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MINI-REVIEW

Cell Regulation by Sphingosine and More Complex Sphingolipids[1]

Alfred H. Merrill, Jr.[2]

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Abstract

Sphingolipids have the potential to regulate cell behavior at essentially all levels of signal transduction. They serve as cell surface receptors for cytoskeletal proteins, immunoglobulins, and some bacteria; as modifiers of the properties of cell receptors for growth factors (and perhaps other agents); and as activators and inhibitors of protein kinases, ion transporters, and other proteins. Furthermore, the biological activity of these compounds resides not only in the more complex species (e.g., sphingomyelin, cerebrosides, gangliosides, and sulfatides), but also in their turnover products, such as the sphingosine backbone which inhibits protein kinase C and activates the EGF-receptor kinase, *inter alia*. Since sphingolipids change with cell growth, differentiation, and neoplastic transformation, they could be vital participants in the regulation of these processes.

Key Words: Sphingosine; ceramide; sphingolipids (sphingomyelin, gangliosides); protein kinase; receptor kinase; signal transduction.

Introduction

Sphingolipids have long been associated with cell surface phenomena relevant to signal transduction (Fishman and Brady, 1976; Sweeley, 1980; Hakomori, 1981, 1984; Kanfer and Hakomori, 1983; Feizi and Childs, 1985; Sweeley, 1985; Hannun and Bell, 1989; Hakomori, 1990). They are found primarily on the external leaflet of the plasma membrane; affect the properties of cell surface receptors (and serve as the receptors for a number of bacterial toxins); undergo changes with cell growth, differentiation, and neoplastic

[1] This review is dedicated to Professor Herbert E. Carter on the occasion of his 80th birthday.

[2] Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322.

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transformation; bind cytoskeletal elements and lectins; participate in cell–cell communication and cell–substratum interactions; serve as cell surface antigens; and alter the behavior of cellular protein kinases. The underlying mechanisms for sphingolipid action are not fully understood; however, recent findings have been consistent with two complementary paradigms: (1) complex sphingolipids (such as gangliosides) may interact directly with receptors to modulate their function, and (2) sphingolipid turnover may remove one type of bioactive compound to produce products (including sphingosine, lysosphingolipids, and ceramides) that serve as lipid second messengers. This article focuses on recent findings pertinent to these two hypotheses, but will begin with a brief introduction to sphingolipids and sphingolipid metabolism.

Sphingolipid Structures, Occurrence, and Metabolism

Sphingolipids are defined as compounds having a long-chain (sphingoid) base as the backbone. The prevalent long-chain base of most mammalian tissues is an 18-carbon compound called *trans*-4-sphingenine or sphingosine (Fig. 1) (the latter name is often used generically for long-chain bases, which encompass chain length homologs), sphinganines (without the 4,5-double bond), and 4-hydroxysphinganines or "phytosphingosines" (which have a third hydroxyl at position 4). Free long-chain bases occur in tissues in small amounts (Kobayashi *et al.*, 1988b; Merrill *et al.*, 1988); most exist as ceramides with an amide-linked fatty acid (typically saturated with an alkyl chain length of 16 to 24 carbon atoms, and in some cases with a hydroxyl at the α carbon). Complex sphingolipids have a headgroup at position 1 (Fig. 1), such as a phosphorylcholine in sphingomyelin (the major phosphosphingolipid in mammals) and simple to complex carbohydrates of cerebrosides, gangliosides, sulfatides, etc.

Occurrence of Sphingolipids

One might infer that sphingolipids are specific to neuronal tissues because of their names (ceramide, sphingomyelin, gangliosides, etc.), but this merely reflects their initial discovery in brain. They are significant constituents of all cell types, where they occur mainly in the plasma membrane and cellular organelles that are functionally associated with the plasma membrane, such as the Golgi apparatus, endosomes, and lysosomes. They are components of lipoproteins (Chapman, 1986), milk (Zeisel *et al.*, 1986), and abnormal structures such as atherosclerotic plaques (Barenholz and Thompson, 1980; Prokazova *et al.*, 1987).

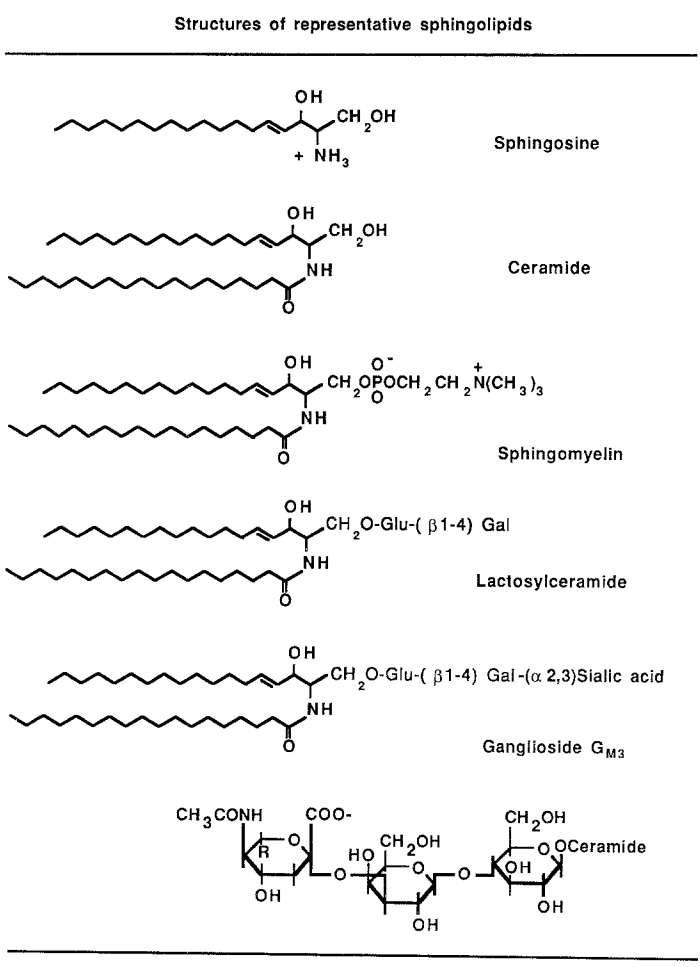

Fig. 1. Structures of sphingosine, ceramide, and examples of more complex sphingolipids. Sphingosine (*trans*-4-sphingenine, shown) and related long-chain (sphingoid) bases are the characteristic backbones of this class of compounds. Most sphingolipids have an amide-linked, long-chain fatty acid attached to the long-chain base; hence, they are derivatives of ceramide. More complex sphingolipids have various headgroups at position 1, such as: phosphorylcholine for sphingomyelin; lactose (GalGlu) for lactosylceramide (a neutral glycolipid); and *N*-acetylneuraminic acid (or sialic acid) plus lactose for ganglioside G_{M3}. The structure of the carbohydrate headgroup of G_{M3} has also been shown.

