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SPHINGOSYLPHOSPHORYLCHOLINE IS A REMARKABLY POTENT MITOGEN FOR A VARIETY OF CELL LINES

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SUMMARY: The effect of sphingosylphosphorylcholine on cellular proliferation was investigated in a variety of cell types. Sphingosylphosphorylcholine at low concentrations greatly stimulated DNA synthesis and cell division in quiescent Swiss 3T3 fibroblasts. The increased DNA synthesis was also accompanied by pronounced morphological alterations. Sphingosylphosphorylcholine was remarkably more potent than other known growth factors and also acted synergistically with insulin, epidermal growth factor, fibroblast growth factor, and the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate, to induce cellular proliferation. Sphingosylphosphorylcholine was less effective in stimulating DNA synthesis in rapidly growing normal and transformed cells. Sphingosylphosphorylcholine appears to be a new type of potent, wide-spectrum growth promoting agent.

The biological roles of sphingolipids and their metabolites have been the subject of extensive studies (1-3). It has been suggested that this complex class of lipids may play important roles in cellular growth regulation (1-4). Sphingosine and lysosphingolipids, the breakdown products of more complex sphingolipids, have been proposed to function as endogenous negative regulators of proliferation by inhibiting protein kinase C, a pivotal regulatory enzyme in cell growth (5-7). Surprisingly, we found that sphingosine at low concentrations stimulates cell proliferation of quiescent 3T3 fibroblasts via a protein kinase C-independent pathway (8). More recently, we have demonstrated that the mitogenic effect of sphingosine is mediated by a rapid rise in the levels of phosphatidic acid (9) and of sphingosine-1-phosphate (10) which are both potent mitogens for 3T3 cells (9-11). Our findings suggested that sphingosine may function as a positive regulator of cell growth acting through a novel pathway.

Recently, sphingosylphosphorylcholine (SPC) or lysosphingomyelin, was shown to release calcium from insoitol trisphosphate-sensitive and insensitive intracellular pools in permeabilized smooth muscle cells (12). Since an increase in $[Ca^{+2}]_i$ appears to be an early and general response to mitogenic stimuli, we have examined the effects of SPC on the growth of Swiss 3T3

Abbreviations: SPC, sphingosylphosphorylcholine; TPA, 12-o-tetradecanoyl-phorbol 13-acetate; EGF, epidermal growth factor; FGF, fibroblast growth factor; BSA, bovine serum albumin; CS, calf serum; PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle's medium.

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fibroblasts, a convenient model system for the study of cell activation and growth (13), as well as on various other types of cells. We have found that SPC is an unusually potent, wide-spectrum growth promoter.

MATERIALS AND METHODS

Materials - [methyl^{3}H]Thymidine (55 Ci/mmol) was purchased from Amersham. Epidermal growth factor (EGF), insulin, fibroblast growth factor (FGF) and transferrin were from Collaborative Research. TPA, and SPC (free base) were from Sigma Chemical Co.
Cell Culture - Swiss 3T3 (CCL 92), NIH 3T3 (CRL 1658), Moloney MSV 3T3 transformed (CRL 1568), BALB/c (TIB 80), and preadipocyte 3T3 L1 (CCL 92.1) fibroblasts; human astrocytoma (CRL 1718), HeLa S3 epitheloid cervix carcinoma (CCL2.2), MDCK epithelial like kidney cells (CCL34), HIT Syrian hamster β cell line (CRL1777), rat adrenal pheochromocytoma PC12 (CRL1721), were from the American Type Culture Collection. NIH 3T3 cells transformed with a plasmid bearing the gene for FGF were from Dr. F. Kern, Lombardi Cancer Center. Rat glioma C6-2B cells were from Dr. G. Brooker. Stock cultures of cells were routinely maintained either as recommended by ATCC or as previously described (14). To obtain quiescent cultures, the cells were subcultured at a density of 1.5 x 10^4 cells/cm^2 in DMEM (JRH Biomedicals) supplemented with 2 mM glutamine, 1 mM pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% calf serum (Colorado Serum Co.). The cells were used 7 days later when they were confluent and quiescent (15). Transformed cells were seeded at the same density and used 1-2 days later. Rapidly growing cells were subcultured at a density of 2.5 x 10^3 cells/cm^2 and used 2-3 days later.
Assay of DNA Synthesis - Cultures were washed with DMEM to remove residual serum and 1 ml of DMEM supplemented with 20 μg/ml BSA and 5 μg/ml transferrin was added (15). The cells were treated with various growth factors or SPC and were pulsed after 18 h with 1.0 μCi of [^{3}H]thymidine for 6 h. The [^{3}H]thymidine incorporation into trichloroacetic acid-insoluble material was measured as described (14). Values are the means of triplicate determinations. Standard errors were routinely less than 10% of the mean.
Measurement of cell numbers- Cells were cultured and treated with mitogens as described above. Viable cells were released from the dishes by trypsinization and counted (Coulter Model ZBI). Data represent the mean ± SD of three independent cultures.

RESULTS AND DISCUSSION

SPC Significantly Stimulates Proliferation of Quiescent 3T3 Fibroblasts

SPC stimulated DNA synthesis in quiescent Swiss 3T3 fibroblasts grown in chemically defined medium as measured by [^{3}H]thymidine incorporation (Fig. 1). A mitogenic effect was observed at a concentration of SPC as low as 0.5 μM and maximum stimulation of 15-fold was achieved at 10 μM. Up to this concentration, there was no loss of cell viability and more than 95% of the cells were viable. At high concentrations of SPC (about 100 μM), most of the cells were detached from the plates. SPC alone at optimal concentrations is significantly more mitogenic than insulin, EGF, and even TPA, which is an exceptionally potent growth stimulator for Swiss 3T3 cells (Table 1). Similar to the synergistic effect between insulin and other growth factors, the mitogenic response to SPC was also potentiated by insulin, EGF, FGF, and TPA. This synergistic interaction between SPC 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 SPC caused a further potentiation of [^{3}H]thymidine incorporation (Table 1).

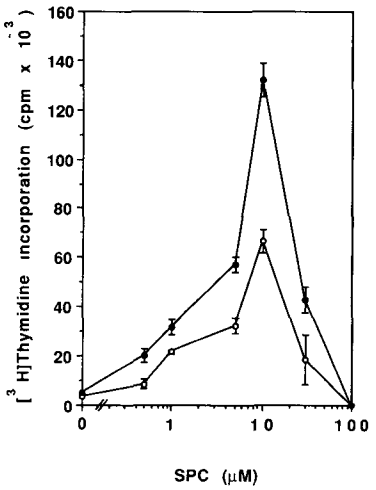

Figure 1. Dose response for SPC-dependent stimulation of DNA synthesis in Swiss 3T3 cells. Confluent and quiescent cultures of Swiss 3T3 cells were incubated with various concentrations of SPC in the absence (o) or presence of (•) insulin (2 μg/ml) and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. Similar results were obtained in at least seven additional experiments. All concentrations of SPC above 0.5 μM were statistically significant (Student's t test), $p \leq 0.01$ compared to the untreated cells.

SPC not only stimulated DNA synthesis, but also caused an increase in cell number (Fig. 2A). The increases in cell numbers were well correlated with the increases in DNA synthesis (Figs. 1A and 2A). A maximal increase in cell numbers of 100% was observed after 48 h exposure to SPC and was comparable to the increase mediated by 10% calf serum. In contrast, other known mitogens, such as EGF, induced only a 30% increase in cell number.

Similar to other potent mitogens for these cells, SPC also induced large morphological transformations. Fig. 3 shows the morphological effects on cells treated with either the tumor

TABLE 1. EFFECTS OF SPC ON DNA SYNTHESIS IN QUIESCENT CULTURES OF SWISS 3T3 FIBROBLASTS

Stimulants	[^{3}H]Thymidine Incorporation (cpm x10^{-3} / well)	
SPC	(-)	(+)
None	4.9±0.5	93.3±5.6
Insulin	12.1±0.8	317.9±23.6
TPA	50.7±3.1	198.6±6.6
TPA plus Insulin	209.9±15.8	340.3±24.2
EGF	26.2±1.4	332.8±15.6
EGF plus Insulin	172.7±6.3	437.8±16.1
FGF	19.5±4.0	204.0±12.1

Confluent and quiescent Swiss 3T3 fibroblasts were exposed to the indicated mitogens in the absence (-) or presence (+) of SPC 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 ten additional experiments. The concentrations of the mitogenic agents were as follows: SPC, 10 μM; Insulin, 2 μg/ml; TPA, 100 nM; EGF, 10 ng/ml; FGF, 25 ng/ml.

