see Merrill, 1991). We have previously reported that sphingosine possesses growth-promoting effects in 3T3 fibroblasts that are clearly independent of PKC (Zhang et al., 1990*a*). The mitogenic action of sphingosine is coupled to an increase in the levels of phosphatidic acid (PA) (Zhang et al., 1990*b*) and mobilization of calcium from internal stores (Zhang et al., 1991).

Metabolites of sphingosine have been shown to be produced in cells and to have potent effects on cell growth (Hakomori, 1990; Zhang et al., 1991). Hakomori et al. found that a methylated derivative of sphingosine, *N,N*-dimethylsphingosine, was much more effective than sphingosine in inhibition of PKC and stimulation of src-kinase and EGF receptor tyrosine kinase activity (Igarashi et al., 1990; Felding-Habermann et al., 1990; Hakomori, 1990). In addition, experiments performed in our laboratory have shown that a different sphingosine-derived metabolite, sphingosine-1-phosphate (SPP), which mimics the effects of sphingosine on PA and calcium release, is also a very potent mitogen for 3T3 fibroblasts (Zhang et al., 1991; Desai et al., 1992).

Recently, we reported that another sphingoid base, sphingosylphosphorylcholine (SPC) or lysosphingomyelin, is a powerful mitogen that stimulates DNA synthesis and cellular proliferation of Swiss 3T3 fibroblasts and a variety of other cell types (Desai and Spiegel, 1992). In the present study, we attempted to elucidate the signal transduction pathways

Please address all correspondence to Dr. Sarah Spiegel, Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, 307 Basic Science Building, 3900 Reservoir Road NW, Washington, DC 20007.

1. *Abbreviations used in this paper*: AA, arachidonic acid; $[Ca^{2+}]_i$, intracellular free calcium; BK, peptide hormone bradykinin; $InsP_3$, inositol trisphosphate; PA, phosphatidic acid; PDBu, phorbol 12, 13-dibutyrate; PKC, protein kinase C; SPC, sphingosylphosphorylcholine; SPP, sphingosine-1-phosphate; TG, thapsigargin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

underlying the mitogenic action of SPC. Initiation of DNA synthesis is considered to be triggered by several independent signal transduction pathways that act synergistically (Rozengurt, 1986). The most well known pathways are associated with intracellular messengers such as cAMP, diacylglycerol, an endogenous activator of PKC (Nishizuka, 1992), and inositol trisphosphate ($InsP_3$), which causes a release of Ca^{2+} from intracellular stores (Berridge and Irvine, 1989). Other less well-defined second messengers include PA (Exton, 1990) and arachidonic acid (AA) (Irvine, 1982; Burch and Axelrod, 1987) generated by receptor-coupled activation of phospholipases D and A_2, respectively. We report here that of these potentially important signaling pathways for mitogenesis, SPC induced changes only in intracellular calcium levels and AA metabolism in Swiss 3T3 fibroblasts, suggesting that the ability of SPC to act as a potent mitogen may be due to activation of unique signaling pathways distinct from those used by sphingosine or SPP.

Materials and Methods

Materials

[methyl^{3}H]Thymidine (55 Ci/mmol) and myo-[2-^{3}H]inositol (15 Ci/mmol) and [*N*-methyl-^{14}C]sphingomyelin (50–60 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). EGF, insulin, and transferrin were from Collaborative Research (Lexington, MA). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), D-sphingosine, and SPC were from Sigma Chem. Co. (St. Louis, MO). Thapsigargin was from Calbiochem (San Diego, CA). The various standard phospholipids were from Avanti Polar Lipids (Birmingham, AL). Silica Gel 60 plates were from EM Sciences (Cherry Hill, NJ). DMEM and PBS were from Advanced Biotechnologies (Silver Spring, MD), and Waymouth medium was from GIBCO BRL (Gaithersburg, MD). Fura-2/AM was from Molecular Probes Inc. (Eugene, OR). SPP was prepared as previously described (Zhang et al., 1991).

Cell Culture

Swiss 3T3 cells from the American Type Culture Collection, Rockville, MD (CCL 92) were cultured as previously described (Spiegel, 1989). For measurement of DNA synthesis, cAMP accumulation, and AA release, the cells were seeded and grown on multicluster plastic tissue culture dishes (24 × 16 mm wells, from Costar Corp., Cambridge, MA). For phospholipid analysis and phosphoinositide assays, the cells were cultured in 6-well cluster tissue culture dishes (6 × 34 mm wells, from Costar Corp.) For measurements of intracellular free Ca^{2+} ($[Ca^2]_i$), cells were grown on glass coverslips in 6-well clusters. The cells were subcultured at a density of 1.5×10^4 cells/cm^2 in DMEM supplemented with 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% calf serum, refed with the same medium after 2 d, and used 5 d later when the cells were confluent and quiescent (Spiegel and Panagiotopoulos, 1988).

Assay of DNA Synthesis

DNA synthesis was measured as previously described (Spiegel and Panagiotopoulos, 1988). After 18 h of incubation with test agents, 1 μCi of [^{3}H]thymidine was added, the cells were incubated an additional 6 h, and the incorporation of radioactivity into TCA-insoluble material was measured (Spiegel and Panagiotopoulos, 1988). Values are the means of triplicate determinations. Standard errors were routinely <10% of the mean.

Uptake and Metabolism of Radiolabeled SPC

[*N*-methyl-^{14}C]sphingomyelin was converted to [*N*-methyl-^{14}C]SPC by treatment with 0.5 M HCl in methanol (1 μCi/ml) at 65°C for 3 h and extracted with chloroform/methanol/water (8:4:3, vol/vol). The aqueous phase was removed, the pH was adjusted to 12, and the lipids reextracted with an equal volume of chloroform. The radioactivity present in the organic phase was solely SPC as determined by TLC. After evaporation of the chloroform, the labeled SPC was solubilized in DMEM containing 10 μM unlabeled SPC to a final specific activity of 1 mCi/mmol. Two ml of this solution were added to confluent and quiescent 3T3 cells in 6-well clusters and incubated at 37°C for various times. The medium was removed and the cells were washed three times with 2 ml of ice cold DMEM. Medium and cells were extracted with chloroform/methanol/water (8:4:3, vol/vol) and the pH of the aqueous phase was increased to 12 with 10 M KOH to optimize recovery of SPC in the organic phase. The extracted lipids were analyzed by TLC (solvent system: chloroform/methanol/water (70:35:8, vol/vol).

Mass Measurement of Ceramide and Sphingosine

The mass amounts of ceramide in cellular extracts were measured by the use of the DAG kinase enzymatic method (Schneider and Kennedy, 1973; Dressler and Kolesnick, 1991). Cells were treated with SPC or vehicle for various incubation periods. To quantitate ceramide levels, lipids were extracted, and then subjected to the action of *E. coli* DAG kinase as described previously (Olivera et al., 1992), and ceramide-1-phosphate was resolved by TLC with chloroform/methanol/acetic acid (65:15:5, vol/vol). Known amounts of ceramide standards were included with each assay. Sphingosine levels were measured by a similar enzymatic assay using sphingosine kinase partially purified from Swiss 3T3 fibroblasts (Olivera et al., 1992).