Sphingolipid Metabolism

A simplified scheme for sphingolipid metabolism is given in Fig. 2; additional information is available from recent biochemistry texts (Vance, 1988; Mathews and van Holde, 1990; Voet and Voet, 1990) and reviews (Barenholz and Gatt, 1982; Kanfer and Hakomori, 1983; Kishimoto, 1983;

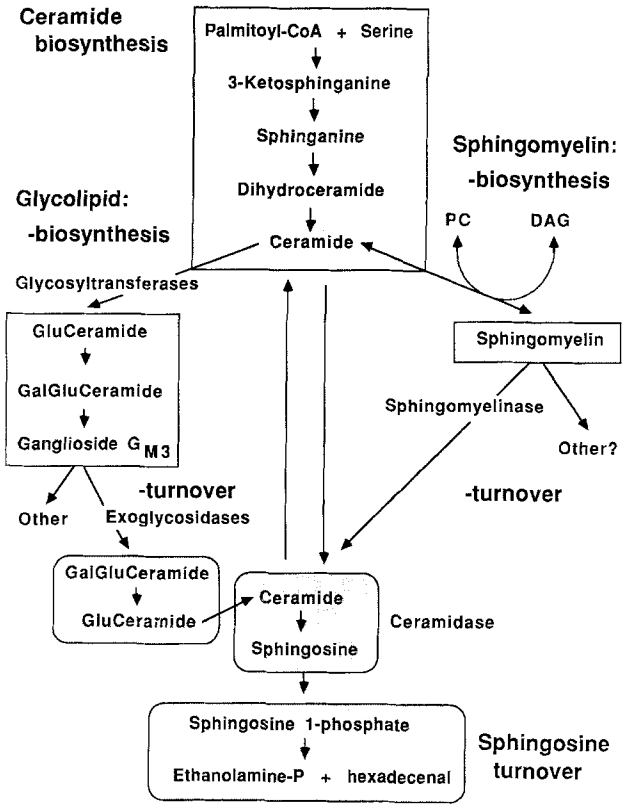

Fig. 2. Sphingolipid metabolism. These blocks illustrate an overall scheme for sphingolipid metabolism using sphingomyelin and ganglioside G_{M3} as examples. For simplicity, many of the co-substrates and products (e.g., fatty acyl-CoA's, CoASH, UDP-glucose, CMP-siliac acid, etc.) have been omitted, and only some of the key enzyme names are given. For more detailed information, consult Sweeley (1986) and the other references cited in this review.

Sweeley, 1985; Merrill and Jones, 1990). For brevity, specific citations will not be given below unless the point is relevant to signal transduction.

Sphingolipid Biosynthesis. The initial precursors of *de novo* sphingolipid biosynthesis are palmitoyl-CoA and serine, which are combined to form 3-ketosphinganine by serine palmitoyltransferase, a pyridoxal 5′-phosphate-dependent enzyme. This appears to be the rate-limiting reaction of long-chain base biosynthesis because the product is rapidly reduced to sphinganine by an NADPH-dependent reductase and converted to a (dihydro)ceramide by addition of a fatty acid. The introduction of the 4-*trans*-double bond appears to occur after addition of the amide-linked fatty acid (Ong and Brady, 1973; Wang and Merrill, 1986); if so, free sphingosine (i.e., sphingenine) is not an intermediate of the *de novo* biosynthetic pathway. Sphingomyelin biosynthesis

involves transfer of the phosphorylcholine headgroup from phosphatidylcholine to ceramide (Fig. 2). Since this potentially reversible reaction involves diacylglycerols as precursors or products, Hampton and Morand (1989) have speculated that the turnover of glycerolipids in response to agonist-induced phospholipases could affect sphingomyelin metabolism and vice versa.

Glycosphingolipid biosynthesis involves the stepwise addition of carbohydrate groups, as depicted for a few examples in Fig. 2. The regulation of glycolipid synthesis appears to reside in the activity of glycosyltransferases at key steps of the pathways, and in later modification reactions (such as addition of sulfate, addition or removal of an *N*-acetyl group, turnover, etc.). Some of the factors that alter the activities of glycosyltransferases are of particular relevance to signal transduction; for examples, phorbol esters increase the activity of CMP-sialic acid: lactosylceramide sialyltransferase in V79 cells (Burczak *et al.*, 1983), HL-60 cells (Momoi *et al.*, 1986), and NIL8-HSV cells (Moskal *et al.*, 1987), and retinoic acid increases the sialyltransferase in the latter. Dexamethasone also increases this enzyme and UDP-galactosyl-lactosylceramide galactosyl-transferase in Golgi membranes (Dudeja *et al.*, 1988). Srinivas *et al.* (1982) have reported that transformation of JB6 cells with PMA decreases the synthesis of trisialoganglioside, which appears to be required for anchorage-dependent growth, and glycolipid profiles are altered in *ras*-transfected NIH 3T3 cells (Matyas *et al.*, 1987). An inhibitor of glycolipid biosynthesis prolongs the survival of tumor-bearing mice (Inokuchi *et al.*, 1987).

Sphingolipid Turnover. The catabolism of cellular sphingolipids has been studied extensively because several genetic diseases arise from defects in sphingolipid hydrolysis. Sphingolipid turnover involves removal of the sugars by exoglycosidases and the phosphorylcholine of sphingomyelin by phospholipases. These processes can be complex (for example, accessory proteins are sometimes required) and the simplified scheme in Fig. 2 does not depict the possibility of alternative pathways nor of multiple cellular sites. For example, cells contain an acidic (lysosomal) sphingomyelinase as well as an enzyme with a neutral pH optimum, a requirement for magnesium, and a localization on the extracellular side of the plasma membrane (Das *et al.*, 1984). The recycling of sphingolipids in whole or part has been seen in many studies (Sonderfeld *et al.*, 1985; Trinchera *et al.*, 1988; Kok *et al.*, 1989).

After removal of the sphingolipid headgroup, the next step is thought to be cleavage of the amide-linked fatty acid by three ceramidases with acidic, neutral, and alkaline pH optima (Spence *et al.*, 1986). Ceramides may escape degradation and be reutilized for complex sphingolipid formation, or undergo phosphorylation by a ceramide kinase (Bajjalieh *et al.*, 1989), the purpose of which is unknown. Likewise, the sphingosine that is released by hydrolysis of ceramide can be converted back to ceramides or degraded, which involves

phosphorylation at position 1 followed by lytic cleavage to ethanolamine phosphate and *trans*-2-hexadecanal. Sphingosine must leave the lysosomes to interact with the kinase and lyase, which are cytosolic, and therefore has the potential to interact with other sites in the cell. Removal of sphingosine 1-phosphate is apparently important because administration of even small amounts of the related phosphonate (i.e., 1-desoxysphinganine 1-phosphonate) is lethal (Stoffel and Grol, 1974). Igarashi and Hakomori (1989) have also observed methylation of sphingosine *in vitro* by a mouse brain homogenate.

Complex Sphingolipids as Modulators of Cell Behavior

Much of the initial evidence that complex sphingolipids might be involved in the regulation of cell growth and differentiation was based on the observation that glycolipid profiles change as cells grow or express a new phenotype (Hakomori, 1981, 1984; Kanfer and Hakomori, 1983). The majority of the cellular glycolipids and sphingomyelin reside in the external leaflet of the plasma membrane (Miller-Podraza *et al.*, 1982). This allows interaction with cell surface receptors, extracellular cytoskeletal elements, and other factors (e.g., antibodies), which raises the question of how changes in sphingolipids are translated into intracellular responses.

Since gangliosides form water-soluble micelles and are relatively easy to administer to cells, it has been possible to demonstrate directly that they can affect growth and differentiation, rather than merely change as consequences of them. Exogenous addition of gangliosides G_{M3} or G_{M1} inhibits growth through extension of the G_1 phase of the cell cycle (Laine and Hakomori, 1973; Keenan *et al.*, 1975) and can make cells refractory to growth stimulation by growth factors; G_{M3} and G_{M1} inhibit fibroblast growth factor (FGF) stimulated growth with BHK cells (Bremer and Hakomori, 1982); platelet-derived growth factor (PDGF) stimulation of 3T3 cell growth (Bremer *et al.*, 1984); and epidermal growth factor (EGF) stimulation of growth of KB cells (Bremer *et al.*, 1986). Addition of ganglioside G_{M3} induces HL-60 cells to differentiate into monocyte-like cells (Saito *et al.*, 1985), and various exogenous gangliosides stimulate neurite outgrowth and synapse formation (Roisen *et al.*, 1981; Ferrari *et al.*, 1983; Ledeen, 1984; Tsuji *et al.*, 1988a,b; Unsicker and Wiegandt, 1988).

Complementary to these findings, removal of ganglioside G_{M1} by adding the β-subunit of cholera toxin (which binds to this ganglioside) is mitogenic for rat thymocytes (Spiegel *et al.*, 1985) and quiescent 3T3 cells, and potentiates the response of the latter to EGF, PDGF, and insulin (Spiegel and Fishman, 1987). Similar effects have been seen with antibodies to gangliosides. In contrast, the β-subunit is inhibitory for rapidly growing 3T3 cells

and *ras*-transformed 3T3 cells; therefore, Spiegel and Fishman (1987) have suggested that gangliosides are bimodal regulators of cell growth.

These observations are consistent with the idea that sphingolipids interact with receptor(s) to alter the responsiveness to growth factors and potentially other agonists. It is also possible that glycolipids bind some factors directly (as bacterial toxins) (Fishman, 1982), or interact with cytoskeletal elements or receptors on neighboring cells in ways that infuence the responsiveness of the target cell. Additionally, one may envision that some conditions could cause at least partial hydrolysis of sphingolipids to release smaller compounds that alter cell behavior.

Effects of Gangliosides on Growth Factor Receptors

Bremer *et al.* (1984) have shown that G_{M3} and G_{M1} inhibit PDGF binding and PDGF-induced protein phosphorylation (on tyrosine); therefore, inhibition of 3T3 cell growth could be due to interference with both growth factor binding and the cellular response. On the other hand, G_{M3} inhibits EGF-induced phosphorylation (on tyrosine only) without inhibition of EGF binding with KB and A431 cells (Bremer *et al.*, 1986). In studies with a subclone of A431 cells that is only weakly responsive to EGF (A5S cells show weak growth stimulation by EGF), Hanai *et al.* (1988b) found that the affinity-purified EGF receptor contains G_{M3}. In contrast, G_{M3} did not copurify with the EGF receptor of a subclone (A5I) that exhibits growth inhibition in response to EGF, although more polar gangliosides were obtained. LysoG_{M3} (which lacks the amide-linked fatty acid) also inhibited the receptor kinase, and was found in small amounts in the cells.

In related experiments, de-*N*-acetyl-G_{M3} (with loss of the *N*-acetyl group from the sialic acid) strongly enhanced the EGF receptor kinase activity and was growth stimulatory, without altering the binding of EGF (Hanai *et al.*, 1988a). This compound was found in small amounts in the cells. Hanai *et al.* (1987, 1988a,b) have suggested that gangliosides and their *N*-deacetylated derivatives (i.e., lacking an *N*-acetyl group from the sugar or a fatty acid from ceramide) may represent a mechanism for stimulating and inhibiting growth. Long-chain bases, other products of sphingolipid metabolism, stimulated DNA synthesis and growth at low concentrations (Merrill, 1983; Zhang *et al.*, 1990; Stevens *et al.*, 1990a). The effect is particularly potent with quiescent Swiss 3T3 fibroblasts (Zhang *et al.*, 1990), and sphingosine acts synergistically with known growth factors to induce proliferation. Thus, it is possible that free sphingosine is also involved in growth regulation.

Usuki *et al.* (1988a,b) have associated the turnover of G_{M3} with cell growth via a different mechanism, removal of the sialic acid by a G_{M3} sialidase activity found in the culture medium of human fibroblasts. Preconfluent cells

have G_{M3} sialidase activities with pH optima of 4.5 and 6.5; however, the latter is virtually absent from the medium of contact-inhibited cells. Double labeling of the cellular G_{M3} with precursors for sialic acid and ceramide revealed that about 35% of the sialic acid was turned over during a 24-h chase with no measurable loss of labeled ceramide. To assess the importance of extracellular sialidase(s) to cell growth, Usuki *et al.* (1988a) incubated fibroblasts with 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, a potent sialidase inhibitor. This compound reversibly inhibited growth and the turnover of labeled G_{M3}. Thus, they conclude that G_{M3} turnover to lactosylceramide occurs at some point in the cell cycle of growing cells and that this may release them from the growth inhibitory effects of the intact ganglioside. G_{M3} turnover is reduced and the inhibition of growth factor-induced growth would be expected to be greatest at confluence, when the neutral sialidase activity is low.

Effects of Gangliosides on Cellular Kinases

In addition to inhibition of these receptor kinases, gangliosides have been found to have complex effects on protein phosphorylation in membranes from brain (Goldenring *et al.*, 1985), including stimulation of Ca^{2+}/calmodulin-dependent kinase system (Goldenring, 1985; Tsuji *et al.*, 1985; Cimino *et al.*, 1987), and in muscle (Chan, 1989). A ganglioside-stimulated protein kinase has been purified from guinea pig brain (Chan, 1987). The enzyme is activated most potently by G_{T1b} and G_{D1a} (and slightly less so by G_{D1b} and G_{M1}) and phosphorylates exogenous polypeptides such as Leu-Arg-Arg-Ala-Ser-Leu-Gly, as well as its own autophosphorylation. The kinase does not require Ca^{2+} for activity, and appears to be distinct from *c*AMP-dependent protein kinase, protein kinase C, and Ca^{2+}/calmodulin-dependent kinase(s). Gangliosides have, however, been reported to inhibit protein kinase C (Kim *et al.*, 1986; Kreutter *et al.*, 1987) in the order of potency $G_{T1b} > G_{D1a} = G_{D1b} > G_{M3} = G_{M1}$ using histones, myelin basic protein, or synthetic substrates.

Tsuji *et al.* (1988a,b) have noted that addition of nanomolar concentrations of ganglioside G_{Q1b} to a human neuroblastoma cell line (GOTO) stimulated the phosphorylation of at least three cell surface-associated proteins (50, 60 and 64 kDa) by ecto-type kinase(s). Since G_{Q1b} was effective at low concentrations, exhibited a ganglioside specificity resembling that for neuritogenesis, and activated kinase(s) on the outer surface of the cell, where most cellular gangliosides are thought to reside, Tsuji and coworkers suggest that this may represent the system that is associated with the neuritogenic effect of this ganglioside.

Spiegel and coworkers have also explored the mechanism of action of the β-subunit of cholera toxin, which stimulates DNA synthesis in Swiss 3T3

fibroblasts. The β-subunit induces a rapid increase in intracellular Ca^{2+} that is sensitive to the extracellular calcium concentration, which suggests that the increase does not arise from intracellular stores (Spiegel and Panagiotopoulos, 1988). The *c*AMP-dependent protein kinase and protein kinase C did not appear to be involved since there were no elevations in *c*AMP, adenylate cyclase activity, or inositol triphosphate, and the response occurred in cells pretreated with phorbol ester to down-regulate protein kinase C. A GTP-binding protein does appear to be involved, since pertussis toxin markedly inhibits this mitogenesis by the β-subunit (Spiegel, 1989a), but it does not appear to be one of the G proteins coupled to adenylate cyclase or phospholipase C. In contrast to these stimulatory effect, the β-subunit inhibited DNA synthesis in response to phorbol esters although it did not modify the ability of PMA to activate protein kinase C nor phorbol dibutyrate binding (Spiegel, 1989b); this was not affected by pertussis toxin. Inhibition by the β-subunit was maximal when added 2 h after PMA, which suggests that it affects a late step in mitogenesis, and underscores the complexity of the effects of agents that interact with gangliosides. As another example, monoclonal antibodies to G_{D3} and G_{D2} augment T cell responses to various stimuli without changes in intracellular Ca^{2+}, but with increased *c*GMP and protein kinase C (Hersey *et al.*, 1989).

Sphingosine as a Bioactive Product of Sphingolipid Turnover

Another provocative link between sphingolipids and signal transduction was uncovered by Bell and coworkers (Hannun *et al.*, 1986) in the discovery that sphingosine and other long-chain bases inhibit protein kinase C *in vitro* and cellular responses to protein kinase C activators in platelets (Hannun *et al.*, 1986), neutrophils (Wilson *et al.*, 1986), and HL-60 cells (Merrill *et al.*, 1986). Unlike most other inhibitors, which act as substrate analogs, sphingosine is competitive with diacylglycerol, phorbol dibutyrate (PDB), and Ca^{+2}, and sphingosine also blocks protein kinase C activation by unsaturated fatty acids and other lipids (Wilson *et al.*, 1986; Oishi *et al.*, 1988; El Touny *et al.*, 1990). Sphingosine is one of the more potent inhibitors of protein kinase C and acts on an equimolar basis with 1,2-dioleoylglycerol (Hannun *et al.*, 1986). The inhibition depends on the mole percentage of the sphingosine with respect to the other lipids or detergents, as typically occurs with molecules that partition into membranes.

The mechanism of protein kinase C inhibition is not known; however, since acidic lipids (e.g., phosphatidylserine) are required for maximal activity of protein kinase C, positively charged long-chain bases may localize in the same region of the membrane and block binding and/or activity (Hannun

et al., 1986; Bazzi and Nelsestuen, 1987; Rando, 1988; Merrill *et al.*, 1989). Sphingosine appears to interact with the membrane-binding domain of protein kinase C because it is not inhibitory for catalytic fragment when lipids are present (Nakadate *et al.*, 1988).

The inhibition is roughly the same with all of the major long-chain bases that are found naturally (Hannun *et al.*, 1986; Merrill *et al.*, 1989), including some lysosphingolipids (Hannun and Bell, 1987). Recent studies of other structure/function relationships (Merrill *et al.*, 1989) revealed that the most potent inhibitors have an alkyl chain length of 18 carbon atoms, which is the prevalent chain length *in vivo* (Karlsson, 1970). Subtle differences among the four stereoisomers of sphingosine, *N*-methyl derivatives, and simpler alkylamines (e.g., stearylamine) have been seen. Igarashi *et al.* (1989) have made basically similar observations; however, they reported that commercial sphingosine (Sigma) was more potent than synthetic D-*erythro*-sphingosine, presumably because the former contains a more effective inhibitor as a contaminant. We have not seen this distinction in side-by-side comparisons of synthetic sphingosine (Nimkar *et al.*, 1988) and sphingosine from Sigma; however, this might be due to variability in the amounts of contaminants in the commercial preparations.

One of the puzzling findings of both studies (Merrill *et al.*, 1989; Igarashi *et al.*, 1989) is that the inhibition of protein kinase C is not limited to the D-*erythro*-sphingosine, the major naturally occurring stereoisomer. This may mean that long-chain bases do not interact directly with protein kinase C; however, it is premature to draw this conclusion because all four stereoisomer provide the same headgroup conformation (see Fig. 8 in Merrill *et al.*, 1989) and vary only in the position of the alkyl chain. Protein kinase C may recognize mainly the headgroup of sphingosine, in analogy to its specificity for the headgroup of phosphatidylserine but not for the hydrophobic domain (e.g., both 1,2- and 1,3-diacyl compounds are effective as activators) (Lee and Bell, 1989).

It has been concluded that sphingosine inhibits protein kinase C in the protonated form (Hannun *et al.*, 1986; Hannun and Bell, 1989; Merrill *et al.*, 1989; Bottega *et al.*, 1989). Sphingosine has a low pK_a (i.e., ~ 7 in Triton X-100 or octyl-β-D-glucopyranoside micelles) due to intramolecular H-binding (Merrill *et al.*, 1989). Thus, long-chain bases are present in both neutral and ionized forms at physiological pH, which probably accounts for the rapidity of cellular uptake of sphingosine and its movement across the cell membrane to inhibit protein kinase C. A recent NMR study (Bottega *et al.*, 1989) has suggested that the pK_a of sphingosine is 8.5; however, the lower estimate agrees better with model compounds (Jencks and Regenstein, 1975; Merrill *et al.*, 1989) as well as the pH dependence of protein kinase C inhibition (Bottega *et al.*, 1989).

Cellular Effects of Sphingosine

Sphingosine is an inhibitor of protein kinase C in intact cells, as has been reviewed recently (Bell *et al.*, 1988; Hannun and Bell, 1989; Stevens and Merrill, 1989). It inhibits protein kinase C activity, protein phosphorylation, and cellular responses dependent on protein kinase C, blocks the Ca^{2+}-mediated translocation of phorbol ester binding sites from the supernatant to the pellet fraction. (Vaccarino *et al.*, 1987; Kolesnick and Clegg, 1988), and protects protein kinase C from proteolytic down-regulation (Grove and Mastro, 1988). The chemical properties of this amphipathic molecule are well suited for studies with cells because it dissolves readily in water-miscible solvents (such as DMSO and ethanol) and can be further stabilized by formation of a 1 : 1 molar complex with fatty acid-free bovine serum albumin which, if desired, can be dialyzed and retain the bound sphingosine. In addition, it is rapidly taken up by cells (Merrill *et al.*, 1986) and moves between membranes (Merrill and Wang, unpublished observations). The following sections present some of the findings that have resulted from studies with intact cells.

Platelet Aggregation and Other Functions. Sphingosine inhibits [^{3}H]PDB binding (Hannun *et al.*, 1986); thrombin, PMA, and diacylglycerol-dependent phosphorylation of the 40-kDa (47 kDa) protein (Hannun *et al.*, 1989); platelet secretion and second phase aggregation in response to ADP, γ-thrombin, collagen, arachidonic acid, and PAF, but not diacylglycerol generation in response to thrombin nor the first phase of aggregation and the initial shape change of platelets in response to these agonists (Hannun *et al.*, 1987). It also blocks release of α-granule contents upon assembly of the C5b-9 proteins of the complement system on the platelet surface (Wiedmer *et al.*, 1987), and the thrombin-induced rise in cytoplasmic pH, consistent with a role for protein kinase C in the regulation of the Na^+/H^+ exchanger (Sanchez *et al.*, 1988; Siffert and Akkerman, 1988).

Neutrophil Respiratory Burst and Other Functions. One of the most thoroughly studied systems using sphingosine is the human neutrophil (Lambeth, 1988). Sphingosine inhibits [^{3}H]PDB binding and the neutrophil respiratory burst in response to phorbol esters, diacylglycerol, formylmethionylleucylphenylalanine (fMLP), arachidonic acid, and opsonized zymosan (Wilson *et al.*, 1986). The effective concentration of sphingosine is the same for different agonists, consistent with a common site of action at protein kinase C, and structure/function studies reveal that the same compounds that block superoxide generation also inhibit protein kinase C *in vitro* (Wilson *et al.*, 1986, 1987; Merrill *et al.*, 1989). Under these conditions, sphingosine does not diminish cell viability, phagocytosis, or agonist-induced increases in diacylglycerol, inositol phosphate, and Ca^{2+} (Lambeth *et al.*, 1988). Sphingosine

inhibits phorbol ester-induced accumulation of alkylacylglycerols (Rider *et al.*, 1988); secretion of the specific granule component lactoferrin when PMA or fMLP is used as the agonist (in contrast to fMLP-induced secretion of lysozyme, a component of specific and azurophilic granules, which is only inhibited 50% by sphingosine) (Wilson *et al.*, 1987); the "priming" of the cells by nonactivating levels of agonists (Lambeth *et al.*, 1988); the synergistic activation of superoxide release by 5-hydroxy-6,8,11,14-eicosatetraenonate (5-HETE) (Badwey *et al.*, 1988); the synthesis of platelet-activating factor and leukotriene B_4 (LTB_4) in neutrophils stimulated with A23187 (McIntyre *et al.*, 1987); the incorporation of oleate, linoleate, and palmitate into phosphatidylcholine (although labeling of phosphatidylinositol and phosphatidic acid were increased) (Reinhold *et al.*, 1989); and PMA- and opsonized zymosan-induced PAF synthesis (Leyravaud *et al.*, 1989). These findings are entirely consistent with sphingosine acting at protein kinase C, although additional effects at sites after this enzyme have not been excluded. Sphingosine has other effects on the host defense system, such as suppression of normal and interleukin-2 activated NK killing of K562 cells (Chow *et al.*, 1988) and macrophage spreading (Petty, 1989).

Differentiation of Promyelocytic Leukemic Cells. As a model for long-term effects of sphingosine on cells, extensive studies have been conducted with HL-60 cells, which can be induced to differentiate into cells with characteristics of monocytes and macrophages upon treatment with PMA, 1α,25-dihydroxyvitamin D_3, and ganglioside G_{M3}, and into granulocytes by retinoic acid or DMSO. Long-chain bases inhibit [^{3}H]PDB binding, PMA-induced adherence and growth inhibition, and other markers for differentiation (Merrill *et al.*, 1986), especially when added daily to overcome removal of the long-chain base by metabolism (Stevens *et al.*, 1989). This effect has also been shown when the cells were treated with sphingomyelinase to release sphingosine (Kolesnick, 1989). In contrast, there was no inhibition of the differentiation of HL-60 cells to monocytes by 1α,25-dihydroxyvitan D_3 or ganglioside G_{M3} (Stevens *et al.*, 1989), nor the retinoic acid-induced differentiation to granulocytes (Stevens *et al.*, 1990b). Sphinganine increased some markers for monocyte differentiation, and when added with retinoic acid enhanced the percentage of differentiated cells and the respiratory burst. Okazaki *et al.* (1989) have found that 1α,25-dihydroxyvitamin D_3 induces sphingomyelin turnover in these cells; thus, changes in sphingolipid metabolism may be an integral part of differentiation.

Phorbol Ester- and Insulin-Induced Changes in Hexose Uptake and Metabolism. Nelson and Murray (1986) reported that long-chain bases inhibit PMA- and insulin-stimulated uptake of 2-deoxyglucose in 3T3-L1 cells, and suggested that sphingosine may contribute to the action of glucocorticoids (Nelson and Murray, 1988; Ramachandran *et al.*, 1990). Similar

results have been seen with isolated adipocytes (Cherqui *et al.*, 1989; Robertson *et al.*, 1989); the latter study found that the effectiveness of sphingosine depends on the amount of cellular lipid. Other responses to insulin are also inhibited (Civan *et al.*, 1988; Dawson and Cook, 1988; Smal and De Meyts, 1989).

Growth Inhibition and Cytotoxicity of Long-chain Bases. A common hindrance in such studies is that long-chain bases can be growth inhibitory and cytotoxic. For CHO cells, inhibition of protein kinase C appears to be involved based on the concentration dependency, structural specificity, protein phosphorylation patterns (especially when wild-type cells were compared to a cell line selected for partial resistance to the toxicity of long-chain bases), and the apparent *lack* of perturbation of cellular acidic compartments—the most likely site of an artifactual effect of these types of compounds (Merrill *et al.*, 1989; Stevens *et al.*, 1990a). It has been known for some time that even simpler long-chain bases such as stearylamine (Campbell, 1983), which have been used in preparing stable liposomes for drug delivery, can be cytotoxic. While it is difficult to define the specific molecular events of cell death, protein kinase C is thought to influence growth, ion transporters, and other systems that could cause this phenotype. Virus replication, as another type of "growth", is also affected by sphingosine. Nutter *et al.* (1987) have reported that sphingosine inhibits the induction of DNA polymerase and DNase activities in Epstein-Barr virus-infected cells treated with phorbol esters and *n*-butyrate.

Effects of Sphingosine on Tumorigenesis. As an inhibitor of protein kinase C, which is believed to participate in some aspects of tumorigenesis, long-chain bases have been suggested to be potential antitumor agents (Hannun *et al.*, 1986). Gupta *et al.* (1988) and Enkvetchakul *et al.* (1989) have observed that sphingosine blocks the induction of ornithine decarboxylase by phorbol esters in mouse skin, one biochemical marker of tumor promotion. However, follow-up experiments (Birt *et al.*, unpublished) have not indicated that sphingosine reduces the number of tumors in mice, although these findings may be a consequence of the doses that have been tested thus far. Other studies (Borek and Merrill, unpublished observations) have found that long-chain bases reduce C3H10T1/2 cell transformation in response to gamma irradiation and PMA. Until more is known about these compounds, it would be prudent to handle them cautiously.

Sphingosine Affects Many Systems in Addition to Protein Kinase C

Sphingosine is not exclusively an inhibitor of protein kinase C even though it is selective (i.e., it does not appear to inhibit the *c*AMP-dependent kinase or myosin light chain kinase) (Hannun and Bell, 1989; Merrill and Stevens, 1989). The first reports of additional effects were made with the EGF

receptor kinase by Davis and coworkers (Faucher *et al.*, 1988; Davis *et al.*, 1988; Northwood and Davis, 1988), who observed that all of the cellular effects of sphingosine were not reversed by PMA, and there were inconsistencies in the time course and stoichiometry of phosphorylation. Sphingosine appears to stimulate a protein kinase C-independent pathway of protein phosphorylation and increase the affinity and number of cell surface EGF receptors. Wedegaertner and Gill (1989) have also shown that 5 μM sphingosine increases the activity of the cytoplasmic tyrosine kinase domain of the EGF receptor to equal or greater than that of the ligand-activated holo EGF receptor, and have suggested that sphingosine may mimic the effect of EGF to induce a conformation that is optimal for tyrosine kinase activity. As already mentioned, long-chain bases are mitogenic at low concentrations, and Zhang *et al.* (1990) have shown that these are below the levels where competition for phorbol dibutyrate binding occurs. Sphingosine stimulates the hydrolysis of phosphatidylethanolamine (Kiss and Anderson, 1990); it also inhibits purified diacylglycerol kinases (Sakane *et al.*, 1989) and, thus, has the potential to interfere with a major mechanism for removing an activator of protein kinase C.

Sphingosine inhibits the Na,K-ATPase with approximately the same dose response as for protein kinase C inhibition (Oishi *et al.*, 1990). Long-chain bases have also been reported to affect several ion transport systems that are thought to be regulated by protein kinase C (Connor *et al.*, 1988; Soliven *et al.*, 1988; Conn *et al.*, 1989; Gillies *et al.*, 1989; Ling and Eaton, 1989) as well as ones without an evident link to protein kinase C (Ghosh *et al.*, 1990). Sphingosine inhibits coagulation initiated by lipopolysaccharide-stimulated human monocytes by inhibiting Factor VII binding (Conkling *et al.*, 1989). It inhibits thyrotropin-releasing hormone binding to GH_3 cells (Winicov and Gershengorn, 1988), but the IC_{50} is fairly high (i.e., 63 μM compared to a typical IC_{50} of 1 to 3 μM for protein kinase C in a comparable number of cells) and exhibits an atypical difference in potency for sphinganine versus sphingosine (Merrill *et al.*, 1989). Sphingosine inhibits a calmodulin-dependent kinase (Jefferson and Schulman, 1988); however, the cellular effects were only reported with very high concentrations of sphingosine (suggesting that they, too, may be less potent than for protein kinase C); and it has been reported to inhibit c-src and v-src kinases, but at concentrations of 330 and 660 μM (Igarashi *et al.*, 1989). At high concentrations or in the absence of membranes, long-chain bases probably have nonspecific effects due simply to their charge and/or amphipathic nature.

Information Regarding Sphingosine as Another "Lipid Second Messenger"

To be regarded a second messenger, sphingosine must: (1) have biological effects (which it does, as described above), (2) be present in cells and change in

response to stimuli, for which evidence will be summarized here, and (3) cause cell responses when the levels of endogenous sphingosine are altered by stimuli, antagonists, etc. This latter criterion has only been met partially to date.

All cells appear to have at least small amounts of free sphingosine based on recent findings with HL-60 cells (Merrill *et al.*, 1986), neutrophils (Wilson *et al.*, 1988), rat liver and brain (Merrill *et al.*, 1988), mouse tissues (Kobayashi *et al.*, 1988), skin (Wertz and Downing, 1989), and various cultured cells (Van Veldhoven *et al.*, 1989). Lysosphingolipids are also found in certain cells (Hannun and Bell, 1987; Kobayashi *et al.*, 1988). The amount of free sphingosine increases during incubation of human neutrophils and is affected by different factors (Wilson *et al.*, 1988). Activators of protein kinase C were generally found to blunt sphingosine formation, whereas serum (and particularly lipoproteins) increased the amount. The increase was caused by turnover of complex sphingolipids rather than sphingosine synthesis *de novo*; decreases appear to be due to conversion of sphingosine to ceramides. These results suggest that cells contain endogenous sphingosine and a mechanism to increase and decrease the level in response to various factors.

It is difficult to estimate whether or not these levels of sphingosine are high enough to affect protein kinase C, the EGF-receptor kinase, or any of the other systems discussed above. The total cellular concentrations have been found to be in the micromolar range for all of the cells and tissues analyzed; thus, if most is localized in the plasma membrane, the amounts could approach the mole percentage necessary for protein kinase C inhibition (Hannun *et al.*, 1986). One may also compare the cellular amounts versus the levels taken up by cells under conditions where exogenous sphingosine inhibits protein kinase C. On this basis, the endogenous sphingosine of neutrophils is within tenfold of the levels that block protein kinase C and the respiratory burst when added exogenously (Wilson *et al.*, 1986, 1988).

Recent studies (Slife *et al.*, 1989) with rat liver have found that a major portion of the free sphingosine is associated with the plasma membrane, and these membranes turn over sphingomyelin (and perhaps some other complex sphingolipids) using an enzyme system that requires Mg^{2+} and has a neutral to alkaline pH optimum. These characteristics are similar to the neutral sphingomyelinase and ceramidase activities known to be present in plasma membranes (Rao and Spence, 1976; Hostetler and Yazaki, 1979). Therefore, plasma membranes contain at least one enzyme system capable of releasing sphingosine by the turnover of endogenous complex sphingolipids.

To assess if endogenous sphingosine could inhibit protein kinase C, Kolesnick and coworkers have treated cells with exogenous sphingomyelinase. This reduces PMA-induced translocation of protein kinase C in GH_3 pituitary cells and, when added after PMA, restores the cytosolic activity (Kolesnick and Clegg, 1988). Sphingomyelinase also inhibits HL-60 cell

differentiation (Kolesnick, 1989), mimicking the effect of free long-chain bases (Merrill *et al.*, 1986). Thus, endogenous sphingosine has the potential of altering cell behavior. Future work will be needed to establish which, if any, of the "natural" agents known to alter sphingolipid metabolism (e.g., glucocorticoids, growth and differentiation factors, etc.), change the amounts of free sphingosine in ways that affect protein kinase C or any of the other systems that are potently affected by this compound.

Models for Sphingolipids as Pleotrophic Regulators of Cell Function

One can envision many ways in which sphingolipid synthesis and turnover could help regulate cell functions. Some have been depicted in Fig. 3, using sphingomyelin and an unspecified ganglioside, which might be

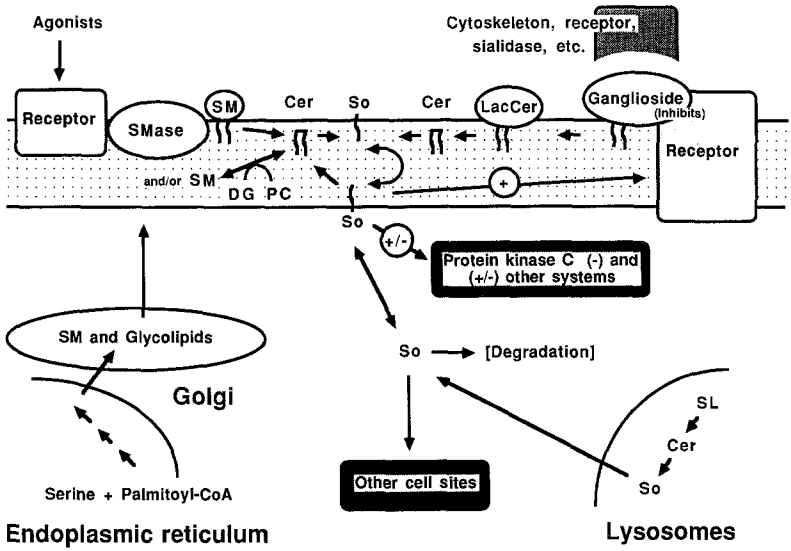

Fig. 3. A model for some of the possible interrelationships among sphingolipids, their turnover products, and signal transduction. The scheme illustrates the approximate localization of sphingolipid biosynthesis (by the endoplasmic reticulum, Golgi, and plasma membranes), catabolism (by lysosomes), and turnover at the plasma membrane, with the representative sphingolipids (SL): sphingomyelin (SM), ceramide (Cer), sphingosine (So), lactosylceramide (LacCer), and an unspecified ganglioside. The scheme illustrates the multiplicity of responses that might accrue from relatively simple metabolic reactions, as well as the possibility of several routes of formation and targets of action for a central compound such as sphingosine. Sphingolipids may be involved in "cycles" of metabolism, as illustrated for the resynthesis of ceramide and sphingomyelin. It has not been possible to include all of the known bioactive sphingolipids (such as other headgroup modifications of gangliosides, various lysosphinglipids, etc.) and signal transduction pathways; for a more detailed description, consult the text.

ganglioside G_{M3} or one of the hundreds of other glycolipids of mammalian cells. An attractive feature of sphingolipids is that unique compounds might be associated with specific receptors and their targets, but they could also be turned over to produce products that act on a large number of additional sites. Thus, sphingolipids could act:

At the surface of the cell, where sphingolipids bind to extracellular receptors (such as bacterial toxins, receptors and lectins from neighboring cells, antibodies, etc.) and to cellular receptors, acting as allosteric modulators of the activity (or affinity for its ligand). This has been exemplified by the interaction between ganglioside G_{M3} and EGF receptor (Bremer *et al.*, 1986; Hanai *et al.*, 1988a,b). Some sphingolipids might serve mainly to define regions of the membrane with particular surface characteristics and thereby aid in receptor binding, responses, internalization, recycling, etc.

The action of a sphingolipid at the cell surface could be altered by metabolism. For example, the extracellular sialidase that cleaves G_{M3} to lactosylceramide will thereby remove an inhibitor of the EGF receptor (Usuki *et al.* (1988a,b). In addition, the neutral sphingomyelinase (which is located on the outer leaflet of the plasma membrane) can be activated by a variety of agents. This activity has most recently been shown to respond to 1α,25-dihydroxyvitamin D_3 in ways that implicate sphingomyelin turnover in HL-60 cell differentiation (Okazaki *et al.*, 1989). In this instance, it appears that ceramide per se may be the active mediator, rather than sphingosine (Okazaki *et al.*, 1990). The interconversion of sphingomyelin and ceramide might also be coupled to phosphatidylcholine and diacylglycerol metabolism (Hampton and Morand, 1989).

In the plasma membrane and/or endosomes, where complex sphingolipids and products of their turnover could stimulate or inhibit other domains of the receptor kinases or the response of G proteins, other kinases, ion transporters, etc. For example, the hydrolysis of sphingolipids to free sphingosine releases an activator of the EGF receptor kinase, thereby producing the opposite effect of intact gangliosides. This would provide a powerful switching mechanism if the degradation product was both an antagonist of effects of the intact sphingolipid and was formed by turnover of the same compound.

Some sphingolipid hydrolysis products could affect both the external and internal leaflets of the plasma membrane. Ceramides are relatively nonpolar and presumably cross membranes readily, and sphingosine has been shown to undergo rapid movement across membranes in the neutral form (Hope and Cullis, 1987). This might be one explanation for the unusual structure of sphingosine (i.e., with hydroxyls flanking the amino group at position 2) since this gives it a pK_a near physiological pH (Merrill *et al.*, 1989).

Because the products of sphingolipid turnover affect more than one target (e.g., sphingosine affects protein kinase C, the EFG receptor kinase,

etc.), this might provide a mechanism for a pleotrophic response to a single signal. Sphingosine may facilitate activation of Ca^{2+}/calmodulin-dependent kinases without activation of protein kinase C. For such effects, the amount of sphingosine may not need to change rapidly. Basal levels could act as a set point to limit the activation of protein kinase C, and this may provide a means to reduce "accidental" activation when cells are exposed to diacylglycerols, fatty acids, etc.

At intracellular sites that are sensitive to products of sphingolipid turnover, or at the plasma membrane but via liberation of sphingosine in lysosomes. Some sphingolipids (e.g., sphingosine) are able to move rapidly among membranes and could affect targets in other cellular compartments. For example, sphingosine might be liberated in the plasma membrane but affect protein kinase C in the nucleus. Sphingosine might also be released at intracellular sites (e.g., during lysosomal catabolism of sphingolipids) and subsequently interact with targets in other locations in the cell. Since many cells internalize a large portion of their surface, this might serve as an internal signal of endocytosis. The stimulation of sphingomyelin turnover by diacylglycerols involves activation of an acidic sphingomyelinase (Kolesnick, 1987); thus, it might occur in lysosomes.

The scheme has not represented the intermediates of sphingolipid biosynthesis as participants in signal transduction (because they are trace constituents and sphingosine does not appear to be a direct intermediate of the *de novo* pathway); however, this potential source should not be forgotten.

Summary

In the strictest sense, no sphingolipid has been proven to be a critical participant in signal transduction. However, the nature of these compounds and the cell behaviors with which they are associated make them promising candidates for this function. The relatively recent findings of many laboratories that sphingolipids interact with cell receptors, protein kinases, and other key sites have provided a sensible mechanism for how they may effect cell regulation. This paradigm strengthens the related hypotheses that the regulation of sphingolipid metabolism will be an important element of their function, and that sphingolipid metabolites serve as another class of lipid second messengers.

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References

Badwey, J. A., Robinson, J. M., Horn, W., Soberman, R. J., Karnovsky, M. J., and Karnovsky, M. L. (1988). *J. Biol. Chem.* **263**, 2779–2786.
Bajjalieh, S. H., Martin, T. F. J., and Floow, E. (1989). *J. Biol. Chem.* **264**, 14354–14360.
Barenholz, Y., and Gatt, S. (1982). In *Phospholipids* (Hawthorne, J. N., and Ansell, G. B., eds.), Elsevier Biomedical Press, Amsterdam, Ch. 4.
Barenholz, Y., and Thompson, T. E. (1980). *Biochim. Biophys. Acta* **604**, 129–158.
Bazzi, M. D., and Nelsestuen, G. L. (1987). *Biochem. Biophys. Res. Commun.* **146**, 203–207.
Bell, R. M., Loomis, C. R., and Hannun, Y. A. (1988). *Cold Spring Harb. Symp. Quant. Biol.* **53**, 103–110.
Bottega, R., Epand, R. M., and Ball, E. H. (1989). *Biochem. Biophys. Res. Commun.* **164**, 102–107.
Bremer, E. G., and Hakomori, S. I. (1982). *Biochem. Biophys. Res. Commun.* **106**, 711–718.
Bremer, E. G., Hakomori, S.-I., Bowen-Pope, D. F., Raines, E., and Ross, R. (1984). *J. Biol. Chem.* **259**, 6818–6825.
Bremer, E. G., Schlessinger, J., and Hakomori, S.-I. (1986). *J. Biol. Chem.* **261**, 2434–2440.
Burczak, J. D., Moskal, J. R., Trosko, J. E., Fairley, J. L., and Sweeley, C. C. (1983). *Exp. Cell Res.* **147**, 281–286.
Campbell, P. I. (1983). *Cytobios.* **37**, 21–26.
Chan, K.-F. J. (1987). *J. Biol. Chem.* **262**, 5248–5255.
Chan, K.-F. J. (1989). *J. Biol. Chem.* **264**, 18632–18637.
Chapman, M. J. (1986). *Methods Enzymol.* **128**, 70–143.
Cherqui, G., Caron, M., Wicek, D., Capeau, J., and Picard, J. (1989). *Mol. Cell Endocrinol.* **65**, 13–25.
Chow, S. C., Ng, J., Nordstedt, C., Fredholm, B. B., and Jondal, M. (1988). *Cell. Immunol.* **114**, 96–103.
Cimino, M., Benfenati, F., Farabegoli, C., Cattabeni, F., Fuxe, K., Agnati, L. F., and Toffano, G. (1987). *Acta Physiol. Scand.* **130**, 317–325.
Civan, M. M., Peterson-Yantorno, K., and O'Brien, T. G. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 963–967.
Conkling, P. R., Patton, K. L., Hannun, Y. A., Greenberg, C. S., and Weinberg, J. B. (1989). *J. Biol. Chem.* **264**, 18440–18444.
Conn, P. J., Strong, J. A., and Kaczmarek, L. K. (1989). *J. Neurosci.* **9**, 480–487.
Connor, J. A., Wadman, W. J., Hockberger, P. E., and Wong, R. K. S. (1988). *Science* **240**, 649–653.
Das, D. V. M., Cook, H. W., and Spence, M. W. (1984). *Biochim. Biophys. Acta* **777**, 339–342.
Davis, R. J., Gironës, N., and Faucher, M. (1988). *J. Biol. Chem.* **263**, 5373–5379.
Dawson, W. D., and Cook, J. S. (1988). *Prog. Clin. Biol. Res.* **258**, 121–133.
Dudeja, P. K., Dahiya, R., Brown, M. D., and Brasitus, T. A. (1988). *Biochem. J.* **253**, 401–408.
El Touny, S., Khan, W., and Hannun, Y. (1990). *J. Biol. Chem.* **265**, 16437–16443.
Enkvetchakul, B., Merrill, A. H., Jr., and Birt, D. F. (1989). *Carcinogenesis* **10**, 379–381.
Faucher, M., Girones, N., Hannun, Y. A., Bell, R. M., and Davis, R. J. (1988). *J. Biol. Chem.* **263**, 5319–5327.
Feizi, T., and Childs, R. A. (1985). *Trends Biochem. Sci.* **10**, 24–29.
Ferrari, G., Fabris, M., and Gorio, A. (1983). *Dev. Brain Res.* **8**, 215–221.
Fishman, P. H. (1982). *J. Membr. Biol.* **69**, 85–97.
Fishman, P. H., and Brady, R. O. (1976). *Science* **194**, 906–915.
Ghosh, T. K., Bian, J., and Gill, D. L. (1990). *Science* **248**, 1653–1656.

Gillies, R. J., Martinez, R., Sneider, J. M., and Hoyer, P. B. (1989). *J. Cell. Physiol.* **139**, 125–130.
Goldenring, J. R., Otis, L. C., Yu, R. K., DeLorenzo, R. J. (1985). *J. Neurochem.* **44**, 1129–1134.
Grove, D. S., and Mastro, A. M. (1988). *Biochem. Biophys. Res. Commun.* **151**, 94–99.
Gupta, A. K., Fischer, G. J., Elder, J. T., Nickoloff, B. J., and Voorhees, J. J. (1988). *J. Invest. Dermatol.* **91**, 486–491.
Hakomori, S.-I. (1981). *Annu. Rev. Biochem.* **50**, 733–764.
Hakomori, S.-I. (1984). *Trends Biochem. Sci.* **9**, 453–455.
Hakomori, S.-I. (1990). *J. Biol. Chem.* **265**, 18713–18716.
Hampton, R. Y., and Morand, O. H. (1989). *Science* **246**, 1050.
Hanai, N., Nores, G. A., Torres-Mendez, C.-R., and Hakomori, S.-I. (1987). *Biochem. Biophys. Res. Commun.* **147**, 127–134.
Hanai, N., Dohi, T., Nores, G. A., and Hakomori, S.-I. (1988a). *J. Biol. Chem.* **263**, 6296–6301.
Hanai, N., Nores, G. A., MacLeod, C., Torres-Mendez, C.-R., and Hakomori, S.-I. (1988b). *J. Biol. Chem.* **263**, 10915–10921.
Hannun, Y. A., and Bell, R. M. (1987). *Science* **235**, 670–674.
Hannun, Y. A., and Bell, R. M. (1989). *Science* **243**, 500–507.
Hannun, Y., Loomis, C. R., Merrill, A., and Bell, R. M. (1986). *J. Biol. Chem.* **261**, 12604–12609.
Hannun, Y. A., Greenberg, C. S., and Bell, R. M. (1987). *J. Biol. Chem.* **262**, 13620–13626.
Hersey, P., Schibeci, S., and Cheresh, D. (1989). *Cell. Immunol.* **119**, 263–278.
Hope, M. J., and Cullis, P. R. (1987). *J. Biol. Chem.* **262**, 4360–4366.
Hostetler, K. Y., and Yazaki, P. J. (1979). *J. Lipid Res.* **20**, 456–463.
Igarashi, Y., and Hakomori, S.-I. (1989). *Biochem. Biophys. Res. Commun.* **164**, 1411–1414.
Igarashi, Y., Hakomori, S.-I., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., and Racker, E. (1989). *Biochemistry* **28**, 6796–6800.
Inokuchi, J.-I., Mason, I., and Radin, N. S. (1987). *Cancer Lett.* **38**, 23–30.
Jefferson, A. B., and Schulman, H. (1988). *J. Biol. Chem.* **263**, 15241–15244.
Jencks, W. P., and Regenstein, J. (1975). In *Handbook of Biochemistry and Molecular Biology, 3rd Edition, Physical and Chemical Data. Vol. I* (Fasman, G. D., ed.), CRC Press, Cleveland, Ohio, pp. 305–351.
Kanfer, J. N., and Hakomori, S., eds. (1983). *Sphingolipid Biochemistry*, Plenum Press, New York.
Karlsson, K.-A. (1970). *Chem. Phys. Lipids* **5**, 6–43.
Keenan, T. W., Schmid, E., Franke, W. W., and Wiegandt, H. (1975). *Exp. Cell Res.* **92**, 259–270.
Kim, J. Y. H., Goldenring, J. R., DeLorenzo, R. J., and Yu, R. K. (1986). *J. Neurosci. Res.* **15**, 159–166.
Kishimoto, Y. (1983). In *The Enzymes* (Boyer, P. D., ed.), Academic Press, New York, Vol. 16, ch. 10.
Kiss, Z., and Anderson, W. B. (1990). *J. Biol. Chem.* **265**, 7345.
Kobayashi, T., Goto, I., Yamanaka, T., Suzuki, Y., Nakano, T., and Suzuki, K. (1988a). *Ann. Neurol.* **24**, 517–522.
Kobayashi, T., Mitsuo, K., and Goto, I. (1988b). *Eur. J. Biochem.* **171**, 747–752.
Kok, J. W., Eskelinen, S., Hoekstra, K., and Hoekstra, D. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 9896–9900.
Kolesnick, R. N. (1987). *J. Biol. Chem.* **262**, 16759–16762.
Kolesnick, R. N. (1989). *J. Biol. Chem.* **264**, 7617–7623.
Kolesnick, R. N., and Clegg, S. (1988). *J. Biol. Chem.* **263**, 6534–6537.
Kreutter, D., Kim, J. Y. H., Goldenring, J. R., Rasmussen, H., Ukomandu, C., DeLorenzo, R. J., and Yu, R. K. (1987). *J. Biol. Chem.* **262**, 1633–1637.
Laine, R. A., and Hakomori, S.-I. (1973). *Biochem. Biophys. Res. Commun.* **54**, 1039–1045.
Lambeth, J. D. (1988). *J. Bioenerg. Biomembr.* **20**, 709–733.
Lambeth, J. D., Burnham, D., and Tyagi, S. R. (1988). *J. Biol. Chem.* **263**, 3818–3822.
Ledeen, R. W. (1984). *J. Neurosci. Res.* **12**, 147–159.
Lee, M.-H., and Bell, R. M. (1989). *J. Biol. Chem.* **264**, 14797–14805.

Leyravaud, S., Bossant, M.-J., Joly, F., Bessou, G., Beneviste, J., and Ninio, E. (1989). *J. Immunol.* **143**, 245–249.
Ling, B. N., and Eaton, D. C. (1989). *Am. J. Physiol.* **256**, F1094–F1103.
Mathews, C. K., and van Holde, K. E. (1990). *Biochemistry*, Benjamin/Cummings, New York, pp. 617–621.
Matyas, G. R., Aaronson, S. A., Brady, R. O., and Fishman, P. H. (1987). *Proc. Natl. Acad. Sci. (USA)* **84**, 6065–6068.
McIntyre, T. M., Reinhold, S. L., Prescott, S. M., and Zimmerman, G. A. (1987). *J. Biol. Chem.* **262**, 15370–15376.
Merrill, A. H., Jr. (1983). *Biochim. Biophys. Acta* **754**, 284–291.
Merrill, A. H., Jr., and Jones, D. D. (1990). *Biochim. Biophys. Acta* **1044**, 1–12.
Merrill, A. H., Jr., Sereni, A. M., Stevens, V. L., Hannun, Y. A., Bell, R. M., and Kinkade, J. M., Jr. (1986). *J. Biol. Chem.* **261**, 12610–12615.
Merrill, A. H., Jr., Wang, E., Mullins, R. E., Jamison, W. C. L., Nimkar, S., and Liotta, D. C. (1988). *Anal. Biochem.* **171**, 373–381.
Merrill, A. H., Jr., Nimkar, S., Menaldino, D., Hannun, Y. A., Loomis, C., Bell, R. M., Tyagi, S. R., Lambeth, J. D., Stevens, V. L., Hunter, R., and Liotta, D. C. (1989). *Biochemistry* **28**, 3138–3145.
Merrill, A. H., Jr., and Stevens, V. L. (1989). *Biochim. Biophys. Acta.* **1010**, 131–136.
Miller-Podraza, H., Bradley, R. M., and Fishman, P. H. (1982). *Biochemistry* **21**, 3260–3265.
Momoi, T., Shinmoto, M., Kasuya, J., Senoo, H., and Suzuki, Y. (1986). *J. Biol. Chem.* **261**, 16270–16273.
Moskal, J. R., Lockney, M. W., Marvel, C. C., Trosko, J. E., and Sweeley, C. C. (1987). *Cancer Res.* **47**, 787–790.
Nakadate, T., Jeng, A. Y., and Blumberg, P. M. (1988). *Biochem. Pharmacol.* **37**, 1541–1545.
Nelson, D. H., and Murray, D. K. (1986). *Biochem. Biophys. Res. Commun.* **138**, 463–467.
Nelson, D. H., and Murray, D. K. (1988). *Endocr. Res.* **14**, 305–318. Nimkar, S., Manaldino, D., Merrill, A. H., and Liotta, D. (1988). *Tetrahedron Lett.* **29**, 3037–3040.
Nimkar, S., Menaldino, D., Merrill, A. H., Jr., and Liotta, D. C. (1988). *Tetrahedron Lett.* **29**, 3037–3040.
Northwood, I. C., and Davis, R. J. (1988). *J. Biol. Chem.* **263**, 7450–7453.
Nutter, L. M., Grill, S. P., Li, J. S., Tan, R. S., and Cheng, Y. C. (1987). *Cancer Res.* **47**, 4407–4412.
Oishi, K., Raynor, R. L., Charp, P. A., and Kuo, J. F. (1988). *J. Biol. Chem.* **263**, 6865–6871.
Oishi, K., Zheng, B., and Kuo, J. F. (1990). *J. Biol. Chem.* **265**, 70–75.
Okazaki, T., Bell, R. M., and Hannun, Y. A. (1989). *J. Biol. Chem.* **264**, 19076–19080.
Okazaki, T., Bielawska, A., Bell, R. M., and Hannun, Y. A. (1990). *J. Biol. Chem.* **265**, 15823–15831.
Ong, D. E., and Brady, R. N. (1973). *J. Biol. Chem.* **248**, 3884–3888.
Petty, H. R. (1989). *Biochim. Biophys. Acta* **1012**, 284–290.
Prokazova, N. V., Orekhov, A. N., Mukhin, D. N., Mikhailenko, I. A., Logtev, L. S., Sadovskaya, V. L., Golovanova, N. K., and Bergelson, L. D. (1987). *Eur. J. Biochem.* **167**, 349–352.
Ramachandran, C. K., Murray, D. K., and Nelson, D. H. (1990). *Biochem. Biophys. Res. Comm.* **167**, 607–613.
Rando, R. R. (1988). *FASEB J.* **2**, 2348–2355.
Rao, B. G., and Spence, M. W. (1976). *J. Lipid Res.* **17**, 506–515.
Reinhold, S. L., Zimmerman, G. A., