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Topical Review

Sphingolipid Metabolites: Members of a New Class of Lipid Second Messengers

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Introduction

In the last decade, many studies have demonstrated that glycerophospholipid metabolites, such as diacylglycerol, inositol 1,4,5-trisphosphate ($InsP_3$), phosphatidic acid, and arachidonic acid, function as second messengers in growth-signaling pathways (Irvine, 1982; Exton, 1990; Nishizuka, 1992; Berridge, 1993). The importance of intracellular signaling molecules derived from another major class of membrane lipids, sphingolipids, has only recently begun to be appreciated. Sphingolipids contain a long-chain sphingoid base backbone of which sphingosine is the most prominent; an amide-linked fatty acid with 16–24 methylene groups; and a polar head group (hydroxyl for ceramide, phosphorylcholine for sphingomyelin, or carbohydrate residues of varying complexity for glycosphingolipids) (Fig. 1). These ubiquitous lipids have long been implicated in cell growth and differentiation, oncogenesis, and cell-cell contact (*reviewed in* Hakomori, 1990; Olivera & Spiegel, 1992). They act as cell surface receptors for some viruses and bacteria; they can modulate the functions of certain growth factors; and may be effectors of protein kinases, ion transporters, and other membrane proteins. However, more recently it has become evident that the biological activity of sphingolipids resides not only in the more complex species (e.g., sphingomyelin, cerebrosides, gangliosides, and sulfatides), but also in their metabolic products, such as ceramide (Hannun, 1994; Kolesnick & Golde, 1994), sphingosine (Zhang et al., 1990a; Merrill, 1991) sphingosine-1-phosphate (Zhang et al., 1991; Desai el at., 1992; Olivera & Spiegel, 1993; Spiegel, 1993; Mattie et al., 1994) lysosphingomyelin or sphingosylphosphorylcholine (Desai & Spiegel, 1991; Desai et al., 1993), and di- and trimethylsphingosine (Igarashi et al., 1990; Hakomori & Igarashi, 1993). The second messenger functions of ceramide mediating the effects of several extracellular stimuli, including TNF-α, vitamin D_3, γ-interferon, and IL-1 and IL-2, has recently been the subject of excellent reviews (Hannun, 1994; Kolesnick & Golde, 1994) and will not be covered here. In this review, we will summarize current knowledge regarding the second messenger role of sphingosine and its metabolites, sphingosine-1-phosphate (SPP)[1] and sphingosylphosphorylcholine (SPC)[1], in the regulation of cellular processes and also discuss possible mechanisms underlying their action.

Overview of Sphingolipid Intermediary Metabolism

The discovery of many human genetic sphingolipid disorders has led to the elucidation of the metabolic pathways for sphingolipid synthesis and degradation. The initial step of the de novo biosynthetic pathway is the condensation of serine with palmitoyl-CoA to form 3-ketodihydrosphingosine, a reaction catalyzed by serine

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[1] The abbreviations used are: SPP, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; PDGF, platelet derived growth factor; BSA, bovine serum albumin; FCS, fetal calf serum

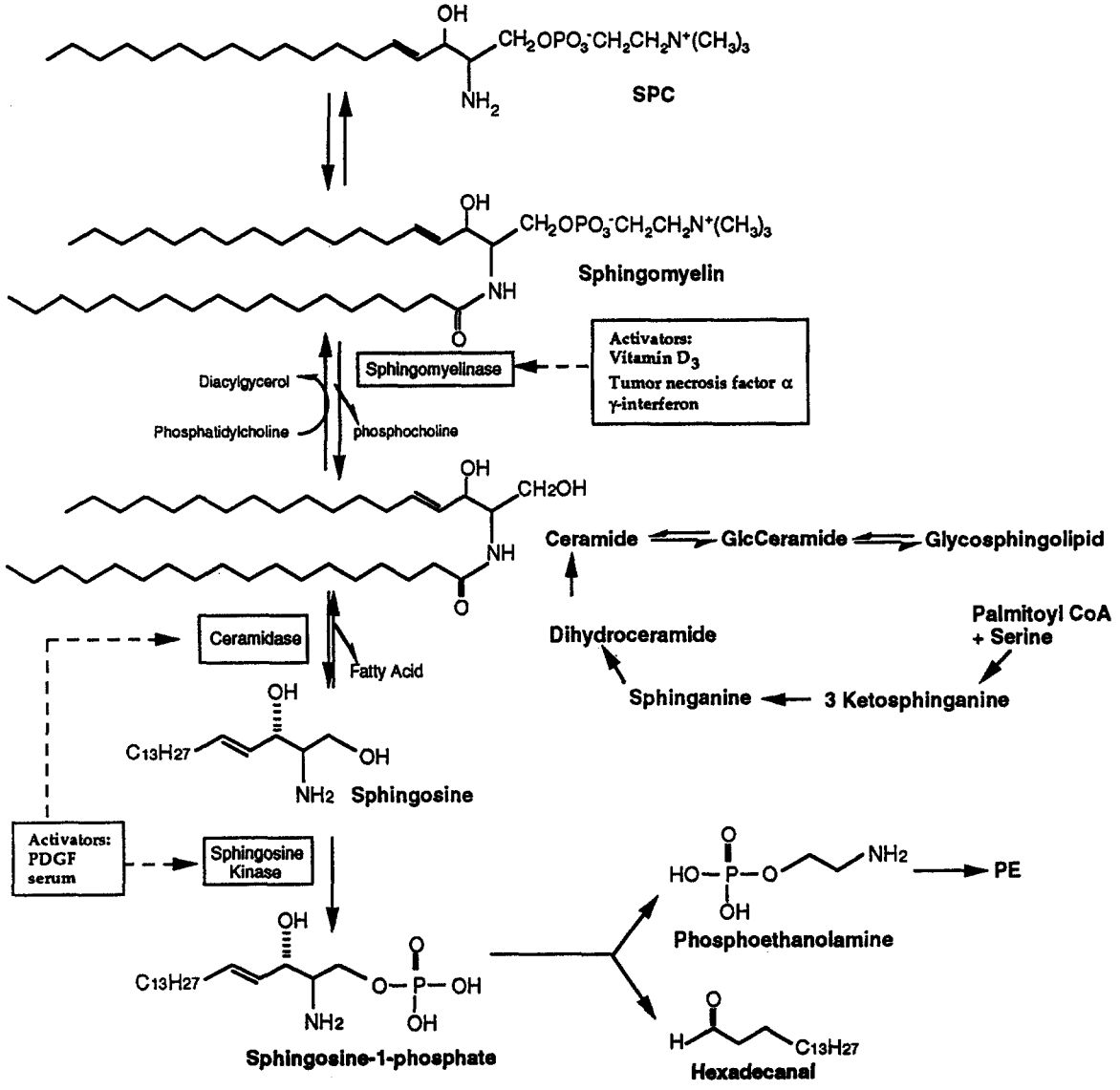

Fig. 1. Sphingolipid metabolic pathways leading to the formation of sphingosine, SPP, and ceramide, putative intracellular second messengers. The influences of growth regulators are indicated. Modified with permission from (Spiegel et al., 1994).

palmitoyl transferase. This product is rapidly reduced by an NADPH-dependent reductase to dihydrosphingosine which is then converted to dihydroceramide by acylation with fatty acyl-CoA. Recent evidence suggests that dihydroceramide is converted directly to ceramide, the precursor of all sphingolipids, by the introduction of a *trans*-4,5-double bond (Merrill & Wang, 1992; Rother et al., 1992). The transfer of a phosphorylcholine head group from phosphatidylcholine to ceramide yields sphingomyelin (Futerman & Pagano, 1991). The sequential addition of carbohydrate groups from the sugar donor, UDP-hexose (except for CMP-sialic acid for gangliosides), to ceramide results in the formation of complex glycosphingolipids (*see* Fig. 1).

The turnover of sphingolipids involves removal of the sugars by exoglyosidases or endoglycosidases and the removal of phosphorylcholine from sphingomyelin by at least three distinct sphingomyelinases. Ceramidase catalyzes the cleavage of the amide-linked fatty acid of ceramide to sphingosine, the back-bone of all sphingolipids. Sphingosine can then be converted back to ceramide by reacylation or metabolized via sphingosine kinase-catalyzed phosphorylation to SPP (Stoffel et al., 1968; Stoffel et al., 1970) followed by cleavage to ethanolamine phosphate and *trans*-2-hexadecanal by a pyridoxal phosphate-dependent lyase (Stoffel et al., 1968; Van Veldhoven & Mannaerts, 1991). This endoplasmic reticulum lyase has been postulated to provide ethanolamine phosphate for the synthesis of phosphatidyl ethanolamine and phosphatidyl choline, and fatty aldehydes

for the synthesis of plamalogens or fatty acids (Stoffel et al., 1968; Keenan, 1972; Van Veldhoven & Mannaerts, 1991). Another potential route for the metabolism of sphingosine has recently been suggested by Hakomori and colleagues which involves methylation to form di- and trimethyl sphingosine (Igarashi et al., 1990; Hakomori & Igarashi, 1993; Okoshi et al., 1991). It is yet not clear if this is a specific metabolic route for sphingosine or a general detoxification pathway.

As discussed below, the metabolism of sphingolipids has some characteristics of a metabolic cycle which are considered to be essential for the production of intracellular messengers and the intermediates of sphingolipid turnover may function in previously unappreciated modes to regulate cellular processes.

Sphingosine and Cell Growth

As knowledge of the mechanisms of cell growth regulation has increased, it has become evident that not all of the intracellular second messengers involved have been uncovered. Numerous studies have demonstrated that polyphosphoinositide hydrolysis is not required for growth factor-induced mitogenesis (Williams, 1989; Margolis et al., 1990) and that activation of phospholipase D, leading to an increase in phosphatidic acid, rather than an increase in the activity of phospholipase C, which generates diacylglyerol and $InsP_3$, correlates with mitogenesis induced by the potent mitogen, platelet-derived growth factor (PDGF)[1] (Fukami & Takenawa, 1992). Furthermore, it has been suggested that PDGF may stimulate the formation of another intracellular second messenger capable of releasing calcium from intracellular sources, since PDGF has virtually no effect on $InsP_3$ levels in Swiss 3T3 fibroblasts (Lopez-Rivas et al., 1987). Recent studies suggest that sphingolipid metabolites, including sphingosine and SPP, could be the potential missing links between the plasma membrane (where the growth factor receptors lie), the intracellular calcium stores, and cellular proliferation.

Previously, it was found that low concentrations of sphingosine stimulated the proliferation of quiescent Swiss 3T3 fibroblasts and also potentiated the mitogenic responses to other growth factors via a protein kinase C-independent pathway (Zhang et al., 1991*a*). Sphingosine has two asymmetric chiral carbon atoms, and can exist as four stereoisomers (D-(+)-*erythro* (2S, 3R), D-(+)-*threo* (2R, 3R), L-(-)-*erythro* (2R, 3S) and L-(-)-*threo* (2S, 3S)). The D-(+)-*erythro* (2S, 3R)sphingosine with a *trans* 4,5 double bond is the naturally occuring isomer. Sphingosine stereoisomers should be useful to determine the specificity for sphingosine targets and may help to define crucial events in sphingosine-activated signaling pathways. In contrast to its known lack of stereospecificity in inhibiting protein kinase C, the mitogenic effect of sphingosine was very specific, since only the D-*erthyro*- isomer of sphingosine stimulated DNA synthesis and structurally related analogs of sphingosine of L-*threo*- stereoisomers were not active (Olivera et al., 1994). These results raised the possibility that this breakdown product of cellular sphingolipids may play an important role as a positive modulator of cell growth acting in a fundamentally different, protein kinase C-independent pathway.

The mitogenic effects of sphingosine have since been confirmed and shown to be widespread but not universal. Sphingosine stimulates cellular proliferation in rodent fibroblasts (Gomez et al., 1994; Schroeder et al., 1994); it has been reported to restore proliferation in HL-60 cells after induction of cell growth arrest by phorbol ester (Merrill et al., 1986; Kolesnick, 1989); and in A431 human epidermoid carcinoma cells (Faucher et al., 1988) and WI38 human fetal lung fibroblasts (Davis et al., 1988), exogenous sphingosine had no effect on proliferation, in spite of sphingosine-induced changes in epidermal growth factor (EGF) receptor activity.

Relevance to Human Diseases

Consumption of corn contaminated with the mold, *Fusarium monilforme* (Sheldon), induces liver cancer in rats and has been correlated with human esophageal cancers (Wang et al., 1991). This organism produces toxins, known as fumonisins, which have structures resembling long chain sphingoid bases. One of these toxins, Fumonisin B, also stimulates DNA synthesis and causes an increase in levels of sphinganine and sphingosine in Swiss 3T3 fibroblasts by inhibition of sphinganine-N-acyltransferase (Schroeder et al., 1994). The effects on sphingolipid metabolism and cell growth could be responsible for the cancer-inducing properties of fumonisins (Schroeder et al., 1994).

Accumulation of sphingosine and other lysosphingoid bases resulting in inhibition of portein kinase C has been proposed as the explanation for the pathology of sphingolidoses, a group of metabolic disorders arising as a consequence of inborn errors in sphingolipid meatbolism (Hannun & Bell, 1987). Cholesterol lipidosis in Niemann-Pick Type C disease results from a defect in post-lysosomal cholesterol transport. It has been found that sphinganine inhibits cholesterol transport and low-density lipoprotein-induced esterification of cholesterol resulting in accumulation of unesterified cholesterol in perinuclear vesicles (Roff et al., 1991). Interestingly, in a murine model for Nieman-Pick type C disease, liver levels of both sphinganine and sphingosine were more than 20-fold higher than normal and liver sphinganine kinase activity was deficient in extracts due to the presence of an inhibitory substance. These results suggest that the accumulation of long chain sphingoid bases

could be the cause of the abnormal intracellular cholesterol trafficking in Niemann-Pick type C disease (Goldin et al., 1992). Furthermore, in intact cells sphingosine has been shown to inhibit activation of cholesterol esterification, which is associated with the degradation of plasma membrane sphingomyelin by sphingomyelinase (Harmala et al., 1993). In this regard, D-*erythro*-sphingosine treatment lowered HMG-CoA reductase activity in CHO-K1 cells, while L-*threo*-sphingosine had no effect (Pinkerton et al., 1993). Since sphingosine has no effect in vitro, this may suggest that a metabolite of sphingosine is responsible for the observed inhibition of cholesteryl ester formation (Harmala, et al., 1993; Pinkerton et al., 1993).

Sphingosine-1-Phosphate and Cell Growth

Recently we have provided new insights into the formation and functions of a metabolite of sphingosine, sphingosine-1-phosphate (SPP), which is rapidly produced in response to mitogenic concentrations of sphingosine (Zhang et al., 1991). Similar to sphingosine, albeit at lower concentrations, SPP induced increased DNA synthesis and cell division in quiescent cultures of Swiss 3T3 fibroblasts (Zhang et al., 1991). Although both sphingosine and SPP acted synergistically with a wide variety of growth factors, there was no additive or synergistic effect in response to a combination of sphingosine and SPP, suggesting that either they both modulate cellular proliferation through a common pathway or that SPP mediates the growth promoting activity of sphingosine (Zhang et al., 1991).

Although sphingosine has positive effects on cell growth, it was originally characterized as a negative regulator of cell growth (Stoffel & Bister, 1973), observations which were later substantiated by others (Merrill, 1983; Stevens et al., 1990). The inhibition of cell growth could be the result of cytotoxicity as a consequence of its detergent properties (Merrill & Stevens, 1989), although it has since been proposed that inhibition of protein kinase C activity may mediate this effect (Merrill & Stevens, 1989; Stevens et al., 1990). Although it is unusual for the same effector to both stimulate and inhibit the growth of cells, it is not unprecedented. Transforming growth factor β (TGF-β) is a bifunctional regulator of cell growth, inhibiting the growth of many tumor cells yet stimulating the growth of non-neoplastic fibroblasts (Sporn & Roberts, 1988). Similarly, the B subunit of cholera toxin stimulates proliferation of quiescent 3T3 fibroblasts, while inhibiting growth of *ras*-transformed or rapidly growing fibroblasts (Buckley et al., 1990; Spiegel & Fishman, 1987). Sphingosine can also have dual effects on cell growth depending on the cell type and the nature of the stimuli (Zhang et al., 1990*a*; Stevens et al., 1990; Chao et al., 1992).

Similar to sphingosine, SPP also has complex effects on cell growth. In striking contrast to the growth promoting effects of SPP on quiescent 3T3 fibroblasts described above, SPP had some inhibitory effects on growth of two human breast cancer cell lines, MCF-7 and MDA-MB-231 (Spiegel et al., 1994). The MCF-7 cell line has functional estrogen receptors and is dependent on estrogen for growth while the MDA-MB-231 cell line lacks estrogen receptors, is unresponsive to antiestrogens and is a model for the more aggressive, hormone-independent class of breast cancers (Lippman & Dickson, 1989). It is interesting to note that SPP more potently inhibited the growth of the more aggressive MDA-MB-231 cells (Spiegel et al., 1994).

Previously it was found that at very low concentrations (10 nM), SPP effectively and specifically inhibited chemotactic motility and invasiveness of several tumor cell lines, including mouse and human melanoma, and human osteosarcoma cells (Sadahira et al., 1992). In contrast, the motilities of other cells (e.g., endothelial and human fibrosarcoma cells) were not affected (Sadahira et al., 1992). Similar to our original studies (Zhang et al., 1991), SPP was much more potent than sphingosine itself. SPP also inhibited extracellular matrix protein-induced haptotactic motility but not adhesion of B16 mouse melanoma cells while other related compounds were much less effective than SPP (Sadahira et al., 1994).

In agreement, we have recently found that SPP significantly reduced invasion of MDA-MB-231 cells in Matrigel-lined Boyden chambers but did not have a strong effect on chemotaxis, as measured by migration through collagen IV-coated polycarbonate filters. In contrast, sphingosine did not have any significant effects (Spiegel et al., 1994). Thus, our results suggest that SPP primarily inhibits the cellular enzymatic activity responsible for degradation of Matrigel. The potent inhibitory effect of SPP on invasion, particularly in certain tumor cell lines, suggests that it could be a useful agent for suppression of metastasis, which represents the major cause of mortality in human cancers.

Modulation of Signal Transduction Pathways by Sphingosine and Sphingosine-1-phosphate

To characterize the role of sphingolipid metabolites as growth mediators, extensive studies have been directed at dissecting the signaling pathways that might be involved. Below is summarized the effects of exogenous long-chain sphingoid bases on many of the intracellular signaling molecules thought to play a role in cell growth regulation including protein kinase C, calcium, phosphatidic acid, $InsP_3$, and cAMP.

Protein Kinase C and other Kinases

The discovery that sphingosine was a negative modulator of protein kinase C, a key regulatory enzyme of many physiological processes, stimulated much interest in its biological role (Hannun & Bell, 1987; Hannun & Bell, 1989; Merrill, 1991) and there are now many examples of sphingosine-mediated phenomena in cells which are apparently due to inhibition of protein kinase C (*reviewed in* Hannun & Bell, 1989; Merrill, 1991). However, more versatile roles for sphingosine have since been uncovered that do not appear to rely solely on its effect on protein kinase C (reviewed in Merrill, 1991; Scheidl et al., 1992; Spiegel, 1993; Spiegel et al., 1993). For example, the mitogenic effect of sphingosine in swiss 3T3 fibroblasts was clearly independent of protein kinase C (Zhang et al., 1990*a,b*; Zhang et al., 1991; Desai et al., 1992). Further support for this independence was provided by the finding that SPP, which mediates part of the mitogenic effect of sphingosine, does not have any effects on protein kinase C in vitro (Van Veldhoven et al., 1989).

There are often large differences between the concentrations of sphingosine required for in vitro inhibition of protein kinase C and the concentrations required for its in vivo effects. These differences could be due to the fact that the apparently high concentration of sphingosine required in mixed micellar assays may be a reflection of the effective concentration of sphingosine in cells caused by membrane sequestration. In addition, different isozymes of protein kinase C may be present in specific cells and may have differing sensitivity to inhibition by sphingosine. In this regard, only protein kinase C-α and -ξ isozymes have been detected in Swiss 3T3 fibroblasts (Mazurek et al., 1994) and if these isozymes are less sensitive to inhibition by sphingosine relative to other isozymes, this could explain the apparent protein kinase C independence of the mitogenic effect of sphingosine in Swiss 3T3 fibroblasts.

Sphingosine and some sphingosine analogues have been shown to modulate the activities of several other kinases. Sphingosine activates some protein kinases, such as casein kinase II (McDonald et al., 1991) and the tyrosine kinase of the EGF receptor (Davis et al., 1988), and also induces in vitro phosphorylation of cytosolic proteins in Jurkat T cells (Pushkavera et al., 1992). On the other hand, sphingosine inhibited insulin receptor tyrosine kinase activity (Arnold & Newton, 1991) and calmodulin-dependent kinases (Jefferson & Schulman, 1988). Sphingosine also induced dephosphorylation of the retinoblastoma gene product, a nuclear phosphoprotein that functions as a tumor suppresser, which was correlated with its ability to inhibit growth and arrest lymphoblastic leukemic cells in the Go/G1 phase of the cell cycle (Chao et al., 1992). Because tyrosine kinases are known to play important roles in the process of cellular proliferation, the reported effects of sphingosine on growth factor receptor tyrosine kinases may be important. Sphingosine increased the number of EGF binding sites and affinity and phosphorylation state of the EGF receptor (Faucher et al., 1988; Davis et al., 1988) and its derivative N,N-dimethyl-sphingosine has a strong and stereospecific enhancing effect on EGF receptor tyrosine kinase activity (Igarashi et al., 1990).

Phosphatidic Acid

The production of phosphatidic acid as a result of growth factor receptor coupled activation of phospholipase D has been suggested to be essential for mitogenic signal transduction cascades (Ben-Av & Liscovitch, 1989; Exton, 1990). Phosphatidic acid regulates the biological action of cellular Ras activity (Tsai et al., 1989; 1990) which plays a central role as a molecular switch in the signal transduction pathways associated with cell growth, differentiation, and neoplasia (Barbacid, 1987). Activation of Ras is an initial and key step of the signaling cascade from the plasma membrane to the nucleus composed of Ras, Raf-1 kinase, which activates the rest of the MAP kinase cascade, culminating in the activation of several transcription factors, including activator protein 1 (AP-1), that have been implicated in cell growth (Pelech & Sanghera, 1992) (Fig. 2). The function of Ras in mammalian cells depends on its interaction with a cytoplasmic GTPase activating protein (GAP) which stimulates its conversion to the inactive form (Trahey & McCormick, 1987). Recently, it has been shown that phosphatidic acid binds and inhibits $p21^{ras}$ GTPase activating protein and also its homolog (Tsai et al., 1989, 1990, 1991). In addition, phosphatidic acid stimulates the cytoplasmic $p21^{ras}$ GTPase inhibitory protein (Tsai et al., 1990). The combined effect is an inhibition of the intrinsic GTPase activity of Ras, stabilizing its active form and positively modulating its activity (Tsai et al., 1989, 1990).

Recently, much attention has been directed to sphingosine-mediated regulation of phosphatidic acid metabolism. The mitogenic effect of sphingosine in Swiss 3T3 fibroblasts has been shown to be accompanied by an increase in the levels of phosphatidic acid (Zhang et al., 1990*b*). Sphingosine stimulated phospholipase D activity in several types of cells, including 3T3 fibroblasts (Lavie & Liscovitch, 1990; Zhang et al., 1990*b*). The major substrate of phospholipase D in NG108-15 neuroblastoma-glioma hybrid cells (Lavie & Liscovitch, 1990) and in Swiss 3T3 fibroblasts (Zhang et al., 1990*b*) is phosphatidylcholine, but in NIH 3T3 fibroblasts, phosphatidylethanolamine can also be hydrolyzed by phospholipase D (Kiss & Anderson, 1990). Sphingosine had

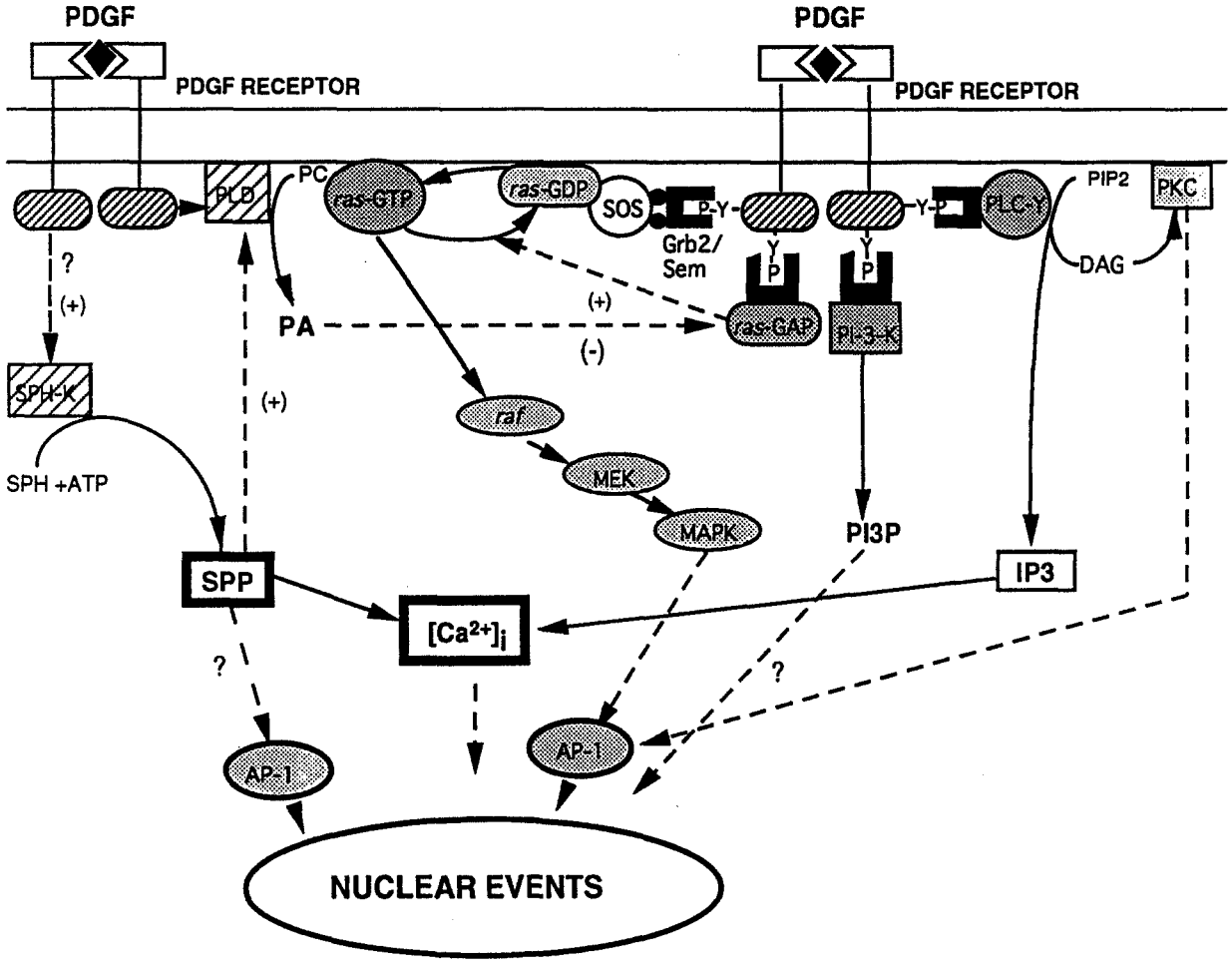

Fig. 2. Growth factor-mediated signaling pathways and possible sites of action of sphingolipid metabolites. Growth factor receptors are linked to and activate other effector systems, including: phosphatidylinositol 3-hydroxy kinase (PI3K); PLD; phospholipase C-gamma (PLCγ1); sphingosine kinase (SPHK); GTPase activating protein (GAP) and the adaptor Grb2-SOS complex. This leads to activation of the signaling cascade involving Ras and Raf-1, which phosphorylates and activates a protein kinase called MEK or MAPK kinase, that then activates mitogen activated protein kinases (MAPKs), and culminates in the phosphorylation of transcription factors (such as Jun/Fos) and other cellular factors that bring about the cellular response. For more detailed information, *see* (Egan et al., 1993; Gale et al., 1993; Rozakis-Adcock et al., 1993). Broken arrows indicate influences on pathways. Stimulatory or inhibitory effects are indicated by (+) or (-), respectively. Modified with permission from (Spiegel et al., 1994).

either potentiating or inhibitory effects on stimulation of phospholipase D activity with 12-O-tetradecanoylphorbol-13-acetate (TPA),[1] depending on the length of incubation and the concentration of TPA (Kiss & Deli, 1992). Sphingosine can also activate the 80 kD form of diacylglycerol kinase in vitro while inhibiting other isoforms (Sakane et al., 1989). Furthermore, in Jurkat T cells, the presence of at least four different isoforms of diacylglycerol kinase have been detected, of which two in the cytosol are activated by sphingosine (Yamada & Sakane, 1993; Yamada et al., 1993). Arachidonyl diacylglycerol-specific kinase was present in membranes although it was not activated by sphingosine (Yamada & Sakane, 1993). Thus, the effect of sphingosine on diacylglycerol kinase, and hence on phosphatidic acid production, could be different depending on the type of diacylglycerol kinase isozyme present in a particular cell. Sphingosine also increased phosphatidic acid levels by inhibition of phosphatidic acid phosphohydrolase in cell lysates from NG108-15 cells (Lavie et al., 1990), neutrophils (Mullmann et al., 1991), in plasma membranes and endoplasmic reticulum fractions from rat liver (Jamal et al., 1991), and also in preparations from the yeast *Saccharomyces cerevisiae* (Wu et al., 1993*b*). In NG108-15 cells, sphingosine and oleic acid caused opposite effects on the redistribution of the cytosolic and microsomal forms of phosphatidic acid phosphohydrolase (Aridor et al., 1992).

In a recent study, we observed that SPP stimulated phospholipase D activity in Swiss 3T3 fibroblasts (Desai

et al., 1992). Conversely, SPP had no effect on phosphatidic acid phosphohydrolase or on diacylglycerol kinase. Therefore, the most likely mechanism for SPP-induced phosphatidic acid accumulation is the activation of phospholipase D.

The molecular mechanisms by which sphingoid bases modulate the activity of phospholipase D, phosphatidic acid phosphohydrolase, or diacylglycerol kinase are still not clear. However, in many cells with different pathways for formation of phosphatidic acid, sphingosine uniformly has a stimulatory effect on phosphatidic acid levels. In view of the prominent role of phosphatidic acid in signal transduction, activation of the Ras cascade, and cellular proliferation, a connection between long chain sphingoid bases and phosphatidic acid, could have important implications for the biological actions of sphingolipid metabolites. The regulation of the GTPase activity of small G proteins could represent a convergence point where signals carried by lipid-derived messengers are integrated. Cross-talk between sphingolipid turnover pathways and the diacylglycerol cycle increases complexity of signaling pathways leading to cellular proliferation and adds additional sites of regulation.

Calcium and Inositol Trisphosphate

Several years ago, Ghosh and colleagues discovered that sphingosine mediated rapid and profound release of Ca^{2+} from intracellular stores in permeabilized smooth muscle cells and proposed that this effect might be indirect and mediated by the phosphorylation of sphingosine to SPP (Ghosh et al., 1990). However, at that time there was no direct experimental evidence that SPP itself could induce release of calcium from internal sources. Subsequently, it was observed that SPP had potent calcium-mobilizing activity in viable 3T3 fibroblasts (Zhang et al., 1991). A number of observations provided strong support for the suggestion that the conversion of sphingosine to SPP was required for the actions of sphingosine on 3T3 fibroblasts: sphingosine only mobilized calcium at 37°C, while SPP was active at low temperatures; there was a time lag for the action of sphingosine while the effects of SPP were much more rapid; SPP was much more potent than sphingosine (Zhang et al., 1991); and finally, inhibition of the conversion of sphingosine to SPP with an inhibitor of sphingosine kinase inhibited sphingosine-induced calcium release (Olivera et al., 1994). Recently, SPP has been shown to release calcium from internal sources in human foreskin fibroblasts (Chao, et al., 1994), REF52 fibroblasts and astrocytoma cells (Randriamampita & Tsien, 1993). It is interesting to note that calcium release occurs at much lower concentrations of SPP in fibroblasts than in other cells. However, in rat pancreatic acinar cells, although sphingosine released calcium from internal sources, SPP was inactive (Yule et al., 1993). Recently, sphingosine was also found to stimulate the release of intracellular calcium in rat parotid acinar cells (Sugiya & Furuyama, 1991). In contrast to its effects on smooth muscle cells and on fibroblasts, the response to sphingosine in these cells was dependent on extracellular calcium (Sugiya & Furuyama, 1991).

It should be noted that the rapid calcium release in response to SPP has many of the hallmarks of a receptor-mediated event. The rapidity, reversibility, and specificity of the effect of SPP all closely resemble the calcium-releasing effect of $InsP_3$ mediated by direct calcium channel activation. One possibility is that SPP interacts directly with calcium channels within the membranes of intracellular stores, such as the $InsP_3$ receptor or the ryanodine receptor. While these calcium channels share sequence homology and tertiary structural features, they can be distinguished physiologically by their responses to ligands and regulators (Taylor & Marshall, 1992). Thus, it is not surprising that sphingosine has differential effects on the two types of calcium channels. In contrast to its effect in permeabilized smooth muscle cells and in Swiss 3T3 fibroblasts (Ghosh et al., 1990; Zhang et al., 1991), sphingosine inhibited the release of calcium from skeletal muscle sarcoplasmic reticulum and directly affected sarcoplasmic reticulum ryanodine receptors, the channels responsible for calcium mobilization in skeletal muscle (Sabbadini et al., 1992). However, ryanodine channels have not been detected in Swiss 3T3 fibroblasts (Mattie et al., 1994). Further evidence for the SPP calcium mobilizing hypothesis has been obtained by studying the effects of this putative second messenger on permeabilized cells where it can gain more direct access to intracellular calcium stores. Similar to its effect on intact cells, SPP potently released calcium in permeabilized cells. Although calcium release by $InsP_3$ was inhibited as expected by heparin, a potent antagonist of $InsP_3$-activated calcium channels (Taylor & Marshall, 1992; Berridge, 1993), it had no effect on SPP-induced calcium release (Mattie et al., 1994).

To add more complexity, sphingosine stimulated phosphoinositide hydrolysis in Swiss 3T3 fibroblasts (Zhang et al., 1990*b*), in primary cultured astrocytes (Richie et al., 1992), and in primary skin fibroblasts (Chao et al., 1994). Sphingosine also potentiated the EGF-induced increase in $InsP_3$ levels in A431 cells without altering the rate of proliferation (Wahl & Carpenter, 1988). Pretreatment of human foreskin fibroblasts with pertussis toxin partially inhibited sphingosine-mediated $InsP_3$ accumulation, suggesting a role for guanine nucleotide binding proteins(s) (G protein) in sphingosine-stimulated phosphoinositol turnover (Chao et al., 1994). Interestingly, SPP stimulated a more rapid release of intracellular calcium than sphingosine in skin fibroblasts, without any effects on phosphoinositol turnover (Chao et al., 1994). Mobilization of intracellular calcium by sphingosine in human neutrophils did not correlate with

$InsP_3$ formation and was not sensitive to pertussis toxin treatment (Wong & Kwan, 1993). Furthermore, sphingosine-induced calcium oscillations in rat pancreatic acinar cells occurred without phosphoinositol hydrolysis or $InsP_3$ production (Yule et al., 1993).

Recently, we found that SPP, like sphingosine, also stimulated the formation of $InsP_3$ in 3T3 fibroblasts (Mattie et al., 1994). However, the mobilization of calcium by SPP apparently proceeded by a previously undescribed mechanism, independent of calcium influx and inositol lipid hydrolysis (Mattie et al., 1994). Several lines of evidence support this conclusion. First, the magnitude of the calcium response decreased with successive SPP challenges, although SPP did not attenuate the response to bradykinin which stimulates phopholipase C and $InsP_3$ formation. Conversely, pretreatment of cells with bradykinin did not diminish the effect of SPP on the calcium signal. Second, although SPP increased $InsP_3$ levels, complete inhibition of inositol phosphate formation by pretreatment of cells with TPA did not inhibit SPP-mediated calcium responses, indicating that formation of $InsP_3$ is not required for release of calcium by SPP (Mattie et al., 1994). In contrast, and in agreement with previous studies (Van Corven et al., 1989), TPA pretreatment suppressed not only $InsP_3$ formation but also calcium responses elicited by lysophosphatidic acid (Mattie et al., 1994). Moreover, as mentioned above, in permeabilized cells, the $InsP_3$ antagonist heparin blocked calcium release induced by exogenous $InsP_3$ but did not significantly diminish the calcium release induced by SPP.

Although the mechanism by which SPP mobilizes internal calcium stores has not yet been elucidated, the structural similarities between SPP and lysophosphatidic acid suggest that SPP might be acting through the lysophosphatidic acid receptor which has recently been shown to be coupled to several G proteins (Van Corven et al., 1989). Furthermore, similar to lysophosphatidic acid, SPP induces inward calcium-activated chloride currents in *Xenopus laevis* oocytes (Durieux et al., 1993). However, as mentioned above, in contrast to lysophosphatidic acid, SPP can release calcium from internal sources in 3T3 fibroblasts without the generation of inositol phosphates. Furthermore, lysophosphatidic acid stimulates arachidonic acid release (Van Corven et al., 1989), while SPP does not (Mattie et al., 1994). Thus, it seems reasonable to assume that SPP may interact directly with calcium channels within the membranes of some intracellular stores which are under the control of the ryanodine receptor or $InsP_3$ receptors. Nevertheless, it is possible that SPP may affect another, yet unknown calcium channel.

Recently, polyunsaturated fatty acids, including arachidonate were found to mobilize calcium from internal sources in Jurkat T cells through a mechanism independent of phosphoinositide turnover or calcium influx (Chow & Jonda, 1990). Thus, it was possible that arachidonate may have been involved in the mechanism by which SPP releases calcium from internal sources. However, this is unlikely, since neither SPP nor sphingosine induced a significant release of arachidonate or its metabolites in Swiss 3T3 fibroblasts (Mattie et al., 1994).

In contrast to the studies described above, it has been shown recently that sphingosine induces a marked rapid decrease of cytosolic calcium concentration in Jurkat T cells stimulated with either CD3 monoclonal antibody, the Ca^{2+}-ATPase inhibitor, thapsigargin, or the calcium ionophore, ionomycin, due to activation of the calcium extrusion process (Breittmayer et al., 1994). To explain these contradictory results, Breittmayer et al. have suggested that the increase in cytosolic free calcium induced by sphingosine or SPP in other cell lines was due to increased phosphatidic acid which in turn can cause the release of calcium from internal sources independent of $InsP_3$ levels (Breittmayer et al., 1991). However, recent results indicate that this is an unlikely explanation since, in Swiss 3T3 fibroblasts there is no correlation between phosphatidic acid accumulation and calcium release. Only D-(+)-*erythro* stereoisomers (cis and trans) stimulated DNA synthesis, whereas neither L-(-)-*threo*-sphingosines (cis or trans) had a significant effect on cellular proliferation. In contrast, all four stereoisomers induced similar increases in phosphatidic acid. However, only the D-(+)-*erythro*- stereoisomers (cis and trans) were effective in releasing calcium from intracellular stores. These results suggest that the formation of phosphatidic acid is not sufficient to mediate sphingosine-stimulated calcium release or DNA synthesis. It is important to note that calcium release is the only early event in sphingosine-induced mitogenesis that has been demonstrated to be stereospecific. Since we have shown that sphingosine-induced release of calcium is mediated primarily through SPP, these findings may imply the existence of a putative intracellular SPP receptor which is involved in calcium release.

As will be discussed below, the fact that cells have mechanisms to regulate their levels of both sphingosine (Merrill, 1991) and SPP (Stoffel, et al., 1973; Zhang et al., 1991; Olivera & Spiegel, 1993), suggest that these sphingolipid metabolites may be important in the regulation of calcium homeostasis.

cAMP

Similar to previous reports that phosphatidic acid induced a dramatic decrease in cAMP levels in isoproterenol-treated 3T3 fibroblasts (Murayama & Ui, 1987), mitogenic concentrations of sphingosine (Zhang et al., 1990*b*) and SPP (K. Goodemote, M.E. Mattie, A. Berger, and S. Spiegel, *unpublished results*) also inhibited cAMP accumulation. Decreases in cAMP were also reported in

S49 lymphoma cells in which sphingosine inhibited adrenaline-, propranolol,- or forskolin-stimulated increases in cAMP (Johnson & Clark, 1990). These results suggested that sphingosine is involved in either inhibition of adenylate cyclase or activation of phosphodiesterase independently of receptor function. Alternatively, sphingosine might alter functions of G proteins. In Swiss 3T3 fibroblasts, activation of phosphodiesterase appears to be an unlikely explanation since sphingosine or SPP inhibited cAMP accumulation in the presence of the phosphodiesterase inhibitor, isobutylmethylxanthine.

Despite more than two decades of study, the precise role of cAMP in regulating cell growth remains controversial. In some cells, such as Swiss 3T3 fibroblasts, cAMP acts as a positive effector of proliferation (Rozengurt, 1986), whereas, in a myriad of other cell types, including rat-1 fibroblasts, neuroblastoma cells and cells transformed by Ras, cAMP is a negative effector of mitogenesis (Wu et al., 1993*a*). In the latter cells, cAMP inhibited signal transduction from Ras to Raf-1 and thereby prevented activation of the MAP kinase cascade (Wu et al., 1993*a*). Due to the complexity of this issue, the relevance of changes in cAMP levels to the mechanism of action of sphingosine or SPP is presently unclear.

Growth Factors Modulate Sphingosine and Sphingosine 1-Phosphate Levels

Sphingosine and SPP were originally considered to be merely metabolites on the degradative pathway of long-chain sphingoid bases (Stoffel & Assmann, 1970). However, as outlined above, these metabolites may indeed play active roles in mediating physiological functions. If sphingosine and SPP function as intracellular second messengers, then their cellular levels should be relatively low and tightly regulated in response to external stimuli. Levels of free sphingosine have been found to be in the range of 10–100 pmol/10^6 cells in a wide variety of cells (Merrill et al., 1986; Van Veldhoven et al., 1989; Merrill, 1991), and can be regulated by several agonists (Wilson et al., 1988; Ramachandran et al., 1990; Merrill, 1991; Olivera & Spiegel, 1993). Dexamethasone induced a modest increase in free sphingosine in 3T3-L1 fibroblasts (Ramachandran et al., 1990). In addition, TPA decreased the level of free sphingosine in human neutrophils, whereas serum, plasma, or serum lipoproteins induced increases (Wilson et al., 1988). In contrast, less is known of basal levels of SPP in cells and until recently it had only been detected after treatment with its precursor, sphingosine (Stoffel et al., 1973; Zhang et al., 1991).

Recently, we found that both PDGF and serum induced a rapid and transient increase in sphingosine and SPP levels in Swiss 3T3 fibroblasts (Olivera & Spiegel, 1993). These responses appeared to be specific for certain growth promoting agents, since EGF did not induce significant changes (Olivera & Spiegel, 1990. Basal levels of SPP in quiescent Swiss 3T3 fibroblasts were 16 $\pm$ 2 pmol/10^6 cells and increased to 34 $\pm$ 4 and 45 $\pm$ 0.3 pmol/10^6 cells in response to PDGF and serum, respectively. Interestingly, similar levels of SPP were detected after addition of mitogenic concentrations (5–10 μM) of sphingosine (40-115 pmol/10^6 cells) (Zhang et al., 1991; Desai et al., 1992). The role of sphingosine in mediating the action of PDGF has also recently been investigated in vascular smooth muscle cells. PDGF increased sphingosine by 1.5-fold with a concomitant decrease in ceramide levels. Furthermore, L-cycloserine, an inhibitor of sphingolipid synthesis, inhibited PDGF-stimulated DNA synthesis (Jacobs & Kester, 1993). The transient increases in levels of intracellular sphingosine and SPP in response to PDGF and serum have important implications for their biological roles in growth factor-activated signal transduction pathways.

To determine whether growth factor-induced elevation in intracellular sphingosine is the sole regulatory factor influencing SPP levels, the effects of various growth factors on the activity of sphingosine kinase were examined. Serum and PDGF induced a transient increase in a specific cytosolic sphingosine kinase activity in Swiss 3T3 fibroblasts (Olivera & Spiegel, 1993). Similarly, other mitogens such as the B subunit of cholera toxin and TPA, significantly increased the activity of this kinase, whereas bombesin, bradykinin, insulin and EGF had little or no effect. Even a combination of insulin and EGF, which induced marked proliferation of quiescent 3T3 fibroblasts, did not activate sphingosine kinase indicating that the effect was restricted to certain growth promoting agents. Recently, it was found that brief treatment of Balb/c 3T3 fibroblasts (A31 variant) with TPA also markedly stimulated sphingosine kinase activity (Mazurek et al., 1994).

The fact that SPP has mitogenic activity when added exogenously to cells, combined with the observation that PDGF and serum can activate sphingosine kinase and consequently regulate endogenous levels of SPP, suggests that SPP has an important role in the signal transduction pathways activated by these growth factors. Furthermore, DL-*threo*-dihydrosphingosine, a known competitive inhibitor of sphingosine kinase, not only inhibited sphingosine kinase activity from 3T3 fibroblasts in vitro, it also inhibited the production of SPP induced by a mitogenic concentration of sphingosine by 63 $\pm$ 2% and the initiation of DNA synthesis in response to sphingosine by more than 55%, supporting the suggestion that SPP mediates the mitogenic effects of sphingosine. Furthermore, this sphingosine kinase inhibitor completely eliminated the production of SPP elicited by PDGF and also reduced DNA synthesis induced by PDGF and serum by 40% and 44%, respectively, consistent with the multiplicity of signal transduction sys-

tems known to be stimulated by these growth factors. As expected, this inhibitor did not abrogate cellular proliferation induced by EGF, which seems to act independently of SPP formation.

Overall, our recent data provide the first clues to the identity of the potential missing link between the plasma membrane (where the growth factor receptors lie), the intracellular calcium stores and cell growth. Thus, sphingosine and SPP have appropriate properties to function as intracellular second messengers: they elicit diverse cellular responses; their turnover is extremely rapid; their levels in cells are very low and increase rapidly and transiently in response to growth factors; they can release calcium from internal sources in a previously unknown, $InsP_3$-independent manner; finally, they could also act in a positive feedback loop to amplify the cascade of events following receptor stimulation via their effect on phosphatidic acid levels, linking growth factor signaling to cellular ras activity.

Sphingosine and Sphingosine-1-Phosphate Stimulate DNA-Binding Activity of AP-1

Despite the evidence that sphingosine and/or SPP may play a role as a second messenger mediating mitogenic effects and the progression of Go-arrested cells through the S phase of the cell cycle, little is yet known of the nuclear events that follow the early responses induced by these sphingolipid metabolites. Many studies have focused on the transcription factor, activator protein-1 (AP-1) which appears to function as a major convergence point, coupling growth factor receptors to long-term cellular phenotypic responses by regulating expression of specific target genes. AP-1, which is one of the transcription factors activated by the Ras pathway, consists of homo and/or heterodimers of the fos and jun gene products, and controls genes that are required for cell growth by binding to AP-1 consensus sequences, named TREs (TPA-responsive elements). Inhibition of either Fos synthesis with a vector expressing antisense c-fos RNA (Holt et al., 1986), or Fos function by microinjection of Fos-specific antibodies (Riabowol et al., 1988), inhibits cell cycle progression, confirming the function of AP-1 protein in controlling cell proliferation. Furthermore, microinjection of oligonucleotides containing consensus AP-1 binding sites blocked DNA synthesis in response to growth factors (Riabowol et al., 1992).

As was previously established for growth factors, sphingosine and SPP, which induced rapid increases in phosphatidic acid (Zhang et al., 1991; Desai et al., 1992), an important activator of Ras (Tsai al., 1989; 1990) and the MAP cascade, also markedly simulated DNA binding activity of AP-1 (Su et al., 1994). The induced AP-1 complex contains both c-Fos and c-Jun proteins. This is important since heterodimer formation of any Jun protein with c-Fos results in the generation of potent transactivators and part of this increased activity is due to the increased stability of these heterodimers (Karin & Smeal, 1992). Interestingly, it was recently found that microinjection of affinity-purified antibodies against different Fos family members, c-Fos, FosB, and Fra-1, into Swiss 3T3 fibroblasts only partially inhibited DNA synthesis. In contrast, DNA synthesis was more effectively inhibited by antibodies against any of the Jun family (Riabowol et al., 1992). Several experiments suggested that the stimulation of DNA synthesis in Swiss 3T3 fibroblasts by SPP was, at least in part mediated by AP-1 (Su et al., 1994). First, the dose-response for activation of AP-1 by SPP correlated closely with the induction of DNA synthesis. Second, although both SPP and sphingosine induced DNA binding activity of AP-1, a competitive inhibitor of sphingosine kinase, DL-*threo*-dihydrosphingosine, which inhibits sphingosine-induced DNA synthesis, also inhibited sphingosine-stimulated AP-1 DNA-binding activity. This result lends further support to our proposal that SPP mediates the mitogenic effect of sphingosine. Finally, SPP induced AP-1 activity within 30 min after stimulation and the activity remained significantly elevated for a least 21 hr. This finding is consistent with the notion that AP-1 may be required for the transcriptional activation of a cascade of genes necessary for the entry of the cells into the S phase by 18 hr after stimulation. AP-1 DNA binding sequences have been localized to the regulatory regions of some genes which are important in the progression of quiescent cells into the S phase, including DNA polymerase α, and AP-1-related sequence in the regulatory region of p53, and a regulatory region within the cell-cycle-specific human H4 histone gene (Angel & Karin, 1991; Karin & Smeal, 1992). AP-1 may be a convergence point where signals carried by sphingolipid-derived messengers are integrated with the well-known intracellular second messages derived from glycerophospholipids, e.g., diacylglycerol. The activation of AP-1 by sphingosine and SPP is the first evidence linking the effects of sphingolipids metabolites on cellular proliferation to gene expression (Su et al., 1994).

Sphingosinephosphorylcholine is a Remarkably Potent Mitogen

To identify the structural components of SPP which are required for the induction of proliferation, many analogues of SPP were tested for mitogenic activity. Of these, only sphingosinephosphorylcholine (SPC) or lysosphingomyelin (a phosphocholine ester of sphingosine), was found to have greater mitogenicity than sphingosine or SPP (Desai and Spiegel, 1991; Desai et al., 1993). SPC-induced proliferation was equal to that of serum and was much greater than all other fibroblast

growth factors, including platelet activating factor, which is structurally similar to SPC. Unlike most peptide mitogens which require long exposure to induce proliferation in fibroblasts, treatment with SPC for only 10 min was sufficient to induce significant DNA synthesis. SPC also stimulated proliferation of many diverse cell types, including preadipocytes, HeLa carcinoma cells, C6 rat glioma cells, transformed 3T3 fibroblasts, human astrocytes, and appears to be a new type of potent, wide-spectrum growth promoting agent (Desai & Spiegel, 1991).

Sphingosinephosphorylcholine Signaling Pathways

SPC induced transient, pancellular increases in $[Ca^{2+}]_i$ in Swiss 3T3 fibroblasts as measured with a digital imaging system (Desai et al., 1993). 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). 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. In agreement, SPC also mediated $InsP_3$-independent Ca^{2+} oscillations in pancreatic acinar cells. This response was not mimicked by addition of SPP, ceramide, or sphingomyelin (Yule et al., 1993). Recently, a novel intracellular SPC-gated calcium permeability channel was detected in rat basophilic leukemia cells. The pharmacologic and electrophysiologic properties of this channel distinguished it from other previously indentified voltage-gated and ligand-gated calcium channels (Kindman et al., 1994).

Previously, we found that sphingosine and SPP stimulated formation of inositol phosphates (Zhang et al., 1990*b*; Mattie et al., 1994). Thus, despite shared structural features, sphingosine and SPP do not share a common mechanism of mitogenic stimulation with SPC (Desai et al., 1993). Although SPC, SPP and sphingosine were all found to stimulate increased intracellular calcium in fibroblasts, SPC did not mimic the other changes in intracellular signaling induced by sphingosine or SPP, including activation of phospholipase D leading to phosphatidic acid accumulation, stimulation of inositol phosphate formation, or inhibition of cAMP production. On the other hand, in contrast to sphingosine and SPP, SPC induced an increase in release of arachidonic acid and may act via both protein kinase C-dependent and independent signaling pathways, whereas the mitogenic activities of sphingosine and SPP in Swiss 3T3 cells were clearly independent of protein kinase C (Zhang et al., 1990*a*, 1991). Furthermore, although the significance of the arachidonic acid released during the mitogenic response to SPC is not clear, it is intriguing to note that protein kinase Cα is the predominant protein kinase C isoform in Swiss 3T3 cells. This isoenzyme can be activated by free arachidonic acid in the presence of calcium (Nishizuka, 1992). The increase in $[Ca^{2+}]_i$ induced by SPC, together with the increase in arachidonic acid levels, might activate protein kinase Cα and could account for the partial protein kinase C dependency observed in these studies. Recently, in contrast to the effect of sphingosine, SPC was found to have dual effects on the phosphorylation of cytosolic proteins in Jurkat T cells (Pushkareva & Hannun, 1994). In this regard, it has previously been shown that activation of casein kinase II by sphingosine was further augmented by SPC (McDonald et al., 1991). Thus, the ability of SPC to act as an extremely potent mitogen may be due to activation of signaling pathway(s) distinct from those utilized by sphingosine or SPP.

Does Endogenous Sphingosylphosphorylcholine Regulate Mitogenesis?

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 does not mediate the SPC-induced increase in intracellular calcium and is probably not involved in the mitogenic effect of SPC. Therefore, SPC itself probably acts as the mediator of mitogenic stimulation. In this regard, it is interesting to note that a significant amount of exogenous SPC was incorporated into Swiss 3T3 cells within a very short time, a result which is consistent with our observation that SPC was a very potent mitogen even after only a brief treatment (Desai et al., 1992).

Although SPC has not yet been detected in normal cells, it has been found in the spleens of patients with Niemann-Pick disease (Strasberg & Callahan, 1987), a form of sphingolipidosis resulting from a deficiency of sphingomyelinase activity (Strasberg & 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 & Callahan, 1988). Earlier, SPC had been proposed to be a potential precursor of sphingomyelin (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 cases, the increases in sphingomyelin were associated with differentiation. Nevertheless, exogenous SPC in 3T3 cells has not been found to be significantly converted to sphingomyelin

(Desai et al., 1992). Furthermore, other studies have clearly demonstrated that most, if not all, sphingomyelin is synthesized in mammalian cells by transfer of phosphorylcholine from phosphatidylcholine to ceramide, suggesting that SPC is probably not a precursor of sphingomyelin (Pagano, 1988; Futerman et al., 1990). Yet, in analogy to receptor-linked phospholipase A_2 which catalyzes liberation of arachidonic acid 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.

Concluding Remarks

Abundant evidence has been accumulated to strongly suggest that sphingolipid metabolites may be a new class of intracellular second messengers. However, many questions remain to be answered. Are sphingosine, SPP, SPC, and ceramide the only active sphingolipids or are there still others remaining to be discovered? Are the effects of these compounds the same or similar in all cells or are their effects limited to specific cells and tissues? More complete descriptions of the mechanisms of regulation of cell growth and other cellular processes should enable us to answer some of these questions.

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References

Angel, P., Karin, M. 1991. *Biochim. Biophys. Acta* **1072:**129–157

Aridor, A., Piterman, O., Lavie, Y., Liscovitch, M. 1992. *Eur. J. Biochem.* **204:**561–568

Arnold, R.S., Newton, A.C. 1991. *Biochemistry* **30:**7747–7754

Barbacid, M. 1987. *Annu. Rev. Biochem.* **56:**779–827

Ben-Av, P., Liscovitch, M. 1989. *FEBS Lett.* **259:**64–66

Berridge, M.J. 1993. *Nature* **361:**315–325

Brady, R.O., Bradley, R.M., Young, O.M., Kaller, H. 1965. *J. Biol. Chem.* **240:**3693–3694

Breittmayer, J.P., Aussel, C., Frahifar, D., Cousin, J.L., Fehlmann, M. 1991. *Immunol.* **73:**134–139

Breittmayer, J.P., Bernard, A., Aussel, C. 1994. *J. Biol. Chem.* **269:**5054–5058

Buckley, N.E., Matyas, G.R., Spiegel, S. 1990. *Exp. Cell Res.* **189:**13–21

Chao, R., Khan, W., Hannun, Y.A. 1992. *J. Biol. Chem.* **267:**23459–23462

Chao, C.P., Laulederkind, S.J., Ballou, L.R. 1994. *J. Biol. Chem.* **269:**5849–5856

Chow, S.C., Jondal, M. 1990. *J. Biol. Chem.* **265:**902–907

Davis, R.J., Girones, N., Faucher, M. 1988. *J. Biol. Chem* **263:**5373–5379

Desai, N.N., Carlson, R.O., Mattie, M.E., Olivera, A., Buckley, N.E., Seki, T., Brooker, G., Spiegel, S. 1993. *J. Cell Biol.* **121:**1385–1395

Desai, N.N., Spiegel, S. 1991. *Biochem. Biophys. Res. Commun.* **181:**361–366

Desai, N.N., Zhang, H., Olivera, A., Mattie, M.E., Spiegel, S. 1992. *J. Biol. Chem.* **267:**23122–23128

Dressler, K.A., Kan, C.C., Kolesnick, R.N. 1991. *J. Biol. Chem.* **266:**11522–11527

Durieux, M.E., Carlisle, S.J., Salafranca, M.N., Lynch, K.R. 1993. *Am. J. Physiol.* **264:**c1360–c1364

Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M., Weinberg, R.A. 1993. *Nature* **363:**45–51

Exton, J.H. 1990. *J. Biol. Chem.* **265:**1–4

Faucher, M., Girones, N., Hannun, Y.A., Bell, R.M., Davis, R.J. 1988. *J. Biol. Chem.* **263:**5319–5327

Fukami, K., Takenawa, T. 1992. *J. Biol. Chem.* **267:**10988–10993

Futerman, A.H., Pagano, R.E. 1991. *Biochem. J.* **280:**295–302

Gale, N.W., Kaplan, S., Lowenstein, E.J., Schlessinger, J., Bar-Sagi, D. 1993. *Nature* **363:**88–92

Ghosh, T.K., Bian, J., Gill, D.L. 1990. *Science* **248:**1653–1656

Goldin, E., Roff, C.F., Miller, S.P., Rodriguez, L.C., Vanier, M.T., Brady, R.O., Pentchev, P.G. 1992. *Biochim. Biophys. Acta* **1127:**303–311

Gomez, M.A., Martin, A., O'Brien, L., Brindley, D.N. 1994. *J. Biol. Chem.* **269:**8937–8943

Hakomori, S. 1990. *J. Biol. Chem.* **265:**18713–18716

Hakomori, S., Igarashi, Y. 1993. *Adv. Lipid. Res.* **25:**147–162

Hannun, Y.A. 1994. *J. Biol. Chem.* **269:**3125–3128

Hannun, Y.A., Bell, R.M. 1987. *Science* **235:**670–674

Hannun, Y.A., Bell, R.M. 1989. *Science* **243:**500–507

Harmala, A.S., Porn, M.I., Slotte, J.P. 1993. *Biochim. Biophys. Acta* **1210:**97–104

Holt, J.T., Gopal, T.V., Moulton, A.D., Nienhuis, A.W. 1986. *Proc. Natl. Acad. Sci. USA* **83:**4794–4798

Igarashi, Y., Kitamura, K., Toyokuni, T., Dean, B., Fenderson, B., Ogawass, T., Hakomori, S. 1990. *J. Biol. Chem.* **265:**5385–5389

Irvine, R. 1982. *Biochem. J.* **204:**3–16

Jacobs, L.S., Kester, M. 1993. *Am. J. Physiol.* **265:**c740–c747

Jamal, Z., Martin, A., Munoz, A.G., Brindley, D.N. 1991. *J. Biol. Chem.* **266:**2988–2996

Jefferson, A.B., Schulman, H. 1988. *J. Biol. Chem.* **263:**15241–15244

Johnson, J.A., Clark, R.B. 1990. *J. Biol. Chem.* **265:**9333–9339

Karin, M., Smeal, T. 1992. *TIBS* **17:**418–422

Keenan, R.W. 1972. *Biochim. Biophys. Acta* **270:**383–396

Kindman, L.A., Kim, S., McDonald, T.V., Gardner, P. 1994. *J. Biol. Chem.* **269:**13088–13091

Kiss, Z., Anderson, W.B. 1990. *J. Biol. Chem.* **265:**7188–7194

Kiss, Z., Deli, E. 1992. *Biochem. J.* **288:**853–858

Kolesnick, R.N. 1989. *J. Biol. Chem.* **264:** 7617–7623

Kolesnick, R., Golde, D.W. 1994. *Cell* **77:**325–328

Lavie, Y., Liscovitch, M. 1990. *Biochem. Biophys. Res. Commun.* **167:**607–613

Lavie, Y., Piterman, O., Liscovitch, M. 1990. *FEBS Lett.* **277:**7–10

Lippman, M.E., Dickson, R.B. 1989. *Rec. Prog. Hormone Res.* **45:**383–439

Lopez-Rivas, A., Mendoza, S.A., Nanherg, E., Sinnett-Smith, J., Rozengurt, E. 1987. *Proc. Natl. Acad. Sci. USA* **84:**5768–5772

Margolis, B., Zilberstein, A., Franks, C., Felder, S., Kremer, S., Ullrich, A., Rhee, S.G., Skorecki, K., Schlessinger, J. 1990. *Science* **247:**607–610

Mattie, M., Brooker, G., Spiegel, S. 1994. *J. Biol. Chem.* **269:**3181–3188

Mazurek, N., Megidish, T., Hakomori, S., Igarashi, Y. 1994. *Biochem. Biophys. Res. Commun.* **198:**1–9

McDonald, O.B., Hannun, Y.A., Reynolds, C.H., Sahyoun, N. 1991. *J. Biol. Chem.* **266:**21773–21776
Merrill, A.H. 1983. *Biochim. Biophys. Acta* **754:**284–291
Merrill, A.H., Sereni, A.M., Stevens, V.L., Hannun, Y.A., Bell, R.M., Kinkade, J.M. 1986. *J. Biol. Chem.* **261:**12610–12615
Merrill, A.H., Stevens, V.L. 1989. *Biochim. Biophys. Acta* **1010:**131–139
Merrill, A.H. 1991. *J. Bioenerg. Biomemb.* **23:**83–104
Merrill, A.H., Wang, E. 1992. *Meth. Enzymol.* **209:**427–437
Mullmann, T.J., Siegel, M.I., Egan, R.W., Billah, M.M. 1991. *J. Biol. Chem.* **266:**2013–2016
Murayama, T., Ui, M. 1987. *J. Biol. Chem.* **262:**5522–5529
Nishizuka, Y. 1992. *Science* **258:**607–614
Okoshi, H., Hakomori, S., Nisar, M., Zhou, Q.H., Kimura, S. Tashiro, K., Igarashi, Y. 1991. *Cancer. Res.* **51:**6019–6024
Olivera, A., Spiegel, S. 1993. *Nature* **365:**557–560
Olivera, A., Spiegel, S. 1992. *Glycoconjugate J.* **9:**109–117
Olivera, A., Zhang, H., Carlson, R.O., Mattie, M.E., Schmidt, R.R., Spiegel, S. 1994. *J. Biol. Chem.* **289:**17924–17930
Pagano, R.E. 1988. *Trends Biochem. Sci.* **13:**202–205
Pelech, S.L., Sanghera, J.S. 1992. *Science* **257:**1355–1356
Pinkerton, F.D., Kisic, A., Wilson, W.K., Schroepfer, G.J. 1993. *Biochem. Biophys. Res. Commun.* **190:**63–69
Pushkareva, M.Y., Hannun, Y.A. 1994. *Biochim. Biophys. Acta* **1221:**54–60
Pushkavera, M.Y., Khan, W.A., Alessenko, A.V., Sahyoun, N., Hannun, Y.A. 1992. *J. Biol. Chem.* **267:**15246–15251
Ramachandran, C.K., Murray, D.K., Nelson, D.H. 1990. *Biochem. Biophys. Res. Commun.* **167:**607–613
Randriamampita, C., Tsien, R.Y. 1993. *Nature* **364:**809–814
Riabowol, K.T., Schiff, J., Gilman, M.Z. 1992. *Proc. Natl. Acad. Sci USA* **89:**157–161
Riabowol, K.T., Vosatka, R.J., Ziff, E.B., Lamb, N.J., Feramisco, J.R. 1988. *Molec. Cell. Biol.* **8:**1670–1676
Roff, C.F., Goldin, E., Comly, M.E., Cooney, A., Brown, A., Vanier, M.T., Miller, S.P., Brady, R.O., Pentchev, P.G. 1991. *Dev. Neurosci.* **13:**315–319
Rother, J., van Echten, G., Schwarzmann, G., Sandhoff, K. 1992. *Biochem. Biophys. Res. Commun.* **189:**14–20
Rozakis-Adcock M., Fernley, R., Wade, J., Pawson, T., Bowtell, D. 1993. *Nature* **363:**83–87
Rozengurt, E. 1986. *Science* **234:**161–166
Sabbadini, R.A., Betto, R., Teresi, A., Fachechi-Cassano, G., Salviati, G. 1992. *J. Biol. Chem.* **267:**15475–15484
Sadahira, Y., Ruan, F., Hakomori, S., Igarashi, Y. 1992. *Proc. Natl. Acad. Sci. USA* **89:**9686–9690
Sadahira, Y., Zheng, M., Ruan, F., Hakomori, S., Igarashi, Y. 1994. *FEBS Lett.* **340:**99–103
Schroeder, J.J., Crane, H.M., Xia, J., Liotta, D.C., Merrill, A.H. 1994. *J. Biol. Chem.* **269:**3475–3481
Scheidl, H., Scita, G., Sampson, P.H., Park, H.Y., Wolf, G. 1992. *Biochim. Biophys. Acta* **1135:**295–300
Spiegel, S., Fishman, P.H. 1987. *Proc. Natl. Acad. Sci. USA* **84:**141–145
Spiegel, S. 1993. *J. Lipid. Mediat.* **8:**169–175
Spiegel, S., Olivera, A., Carlson, R.O. 1993. *Adv. Lipid. Res.* **25:**105–129
Spiegel, S., Olivera, A., Zhang, H., Thompson, E., Su, Y., Berger, A. 1994. *Breast Cancer Res. Treat.* **31:**195–206
Sporn, M.B., Roberts, A.B. 1988. *Nature* **332:**217–221
Stevens, V.L., Nimkar, S., Jamison, W.C., Liotta, D.C., Merrill, A.H. 1990. *Biochim Biophys. Acta* **1051:**37–45
Stoffel, W., Assmann, G. 1970. *Hoppe-Seyler's Z. Physiol. Chem.* **351:**1041–1049
Stoffel, W., Bister, K. 1973. *Hoppe-Seyler's Z. Physiol. Chem.* **354:**169–181
Stoffel, W., Hellenbroich, B., Heimann, G. 1973. *Hoppe-Seyler's Z. Physiol. Chem.* **354:**1311–1316
Stoffel, W., Sticht, G., LeKim, D. 1968. *Hoppe-Seyler's Z. Physiol. Chem.* **349:**1745–1748
Strasberg, P.M., Callahan, J.W. 1987. In Lipid Storage Disorders. Salvayre, R., Douste-Blazy, L., Gatt, S., Eds., Plenum Press, New York, pp. 601–606
Strasberg, P.M., Callahan, J.W. 1988. *Biochem. Cell Biol.* **66:**1322–1332
Su, Y., Rosenthal, D., Smulson, M., Spiegel, S. 1994. *J. Biol. Chem.* **269:**16512–16517
Sugiya, H., Furuyama, S. 1991. *FEBS Lett.* **286:**113–116
Sugiyama, E., Uemura, K.I., Hara, A., Taketomi, T. 1990. *Biochem. Biophys. Res. Commun.* **169:**673–679
Taylor, W., Marshall. 1992. *TIBS* **17:**403–407
Trahey, M., McCormick, F. 1987. *Science* **238:**524–525
Tsai, M., Roudebush, M., Dobrowolski, S., Yu, C., Gibbs, J.B., Stacey, D.W. 1991. *Mol. Cell. Biol.* **11:**2785–2793
Tsai, M., Yu, C., Stacey, D.W. 1990. *Science* **250:**982–985
Tsai, M., Yu, C., Wei, F.S., Stacey, D.W. 1989. *Science* **243:**522–526
Van Corven, E.J., Groenink, A., Jalink, K., Eicholtz, T., Moolenaar, W.H. 1989. *Cell* **59:**45–54
Van Veldhoven, P.P., Foglesong, R.J., Bell, R.M. 1989. *J. Lipids Res.* **30:**611–616
Van Veldhoven, P.P., Mannaerts, G.P. 1991. *J. Biol. Chem.* **266:**12502–12507
Wahl, M., Carpenter, G. 1988. *J. Biol. Chem.* **263:**7581–7590
Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T., Merrill, A.H. 1991. *J. Biol. Chem.* **266:**14486–14490
Williams, L.T. 1989. *Science* **243:**1564–1570
Wilson, E., Wang, E. Mullins, R.E., Uhlinger, D.J., Liotta, D.C., Lambeth, J.D., Merrill, A.H. 1988. *J. Biol. Chem.* **263:**9304–9309
Wong, K., Kwan, Y.L. 1993. *Cell Calcium* **14:**493–505
Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M.J., Sturgill, T.W. 1993a. *Science* **262:**1065–1069
Wu, W.I., Lin, Y.P., Wang, E., Merrill, A.H., Carman, G.M. 1993b. *J. Biol. Chem.* **268:**13830–13837
Yamada, K., Sakane, F. 1993. *Biophys. Acta* **1169:**211–216
Yamada, K., Sakane, F., Imai, S.I., Takemura, H. 1993. *Biochim. Biophys. Acta* **1169:**217–214
Yule, D.I., Wu, D., Essington, T.E., Shayman, J.A., Williams, J.A. 1993. *J. Biol. Chem.* **268:**12353–12358
Zhang, H., Buckley, N.E., Gibson, K., Spiegel, S. 1990*a*. *J. Biol. Chem.* **265:**76–81
Zhang, H., Desai, N.N., Murphey, J.M., Spiegel, S. 1990*b*. *J. Biol. Chem.* **265:**21309–21316
Zhang, H., Desai, N.N., Olivera, A., Seki, T., Brooker, G., Spiegel, S. 1991. *J. Cell Biol.* **114:**155–167