Measurement of Specific Binding of Phorbol Ester

Binding of [^{3}H]PDBu to cells in situ was measured essentially as described (Spiegel, 1989). Briefly, confluent and quiescent cultures were washed and incubated with DMEM containing 25 mM Hepes, pH 7.4, and 0.1% fatty acid free-BSA. Cells were treated with various concentrations of [^{3}H]-PDBu (1–100 nM) in the absence or presence of SPC. After 2 h at 0°C, the cells were washed with 2 × 1 ml of ice cold PBS-0.1% fatty acid free-BSA, dissolved in 0.2 N NaOH, and assayed for bound ligand. Nonspecific binding, measured by the addition of 1 μM unlabeled TPA, was subtracted from the total binding.

Measurements of Intracellular Free Ca^{2+} Concentration

$[Ca^{2+}]_i$ was determined with the fluorescent calcium-sensitive dye fura-2/AM as described previously (De Erausquin et al., 1990; Zhang et al., 1991). 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 mounted in a 35-mm holder. Changes in fura-2 fluorescence in single cells were monitored by dual excitation imaging using a Digital Fluorescence Microscopy System (Atto Instrs. Inc., Rockville, MD). $[Ca^{2+}]_i$ was determined from the ratio of fura-2 fluorescence emissions after excitation at the dual wavelength pair of 334 and 380 nm (De Erausquin et al., 1990). For experiments done at 37°C, the mitogens were dissolved in Locke's solution and perfused over the cells at 1 ml/min with a peristaltic pump. In the experiments where the cells were challenged repeatedly with SPC, each treatment was followed by a 10-min period before the next challenge, to allow the intracellular calcium concentrations to return to basal levels.

Measurement of Phosphoinositide Breakdown

Confluent cultures of Swiss 3T3 cells were prelabeled for the final 60 h of growth with myo-[2-^{3}H]inositol (5 μCi/ml). Cells were rinsed twice with DMEM containing 10 mM LiCl, incubated for 5 min at 37°C to remove free inositol, and then treated with various mitogenic compounds or vehicle. At the end of the incubation period, the medium was removed by aspiration and the reactions terminated by the addition of chloroform/methanol/4N HCl (100:200:2 vol/vol). After extraction, the aqueous phases containing the inositol phosphates were separated on Dowex AG-1 × 8 ion-exchange columns as previously described (Spiegel and Panagiotopoulos, 1988).

Phosphatidic Acid Determination

Confluent and quiescent cultures of 3T3 cells were washed with phosphate-free DMEM and incubated with this medium containing $^{32}P_i$ (40 μCi/ml) for 24 h. Cells were treated with sphingosine, SPC, or vehicle alone for various times, the medium was rapidly removed, and lipids were extracted (Zhang et al., 1990*b*). PA was analyzed by TLC using the organic phase

of the mixture of isooctane/ethyl acetate/acetic acid/water (50:110:20:100 vol/vol) (Pai et al., 1988; Zhang et al., 1990*b*). In this system, PA (R_f = 0.1) was well separated from other phospholipids (R_f = 0). The lipid standards were visualized by spraying with molybdenum blue spray. Phospholipids were located by autoradiography and the radioactivity was quantified by liquid scintillation counting of the corresponding silica gel areas.

Assay for Cyclic AMP

Cells were incubated for 20 min at 37°C in DMEM containing 0.1 mM 1-methyl-3-isobutylxanthine, a phosphodiesterase inhibitor, with or without 10 μM isoproterenol. 10 μM SPC or vehicle was then added, and 20 min later, the medium was aspirated and cAMP extracted from the cells with 1 ml 0.1 M HCl containing 0.1 mM $CaCl_2$ and measured by radioimmunoassay as described previously (Zhang et al., 1990*b*).

Assay for Arachidonic Acid Release

Confluent cultures of Swiss 3T3 cells were prelabeled for 24 h with [^{3}H]AA (0.25 μCi/ml), which resulted in 60–90% incorporation of the label. Cells were washed twice with DMEM containing 20 mM Hepes (pH 7.4). After stimulation with different agents in the presence of BSA (20 μg/ml), aliquots of the medium were taken, centrifuged at 500 *g* for 5 min to remove floating cells, and the resulting supernatants counted for radioactivity. In some experiments, the medium was acidified and extracted twice with an equal volume of ethyl acetate. The extracted lipids were analyzed by TLC (silica gel 60, petroleum ether/diethyl ether/conc. acetic acid (80:20:1 vol/vol) to separate phospholipids, eicosanoids, and free AA, as previously described (Carlson and Levitan, 1990). In other experiments, lipids extracted from cells were analyzed by two-dimensional TLC as previously described (Zhang et al., 1991).

Results

Mitogenicity of SPC Relative to Lysophospholipids and Other Sphingolipids

SPC greatly stimulated DNA synthesis and cell division of quiescent cultures of Swiss 3T3 fibroblasts (Desai and Spiegel, 1991). Consistent with our previous study, SPC caused a dose-dependent increase in [^{3}H]thymidine incorporation into DNA, with maximum effect at 10 μM (Fig. 1). At concentrations greater than 30 μM, SPC inhibited DNA synthesis and was cytotoxic, as determined by Trypan blue dye exclusion. Previously, we have demonstrated that sphingosine and its phosphorylated derivative, SPP, also stimulated proliferation of quiescent 3T3 fibroblasts (Zhang et al., 1990*a*; Zhang et al., 1991). Thus it was of interest to compare the mitogenic properties of these structurally related compounds. As can be seen in Table I, SPC stimulated DNA synthesis to a greater extent than sphingosine, SPP, and other known growth factors. Moreover, in contrast to insulin or EGF, whose action require their continuous presence for at least 6 h (Dicker and Rozengurt, 1978), SPC induced DNA synthesis after a short treatment. Exposure of the fibroblasts to SPC for 10 min, 1 and 3 h, respectively, followed by washing, resulted in 50, 72, and 78% of maximum DNA synthesis induced by 24 h of exposure.

The mitogenic effect of SPC was also compared with the effects of similar concentrations of other lysophospholipids (Table II). None of the compounds tested were as potent as SPC. Only lysophosphatidylcholine and platelet-activating factor had significant mitogenic activity, albeit much less than SPC (Table II). The effect of sphingomyelin, which has a fatty acyl moiety linked at the amino group of SPC on DNA synthesis, was also examined. Since sphingomyelin is relatively insoluble in aqueous solutions, sphingomyelin vesicles were used in these studies (Pagano and Weinstein, 1978). Sphingomyelin (0.1–15 μM) did not stimulate [^{3}H]thymidine incorporation either alone (N = 3), or in the presence of insulin (N = 7), or TPA (N = 8).

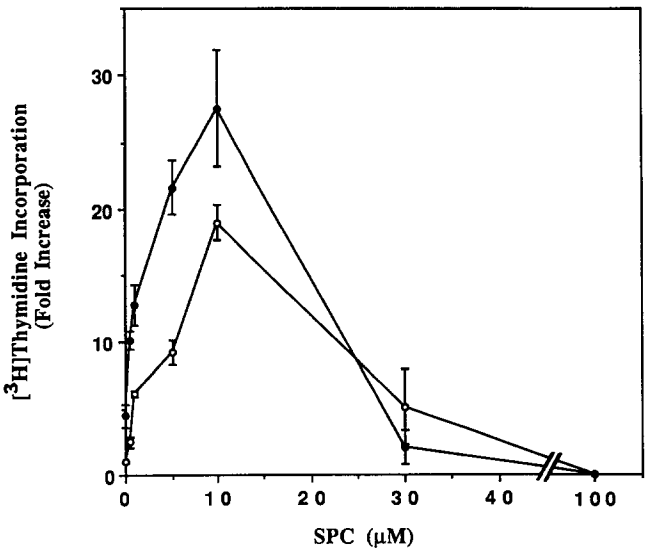

Figure 1. Dose response for stimulation of DNA synthesis in Swiss 3T3 cells by SPC. Confluent and quiescent cultures of Swiss 3T3 cells were incubated with various concentrations of SPC in the absence (○) or the presence of 100 nM of TPA (●) and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. The data are expressed as fold stimulation relative to the untreated control. Similar results were obtained in at least five additional experiments. The amount of [^{3}H]thymidine incorporated in untreated or TPA-treated cells was 3.9 ± 0.4 and 20.4 ± 1.4 (cpm × 10^{-3}/well, respectively.

Uptake and Metabolism of SPC in Swiss 3T3 Fibroblasts

Since SPC is more potent than sphingosine, SPP, spingomyelin (Table I), and ceramide (Olivera et al., 1992) in stimulating DNA synthesis, it seems most likely that SPC itself is the active agent. To examine this point further, we have stud-

Table I. Effects of Sphingosylphosphorylcholine, Sphingosine, and Sphingosine-1-Phosphate on DNA Synthesis

[^{3}H]Thymidine incorporation (cpm × 10^{-3}/well)

	Sphingoid bases			
	None	SPC	Sphingosine	SPP
Mitogens				
None	6	149	11	16
Insulin	13	332	25	40
EGF	42	448	89	101
Insulin plus EGF	251	656	366	429

Quiescent cultures of Swiss 3T3 cells were exposed to the indicated mitogens in the absence or presence of the sphingoid bases. [^{3}H]Thymidine incorporation was measured as described in Materials and Methods. Each value is the mean of triplicate determinations from a representative experiment. SDs were routinely <10%. Similar results were obtained in at least seven additional experiments. The concentrations used were: *Insulin*, 2 μg/ml; *EGF*, 10 ng/ml; *SPC*, 10 μM; *sphingosine*, 20 μM; and *SPP*, 5 μM.

Table II. Stimulation of DNA Synthesis in Quiescent Swiss 3T3 Fibroblasts by Various Lysoglycerophospholipids

Stimulus	[^{3}H]Thymidine incorporation (% of response to SPC)
A SPC	100 ± 4
None	11 ± 3
Lysophosphatidic acid	13 ± 3
Lysophosphatidylcholine	40 ± 3
Lysophosphatidylethanolamine	19 ± 1
Lysophosphatidylserine	22 ± 3
Lysophosphatidylinositol	12 ± 2
B SPC	100 ± 6
None	6 ± 1
Platelet-activating factor	22 ± 2
Lyso-platelet-activating factor	14 ± 1

In two separate experiments (*A* and *B*) quiescent cultures of Swiss 3T3 cells were stimulated with SPC or the indicated lysoglycerophospholipids (10 μM) for 24 h. [^{3}H]Thymidine incorporation was measured as described in Materials and Methods.

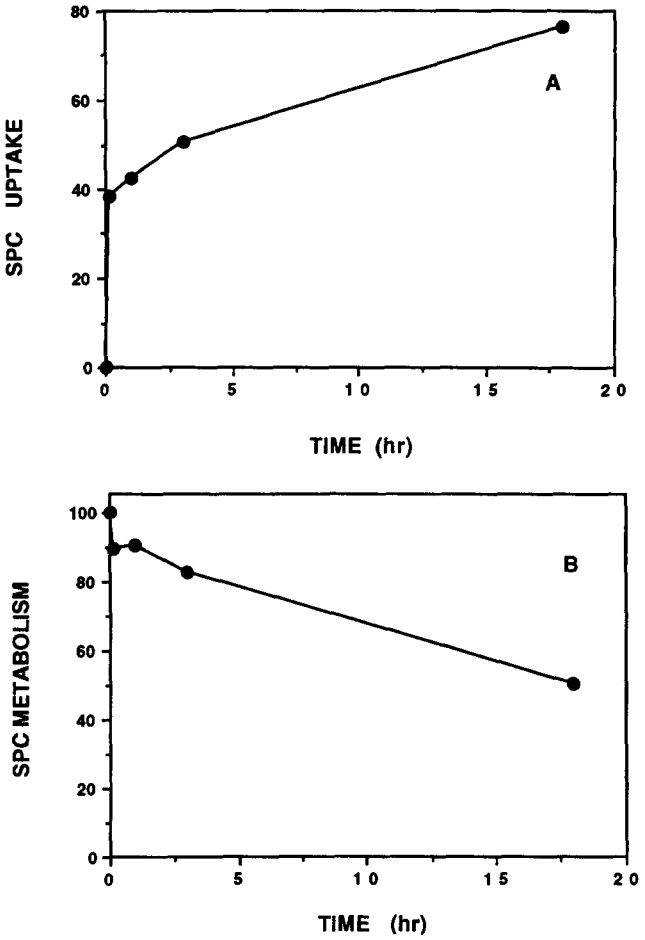

Figure 2. Kinetics of uptake and metabolism of radiolabeled SPC. Confluent and quiescent 3T3 fibroblasts were incubated at 37°C with 10 μM [*N*-methyl-^{14}C]SPC (1 mCi/mmol) for the indicated time periods. After extraction, lipids were separated by TLC and analyzed as described in Materials and Methods. In *A*, the extent of uptake of [^{14}C]SPC is expressed as percent of total [^{14}C]SPC added. In *B*, the percent of the cell-associated radioactivity that comigrated on TLC with authentic SPC is depicted.

ied the cellular uptake and metabolism of [*N*-methyl-^{14}C]SPC. ^{14}C-Labeled SPC (10 μM; 1 mCi/mmol) was rapidly taken up by Swiss 3T3 fibroblasts; 38% of the label was incorporated within 10 min. This was followed by a slower phase of incorporation and by 18 h, 76% of the label was taken up by the cells. After 1 h, ^{14}C-labeled SPC was the only labeled lipid detected by TLC and there was no significant labeling of lysoPC, PC, or sphingomyelin. Over the next 3 h, ~30% of the SPC taken up was broken down or converted to other products and by 18 h 55% of the SPC taken up had undergone metabolism (Fig. 2). Choline or phosphocholine accounted for a significant portion of the SPC metabolic products. Radiolabeled sphingomyelin, which was detectable by 3 h, represented only 1.3% of the total SPC taken up. This increased to 5.9% of the total uptake by 18 h. This production of radiolabeled sphingomyelin may be due to a very slow acylation of [^{14}C]SPC or reutilization of its breakdown products. We also measured the levels of sphingosine and ceramide, which can potentially be produced from SPC by degradation and reacylation. There were no changes in sphingosine levels up to 1 h of treatment with SPC and the level slightly increased after 3 and 18 h to 145 and 158% of control values, respectively. It should be noted that this represents only a very small fraction of the SPC that was taken up. No detectable increase in ceramide was observed.

Role of PKC in the Mitogenic Response Induced by SPC

SPC potentiated the mitogenic effect of TPA in quiescent cultures of Swiss 3T3 cells in a concentration-dependent manner (Fig. 1). This effect was observed even in the presence of insulin, which also potentiated the mitogenic response to TPA (Table III; Desai and Spiegel, 1991). Hannun and Bell found that SPC and other lysosphingolipids inhibited phorbol dibutyrate (DPBu) binding as well as activity of PKC in micellar assays (Hannun and Bell, 1987). To determine whether SPC interacted with PKC in intact Swiss 3T3 fibroblasts, we examined the effects of SPC on PDBu binding.

Table III. Effect of Prolonged Phorbol Ester Pretreatment on Reinitiation of DNA Synthesis Induced by SPC

	[^{3}H]Thymidine incorporation (cpm × 10^{-3}/well)	
	Control	TPA pretreated
Stimulus		
None	2.9 ± 0.2	4.7 ± 0.5
SPC	94.0 ± 3.3	48.0 ± 0.5
TPA	29.9 ± 6.8	6.8 ± 1.9
Insulin	6.7 ± 1.0	29.5 ± 6.0
Insulin + SPC	208 ± 55.0	114.0 ± 5.2
Insulin + TPA	82.0 ± 4.2	16.0 ± 4.8

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 and 5 μg/ml transferrin in the presence of the indicated mitogens. [^{3}H]Thymidine incorporation into DNA 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 mitogens were: *SPC*, 10 μM; *TPA*, 100 nM; and *Insulin*, 2 μg/ml.

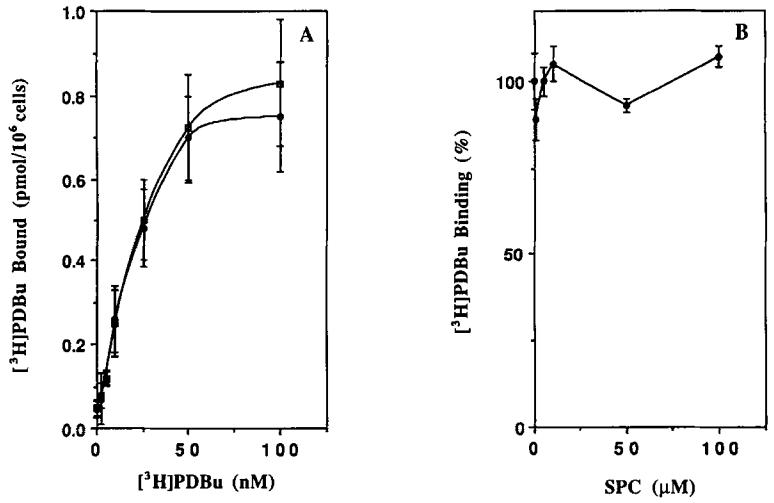

Figure 3. Effect of SPC on specific binding of [³H]-PDBu to Swiss 3T3 cells. (*A*) Confluent and quiescent cultures were incubated in the absence (○) or presence (●) of SPC (10 μM) for 1 h, and [³H]PDBu binding was measured as described in Materials and Methods. In *B*, the cells were pretreated with different concentrations of SPC for 1 h and specific [³H]PDBu binding (25 nM) was measured. The data are expressed as percent of control (100% = 1.12 ± 0.08 pmol/10⁶ cells). Calculated K_d values for control and SPC-treated cells were 32.1 ± 5.6 nM and 26.9 ± 5.5 nM, respectively.

Similar to results previously reported in Swiss 3T3 cells (Dicker and Rozengurt, 1978; Rozengurt, 1986; Zhang et al., 1990*a*), half-maximal [³H]PDBu binding occurred at a concentration of 15 nM (Fig. 3 *A*). Mitogenic concentrations of SPC (≤10 μM) had no effect on the binding of [³H]PDBu to its specific receptors on intact cells (Fig. 3 *A*). Even higher concentrations of SPC did not result in any significant displacement of PDBu (Fig. 3 *B*), indicating that SPC does not compete with PDBu binding sites on PKC.

To further investigate the role of PKC in the mitogenic response induced by SPC, we studied the effects of the selective removal of this enzyme by prolonged treatment of cells with phorbol ester. Prolonged exposure of quiescent cultures of Swiss 3T3 cells to TPA leads to downregulation of PKC enzymatic activity (Rozengurt, 1986; Spiegel, 1989) and [³H]-PDBu binding (Rozengurt, 1986; Zhang et al., 1990*a*; Spiegel, 1989). In agreement with our previous studies, prolonged treatment with TPA abolished the mitogenic effect of TPA (in the absence or presence of insulin, Table III). In contrast, the responses to other mitogens which do not require activation of PKC, such as insulin and sphingosine (Hesketh et al., 1988; Zhang et al., 1990*a*), were not affected. Downregulation of PKC did not eliminate the ability of SPC to stimulate DNA synthesis, however, the response to SPC was reduced by ~50% (Table III).

Effect of SPC on $[Ca^{2+}]_i$

Recently, sphingosine and SPP were shown to cause the release of calcium in viable, quiescent cultures of Swiss 3T3 fibroblasts (Zhang et al., 1991). Furthermore, SPC and sphingosine have been reported to release $^{45}Ca^{2+}$ from permeabilized smooth muscle cells (Ghosh et al., 1990). Using digital imaging fluorescence microscopy, we investigated the effect of SPC on $[Ca^{2+}]_i$ in quiescent Swiss 3T3 fibroblasts. In individual cells, basal $[Ca^{2+}]_i$ varied between 30–120 nM (n = 30), consistent with the values reported by others (Spiegel and Panagiotopoulos, 1988; Hesketh et al., 1988; Zhang et al., 1991). There were genuine variations in the level of $[Ca^{2+}]_i$ from cell to cell, even among quiescent cells, which were morphologically indistinguishable. Application of mitogenic concentrations of SPC induced a wave of $[Ca^{2+}]_i$ which usually spread to cover the entire cell (representative

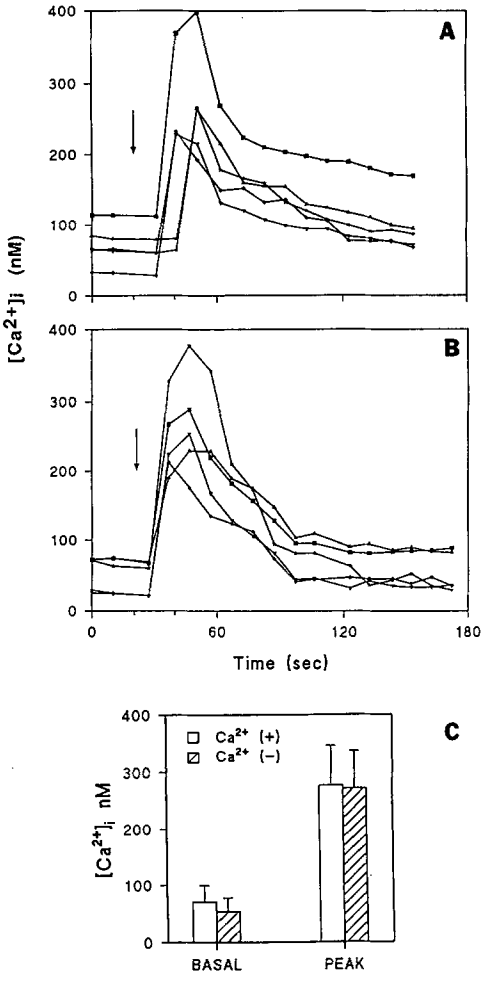

Figure 4. Changes in $[Ca^{2+}]_i$ after addition of SPC. Quiescent cultures of Swiss 3T3 cells were loaded with fura-2/AM, washed, and incubated at 37°C in Locke's buffer containing Ca^{2+} (*A*) or in Ca^{2+}-free buffer containing 2 mM EGTA (*B*). At the indicated times (*arrow*), 10 μM SPC was added. $[Ca^{2+}]_i$ was determined by fura-2 imaging as described in Materials and Methods. In each case, $[Ca^{2+}]_i$ of five responsive cells are shown. In *C*, the mean peak changes in $[Ca^{2+}]_i$ were measured in the absence (−) or presence (+) of 2 mM extracellular calcium. Basal values were determined 10 s before the addition of 10 μM SPC.

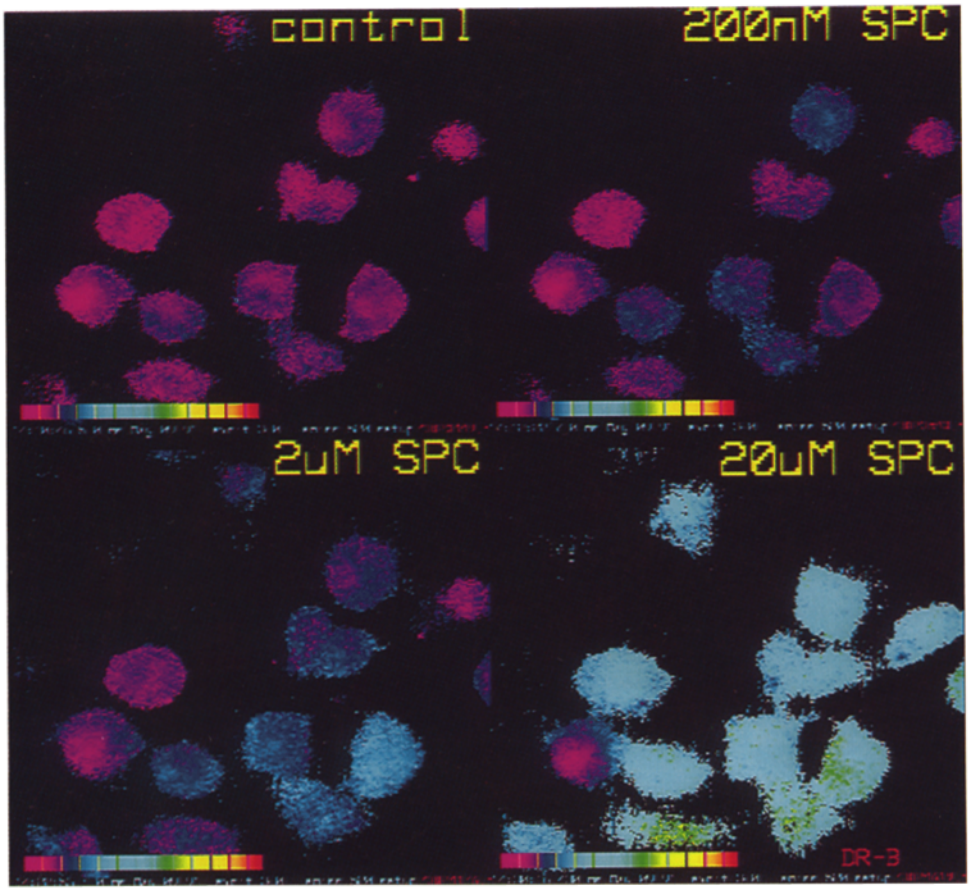

Figure 5. Pseudocolor images of fura-2 fluorescence intensity ratio imaging in Swiss 3T3 cells stimulated with various concentrations of SPC (340 nm image/380 nm image). $[Ca^{2+}]_i$ was visualized by dual fluorescent microscopy imaging using fura-2/AM as described in Materials and Methods. Color scale at bottom depicts pixel intensity of $[Ca^{+2}]_i$ from 0 nM (*pink*) to 800 nM (*red*). Cells were challenged repeatedly with the indicated concentrations of SPC and each treatment was followed by a resting period of 10 min in Locke's buffer before the next challenge. This period allowed $[Ca^{+2}]_i$ to return to basal levels.

examples of cells responsive to 10 μM SPC are depicted in Fig. 4 *A*). The SPC-induced change in $[Ca^{2+}]_i$ peaked within 30 s, and then gradually returned to resting levels over the following 2–3 min. Neither the rate of onset (Fig. 4 *B*) nor the magnitude (Fig. 4 *C*) of this response was altered in the absence of extracellular calcium, suggesting that the SPC-induced change in $[Ca^{2+}]_i$ was because of mobilization of calcium from intracellular pools. However, shorter time was required for $[Ca^{2+}]_i$ to return to baseline levels in the absence of extracellular calcium. Also, lowering the temperature from 37°C to room temperature did not alter the kinetic characteristics of the SPC-induced increase in $[Ca^{2+}]_i$ (data not shown). The response to SPC was heterogeneous. Cells differed significantly in their ability to respond to different concentrations of SPC and some of the cells were entirely unresponsive (Fig. 5). SPC induced a detectable calcium release at a concentration as low as 200 nM (Fig. 5). However, both the magnitude of the response and the fraction of the cells showing a response increased with increasing concentrations of SPC (Fig. 5). Concentrations of SPC above 30 μM were not tested since they were cytotoxic.

Effect of SPC on Phosphoinositide Breakdown

Swiss 3T3 cells have receptors for the peptide hormone bradykinin (BK) which activate polyphosphoinositide hydrolysis leading to the formation of inositol phosphates and the release of calcium from internal stores (Powis et al., 1990; van Corven et al., 1989). In agreement with previous reports, BK (1 μM) induced a transient $[Ca^{2+}]_i$ elevation in Swiss 3T3 fibroblasts. The kinetics of the increase were similar to those evoked by SPC (10 μM); however, the peak values of $[Ca^{2+}]_i$ were higher (Mattie, M. E., G. Brooker, and S. Spiegel, unpublished observations). To investigate the possibility that SPC might stimulate phosphoinositide hydrolysis to generate inositol phosphates which subsequently stimulate increased $[Ca^{2+}]_i$, the effect of SPC on the generation of inositol phosphates was examined. In agreement with previous studies (Powis et al., 1990), stimulation of quiescent Swiss 3T3 fibroblasts with BK (1 μM) for 5 min resulted in a 1.3-fold increase in the levels of inositol phosphates (Table IV). In contrast, an optimal mitogenic concentration of SPC (10 μM) did not stimulate the production of inositol phosphates (Table IV). This concentration of SPC was more than 50-fold greater than that required to elicit a significant calcium response (Fig. 5) and was capable of reinitiating DNA synthesis in quiescent cells as effectively as 10% (vol/vol) FBS (Desai and Spiegel, 1991). However, at higher concentrations, SPC increased the level of $InsP_3$ which was measured directly (data not shown).

Effect of SPC on PA

We have previously demonstrated that the mitogenic effects of sphingosine and SPP are accompanied by an increase in levels of PA (Zhang et al., 1990*b*, 1991). Consistent with our previous study (Zhang et al., 1990*b*), a mitogenic concentration of sphingosine or SPP caused a significant increase in

Table IV. Effect of SPC on Accumulation of Inositol Phosphates and cAMP

Stimulant	None	SPC	BK
[^{3}H]Inositol phosphate accumulation (dpm/well)	18,450 ± 80	17,890 ± 310	23,445 ± 90
cAMP Accumulation (pmol/well)			
(−) Isoproterenol	3.5 ± 0.1	3.9 ± 0.04	ND
(+) Isoproterenol	11.1 ± 0.7	12.4 ± 1.1	ND

Confluent and quiescent cultures of Swiss 3T3 cells were exposed to SPC (10 μM) or BK (1 μM), or vehicle (*None*) for 5 min, and levels of inositol phosphates were determined as described in Materials and Methods. Data are expressed as the total radioactivity incorporated into inositol mono-, bis-, and trisphosphate. Levels of cAMP were measured in duplicate cultures with (+) or without (−) 10 μM isoproterenol. The values are means ± SD. In separate experiments, cAMP levels after treatment with cholera holotoxin (1 μg/ml) were 30.5 ± 5.3 and 55.3 ± 5.0 pmol/well in the absence or presence of 10 μM isoproterenol, respectively. *ND*, not determined.

[^{32}P]PA levels in Swiss 3T3 cells prelabeled to isotopic equilibrium with $^{32}P_i$ (Fig. 6). However, unlike sphingosine or SPP, SPC did not induce a measurable change in the content of radiolabeled PA with incubations as short as 5 min or as long as 6 h (Fig. 6).

Effect of SPC on cAMP Levels

Mitogens, such as insulin and cholera toxin, have been shown to increase intracellular cAMP concentrations and thereby affect cellular metabolism and growth (Rozengurt et al., 1981). In sharp contrast, the mitogenic effects of PA and sphingosine are associated with decreases in cAMP levels (Murayama and Ui, 1987; Zhang et al., 1990*b*). cAMP levels in cells which were treated with mitogenic concentration of SPC were not significantly different from untreated cells (Table IV). In comparison, cAMP levels increased 18- and 3-fold, respectively, after treatment with cholera toxin or isoproterenol. In further experiments, the incubation medium was supplemented with isoproterenol to allow accurate estimation of the cAMP suppression in intact cells, as was done in previous studies with PA and sphingosine (Murayama and Ui, 1987; Zhang et al., 1990*b*). Unlike the effects of sphingosine (Zhang et al., 1990*b*), SPC did not cause any detectable decrease in the cellular cAMP levels (Table IV).

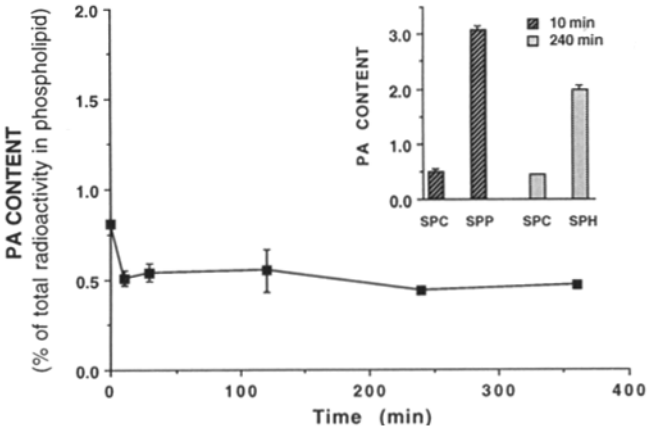

Figure 6. Lack of effect of SPC on PA levels. Confluent and quiescent cultures of Swiss 3T3 cells were prelabeled with $^{32}P_i$ for 24 h and stimulated with SPC (20 μM) for the indicated time periods. After extraction, lipids were separated by TLC and ^{32}P-PA analyzed as described in Materials and Methods. The results are expressed as percent of total radioactivity in phospholipids. The inset bar graph depicts accumulation of ^{32}P-PA in response to SPC (20 μM) or SPP (5 μM) after 10 min (*dark fill*), and in response to SPC (20 μM) or sphingosine (*SPH*, 20 μM) after 240 min (*light fill*). Extensive studies on sphingosine- and SPP-induced PA accumulation have been reported previously (Zhang et al., 1990*a*,*b*, 1991; Desai et al., 1992).

Effect of SPC on Arachidonic Acid Levels

In many cell types, increased generation of unesterified AA and concomitant production of oxygenated metabolites of AA (collectively termed eicosanoids) often accompany increases in $[Ca^{2+}]_i$ (Irvine, 1982). In quiescent 3T3 fibroblasts prelabeled with [^{3}H]AA, SPC induced a concentration- and time-dependent release of radioactivity into the medium (Fig. 7) which was identified as free AA. Loss of radiolabel from phospholipids completely accounted for the release of radioactivity from cells treated with SPC. There was a small but significant decrease of [^{3}H]AA-labeled PC, PE, and PI. In contrast, no significant loss of label from PS was observed.

In contrast to SPC, sphingosine had no significant effect on the release of AA (Fig. 7 *B*). However, it inhibits the SPC-induced AA release (Fig. 7 B). Similarly, sphingosine reduced SPC-induced mitogenesis (Fig. 8). Whereas individually either sphingosine or SPC stimulated [^{3}H]thymidine incorporation in the presence or absence of insulin (Figs. 1 and 8; Tables I and III), when sphingosine and SPC were added simultaneously, the response to SPC was substantially reduced (Fig. 8).

Relationship between Arachidonic Acid, Calcium, and Mitogenesis in Response to SPC

It was possible that the increase in AA induced by SPC was a consequence of the increase in $[Ca^{2+}]_i$. Thapsigargin (TG), an irreversible inhibitor of the ER Ca^{2+}-ATPase that does not inhibit the plasmalemmal enzyme, is a useful agent to deplete intracellular calcium pools (Thastrup et al., 1990). TG stimulates a transient increase in intracellular calcium in 3T3 cells, but does not stimulate a significant increase in release of AA in cells prelabeled with [^{3}H]AA. Furthermore, treatment with TG for 25 min before the addition of SPC completely blocks the ability of SPC (20 μM) to stimulate an increase in $[Ca^{2+}]_i$, but only decreased the SPC-stimulated release of AA from phospholipids by 20%. These results indicate that the rise in calcium induced by

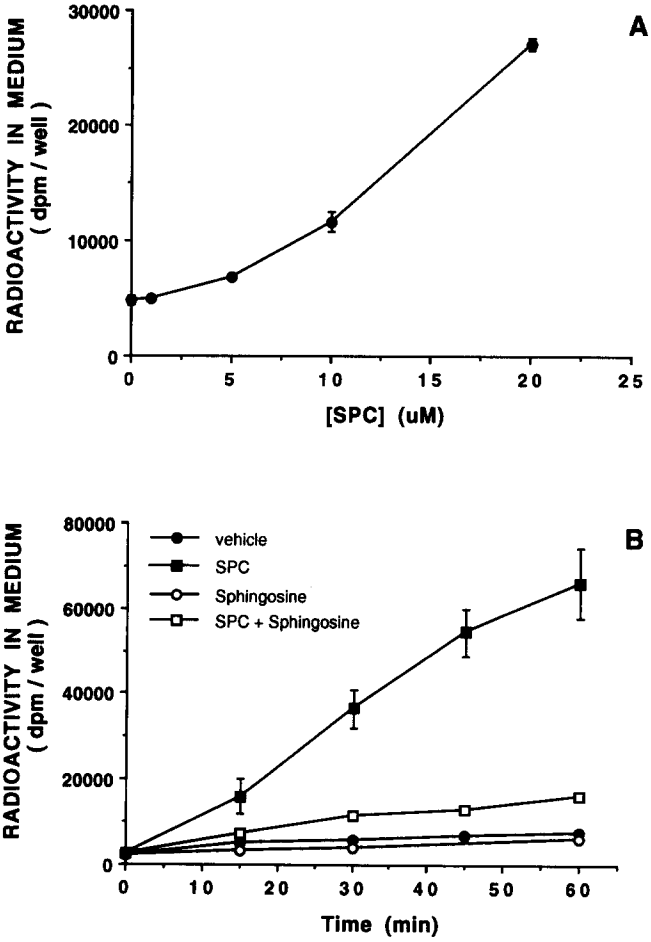

Figure 7. Effect of SPC on AA release in intact Swiss 3T3 fibroblasts. Confluent and quiescent cultures of Swiss 3T3 cells were prelabeled with [^{3}H]arachidonic acid for 24 h and stimulated for 10 min with various concentrations of SPC (*A*). Radioactivity released from the cells (in medium devoid of BSA) was measured as described in Materials and Methods. Values represent means ± standard errors for triplicate determinations. In *B*, Swiss 3T3 cells prelabeled with [^{3}H]arachidonic acid were stimulated (in medium containing 20 μg/ml BSA) with vehicle (*filled circles*), 20 μM SPC (*filled squares*), 20 μM sphingosine (*open circles*), or SPC plus sphingosine (20 μM each, *open squares*).

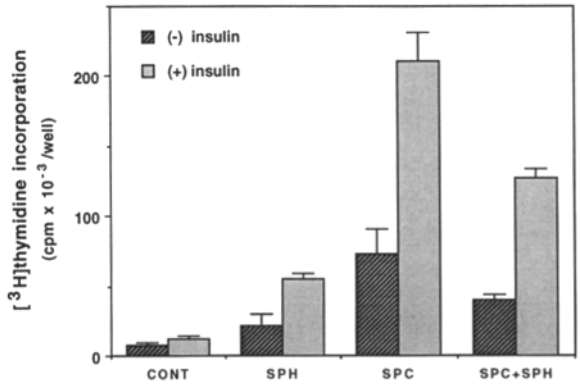

Figure 8. Effect of SPC, sphingosine, or both on DNA synthesis. [^{3}H]Thymidine incorporation was assayed as described in Materials and Methods after stimulation with vehicle (*cont*), 20 μM sphingosine (*SPH*), 20 μM SPC, or SPC plus sphingosine (20 μM each) in the absence (*dark fill*) or presence (*light fill*) of 4 μg/ml of insulin.

SPC is probably not involved in the release of AA. A recent study has demonstrated that AA can stimulate elevation of $[Ca^{2+}]_i$ in Jurkat T lymphocytes (Chow and Jondal, 1990). Thus, it was of interest to examine whether AA can release calcium from internal sources in Swiss 3T3 fibroblasts. High concentrations of exogenous AA (33–66 μM) were found to transiently increase intracellular calcium similar to SPC.

Since the prominent signal transduction pathways that are affected by SPC are calcium mobilization and arachidonate release, we examined the direct effects of calcium mobilization (activated by ionomycin or TG) and exogenous AA on mitogenesis. In 3T3 fibroblasts, in agreement with other cell types (Ghosh et al., 1991), TG inhibits [^{3}H]thymidine incorporation. Similarly, ionomycin (1 μM) which significantly stimulates an increase in intracellular calcium, also decreases [^{3}H]thymidine incorporation and was cytotoxic. Lower concentrations of ionomycin (50–100 nM) were only weakly mitogenic. In agreement with other studies (Millar and Rozengurt, 1990; Gil et al., 1991; Takuwa et al., 1991), AA alone was not mitogenic and only slightly enhanced the mitogenic effect of insulin. Even a combination of AA, insulin, and a low concentration of ionomycin was much less potent than SPC. Thus, none of the combinations of AA and agents which mobilize calcium gave a mitogenic effect that approached that of SPC.

Discussion

SPC Signaling Pathways

Increased levels of SPC have been found in patients with Niemann-Pick disease (Strasberg and Callahan, 1987), a form of sphingolipidosis resulting from a deficiency of sphingomyelinase activity (Strasberg and Callahan, 1988). The potential role of SPC in the pathophysiology of this disease was suggested by its deleterious effects on mitochondrial function and calcium uptake (Strasberg and Callahan, 1988). At high concentrations, SPC has also been shown to induce neurite outgrowth in mouse neuroblastoma cell lines (Sugiyama et al., 1990). More recently, we observed that at low concentrations, SPC was an extremely potent wide-spectrum growth promoting agent for a variety of cell types (Desai and Spiegel, 1991). SPC alone was much more mitogenic for Swiss 3T3 fibroblasts than other known growth factors, such as insulin and EGF, and it was as effective as FBS (Desai and Spiegel, 1991). SPC was also significantly more potent than structurally related sphingolipids, including sphingosine and SPP.

The effect of SPC appeared to be specific, since none of the other lysophospholipids tested were as potent as SPC. Surprisingly, lysophosphatidate was not mitogenic for Swiss 3T3 fibroblasts, in contrast to its potent mitogenic effects reported for quiescent Rat-1 fibroblasts and for diploid human foreskin fibroblasts (van Corven et al., 1989). Of the analogs tested, only lysophosphatidylcholine, platelet-activating factor, and lysoplatelet-activating factor had mito-

genic activity, albeit significantly less than that of SPC. In this regard, a recent study demonstrated that lysophosphatidylcholine potentiated the mitogenic effect of IL2 on T cell proliferation (Asaoka et al., 1991) and EGF-dependent receptor autophosphorylation in A431 cells (Igarashi et al., 1990). Furthermore, lysophosphatidylcholine has been implicated as a putative intracellular messenger (Rustenbeck and Lenzen, 1989; Asaoka et al., 1992). All of the individual structural components of SPC appear essential for its strong mitogenic effect, since either acylation of the amino group with a fatty acid or acetyl group (Sugiyama et al., 1990), or absence of choline and phosphocholine groups (SPP and sphingosine, respectively) reduces its effects (Zhang et al., 1990*a*; 1991).

Involvement of PKC

Sphingosine and other lysosphingolipids have been reported to inhibit PKC activity, modulating the cellular responses dependent on this enzyme (Hannun and Bell, 1987, 1989). However, in contrast to a previous report that SPC inhibits PDBu binding to PKC (Hannun and Bell, 1987), we observed that SPC did not compete with phorbol ester for binding to PKC in intact Swiss 3T3 fibroblasts. SPC also acted synergistically with TPA to stimulate mitogenesis of Swiss 3T3 fibroblasts, suggesting that SPC does not act through inhibition of PKC. These results, together with the ability of SPC to stimulate mitogenesis in 3T3 cells depleted of PKC activity, indicate that PKC cannot be the sole target for SPC-induced mitogenesis. However, we cannot exclude the possibility that PKC plays some role in the mitogenic effect of SPC since there was a significant decrease in the SPC-induced stimulation of DNA synthesis after downregulation of PKC. Furthermore, the synergistic effects between SPC and TPA were not as pronounced as the synergistic effects between SPC and either insulin or EGF, mitogens which are known to act independently of PKC (Hesketh et al., 1988). Thus, SPC may act through both PKC-dependent and -independent signaling pathways. In sharp contrast, we previously demonstrated the mitogenic activities of sphingosine (Zhang et al., 1990*a*) and SPP (Zhang et al., 1991) in Swiss 3T3 cells were clearly independent of PKC.

The Mitogenic Effect of SPC Correlates with $[Ca^{2+}]_i$

Using a digital imaging system for measurement of cytosolic free calcium, we observed that SPC induced transient, pancellular increases in $[Ca^{2+}]_i$. The rapid rise in $[Ca^{2+}]_i$ was independent of the presence of calcium in the external medium, indicating that the response was mainly due to mobilization of calcium from internal stores. Thus, SPC is also a calcium-mobilizing agonist in viable 3T3 fibroblasts, acting in a similar manner as sphingosine and SPP (Zhang et al., 1991). These findings are consistent with recent studies in which sphingosine or SPC were found to stimulate ^{45}Ca fluxes in permeabilized smooth muscle cells (Ghosh et al., 1990). Similar to SPP, and in contrast to sphingosine, the effect of SPC on $[Ca^{2+}]_i$ was not notably altered by lowering the temperature. Although mitogenic concentrations of SPC stimulated a rise in $[Ca^{2+}]_i$ in Swiss 3T3 cells, there was no detectable effect on accumulation of inositol phosphates; therefore, $InsP_3$ probably does not mediate the SPC-stimulated cellular proliferation and may not be involved in mobilization of intracellular calcium induced by SPC. Previously, we found that sphingosine stimulated formation of inositol phosphates (Zhang et al., 1990*b*). These data also suggest that SPC and sphingosine may act through different mechanisms.

The mitogenic action of sphingosine was coupled to an increase in the levels of PA (Zhang et al., 1990*b*). Recently we have shown that SPP can also stimulate PA accumulation, likely through activation of phospholipase D (Desai et al., 1992). PA has been shown to act as an intracellular messenger that can regulate the activity of ras-GAP, a component essential for cellular proliferation (Yu et al., 1988; Exton, 1990; Bocckino et al., 1991). Furthermore, a recent study had demonstrated that there is a PA-sensitive intracellular calcium pool in Jurkat T cells which is independent of $InsP_3$ levels (Breittmayer et al., 1991). In sharp contrast to sphingosine or SPP, SPC did not cause a significant increase in PA levels in Swiss 3T3 fibroblasts. Therefore, whereas PA may mediate the mitogenic effects of sphingosine and SPP, PA is probably not involved in the mechanism of action of SPC. One obvious explanation for this is that the pathways mediating the mitogenic effect of SPC are not identical to those mediating the effects of sphingosine or SPP. Alternatively, changes in PA production may be unrelated to mitogenesis and pathways essential for the mitogenicity of SPC, sphingosine, and SPP have not yet been completely characterized.

The classical intracellular messenger, cAMP, is also not likely involved in the SPC-induced stimulation of mitogenesis or calcium mobilization, since SPC had no effects on the levels of cAMP. Sphingosine (Zhang et al., 1990*b*), and SPP (Mattie, M. E., G. Brooker, and S. Spiegel, unpublished observations) on the other hand, caused a drastic decrease in cellular cAMP levels, representing another point of divergence between the effects of SPC and sphingosine or SPP.

Does Arachidonic Acid Mediate SPC-induced Mitogenesis?

AA has been implicated in the regulation of mitogenesis in 3T3 fibroblasts (Millar and Rozengurt, 1990; Takuwa et al., 1991). The mitogenic peptides bombesin and mastoparan induce mobilization of unesterified AA in Swiss 3T3 fibroblasts (Millar and Rozengurt, 1990; Takuwa et al., 1991; Gil et al., 1991). Furthermore, exogenous AA was shown to potentiate the mitogenic effect of insulin on Swiss 3T3 cells (Takuwa et al., 1991).

Mitogenic concentrations of SPC stimulated release of ^3H-labeled AA which seemed not to be secondary to the increase in $[Ca^{2+}]_i$, since TG blocked the increase in calcium without significant effect on AA release. On the other hand, AA could contribute to the increase in intracellular calcium. However, this also seems unlikely since the effect on calcium is more rapid than the release of AA induced by SPC, and exogenous AA was only weakly mitogenic and required very high concentrations to stimulate an increase in intracellular calcium. The increased AA by itself is not likely the sole mediator of both the SPC-induced increase in intracellular calcium and the SPC-induced mitogenesis.

Although the significance of the AA released during the mitogenic response to SPC is not clear, it is intriguing to note that PKCα is the predominant PKC isoform in Swiss

3T3 cells (McCaffrey and Rosner, 1987; Adams and Gullick, 1989). This isoenzyme can be activated by free AA in the presence of calcium (Nishizuka, 1992). The increase in $[Ca^{2+}]_i$ induced by SPC, together with the increase in AA levels, might activate PKCα and could account for the partial PKC dependency observed in these studies. The inhibition by sphingosine of both SPC-induced AA release and SPC-stimulated mitogenesis provides the most compelling evidence that AA may play a role in mediating SPC mitogenicity. These results also strengthen our conclusion that sphingosine and SPC do not use identical pathways for the stimulation of cell division.

Is Endogenous SPC a Regulator of Mitogenesis?

Since SPC is more potent than sphingosine, SPP, sphingomyelin, and ceramide in stimulating DNA synthesis, it seems most likely that SPC itself is the active agent. Exogenous SPC is readily taken up by cells and metabolized relatively slowly after uptake, primarily to phosphocholine and sphingosine. Based on the time course of sphingosine production from SPC and the magnitude of the increase, it seems likely that sphingosine cannot mediate the SPC-induced increase in intracellular calcium and is probably not involved in the mitogenic effect of SPC. Furthermore, the signal transduction pathway mediated by SPC is distinct from that used by sphingosine or SPP. Therefore, the SPC itself probably acts as the mediator of mitogenic stimulation. In this regard, it is interesting to note that a significant amount of SPC was incorporated into Swiss 3T3 cells within a very short time which is consistent with our observation that SPC is a very potent mitogen even after a brief treatment.

Although SPC has not yet been detected in normal cells, it has been found in the spleens of patients with Niemann-Pick disease (Strasberg and Callahan, 1987). Earlier, SPC had been proposed to be a potential precursor of sphingomyelin. Brady et al. first reported the formation of [^{14}C]sphingomyelin after incubation of SPC and stearoyl-[^{14}C]CoA with rat brain homogenates (Brady et al., 1965). SPC was recently reported to stimulate an increase in sphingomyelin levels in neuroblastoma cells (Sugiyama et al., 1990) and in human leukemia HL-60 cells (Dressler et al., 1991). In both of these studies, the increase in levels of sphingomyelin was associated with differentiation. Nevertheless, exogenous SPC in 3T3 cells was not significantly converted to sphingomyelin.

The formation of SPC from sphingosine and CDP-choline, originally reported by Fujino et al., suggested that cells may have the capacity to synthesize SPC (Fujino and Negishi, 1968; Fujino et al., 1968). In contrast, Stoffel and Melzner were unable to detect SPC synthesis using rat liver microsomes, a preparation which is rich in CDP-choline/ceramide choline phosphotransferase activity (Stoffel and Melzner, 1980). Similarly, phosphatidylcholine/ceramide choline-phosphotransferase activity in plasma membrane fractions from mouse Ehrlich ascite cells, which normally catalyzes formation of sphingomyelin by the transfer of phosphocholine from phosphatidylcholine to ceramide, did not catalyze phosphocholine transfer using sphingosine as an acceptor to form SPC (Marggraf and Kanfer, 1984). Furthermore, recent studies from Pagano's laboratory clearly demonstrated that most, if not all, sphingomyelin is synthesized in mammalian cells by transfer of phosphorylcholine from phosphatidylcholine to ceramide, thus suggesting that SPC is probably not a precursor of sphingomyelin (Pagano, 1988; Futerman et al., 1990). However, in analogy to receptor-linked phospholipase A_2 which catalyzes liberation of AA from phospholipids, a receptor-linked "sphingomyelin deacylase" might function to increase endogenous levels of SPC, which potentially could act as a modulator of cellular proliferation via distinct signaling pathways.

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