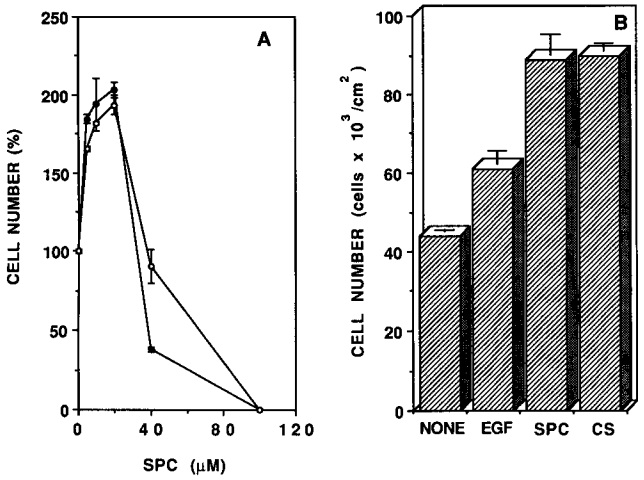

Figure 2. Stimulation of Cell Division by SPC. A. Confluent and quiescent cultures of Swiss 3T3 cells were incubated in DMEM/Waymouth (1:1) supplemented with BSA (20 μg/ml), transferrin (5 μg/ml) and treated with various concentrations of SPC in the absence (o) or in the presence of (•) insulin (2 μg/ml). After 48h, the cells were removed from the dishes and counted as described in Materials and Methods. Data represent the mean ± SD of three independent cultures treated identically. The data are expressed as percent of control values obtained in the absence of added SPC. B. Increase in cell number induced by different mitogens: SPC (20 μM); EGF (10 ng/ml); CS (10%).

promoter TPA or SPC. The flattened appearance of untreated cells is contrasted with the elongated, refractile appearance of cells having long projections after treatment with SPC or TPA (Fig. 3). In SPC-treated cells, there are many foci of intense growth where the cells appear to be overgrowing (Fig. 3D). Cells in these foci may have lost the property of contact inhibition which could cause them to detach from the surface.

SPC Stimulates the Growth of Many Cell Types

The mitogenic activity of SPC is not restricted to confluent and quiescent Swiss 3T3 fibroblasts. SPC stimulated DNA synthesis in other similar contact inhibited cell lines, including BALB/c fibroblasts and preadipocytes (Table 2). It also stimulated DNA synthesis in very divergent cell types, such as HeLa carcinoma cells, C6 rat glioma cells, transformed 3T3 cells, human astrocytoma cells, kidney ethithelial MDCK cells, hamster pancreatic insulinoma HIT cells, and rat pheochromocytoma PC12 cells (Table 2). There was a corresponding increase in cell counts in cases examined (data not shown). The mitogenic effect of SPC on rapidly dividing cells is less than on quiescent cells. It is evident that rapidly growing cells will respond much less to growth promoting agents since they are not arrested in the G_0 phase. In this regard, rapidly growing untransformed 3T3 fibroblasts and other transformed cell lines behaved alike. In general, only 1.5-4 fold stimulation of DNA synthesis was observed for all types of rapidly dividing cells. This is comparable to the effects of other potent growth factors on transformed cell lines. It should be noted that rapidly dividing cells are more sensitive to SPC and show a maximun (albeit lower) increase in DNA synthesis at a lower concentration of SPC than quiescent cells.

In summary, we have shown that SPC is a remarkably potent mitogen for quiescent and contact inhibited cells. The mitogenic activity of SPC was not restricted only to quiescent

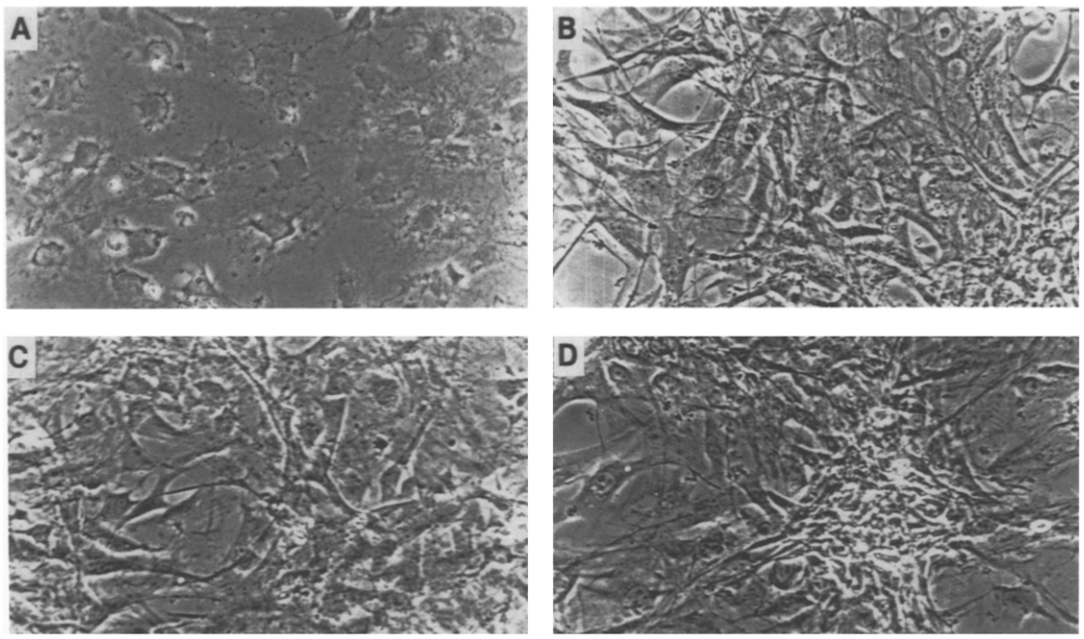

Figure 3. Morphologic alterations of Swiss 3T3 cells induced by SPC or TPA. Confluent and quiescent cultures of Swiss 3T3 cells were incubated in DMEM/Waymouth (1:1) supplemented with BSA (20 μg/ml), transferrin (5 μg/ml), in the absence (A) or in the presence of 100 nM TPA (B), or 10 μM SPC (C,D). Photomicrographs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

TABLE 2. EFFECTS OF SPC ON DNA SYNTHESIS IN VARIOUS CELL TYPES

	[^{3}H]Thymidine Incorporation (fold stimulation)			
Stimulant	**SPC**	**EGF**	**Insulin**	**Insulin + SPC**
A.Untransformed Cells:				
1.Contact Inhibited				
Swiss 3T3 fibroblasts	19.0	5.3	2.5	65.0
BALB/c 3T3 fibroblasts	17.2	ND	12.0	26.1
3T3 L1 preadipocytes	5.9	2.1	8.0	45.6
2.Exponentially Growing				
Swiss 3T3 fibroblasts	3.1	2.0	3.0	5.5
BALB/c 3T3 fibroblasts	4.5	3.9	3.3	7.3
NIH 3T3 fibroblasts	3.5	3.0	2.2	4.8
B.Transformed Cells:				
MSV 3T3	2.0	1.8	2.1	3.7
transformed NIH 3T3	3.7	1.4	3.0	6.8
C6-2B glioma	2.1	ND	0.95	2.3
Astrocytoma	1.6	ND	1.8	2.2
HeLa S3	1.4	0.9	0.9	1.1
MDCK	1.3	1.6	1.3	1.5
HIT insulinoma	1.4	1.4	1.5	1.6

The various cell types were subcultured, incubated in the presence of the indicated mitogens, and DNA synthesis measured as described in Materials and Methods. The data are expressed as [^{3}H]thymidine incorporated relative to the value obtained in the absence of added mitogens. ND, not determined. The concentrations of the mitogenic agents were as follows: Insulin, 2 μg/ml; EGF, 10 ng/ml; SPC, 10 μM for contact inhibited cells and 1 μM for exponentially growing and transformed cells.

fibroblasts, as SPC also stimulated the proliferation of many diverse cell types. Interestingly, Sugiyama et al recently examined the effects of various lysosphingolipids on the growth and differentiation of mouse neuroblastoma cells (16). Although they concluded that SPC is a potent inducer of neurite outgrowth and inhibits growth at concentrations above 100 μM, examination of their results indicates that at lower concentrations, SPC slightly enhanced cell growth. The underlying molecular mechanism of action of SPC has not yet been elucidated. However, it seems likely that its effects may be related to its ability to release calcium from intracellular sources (12). Further studies on the mechanism of action of SPC could be very important in view of its potential therapeutic use in processes that require extensive cellular proliferation, such as wound healing and tissue or organ regeneration.

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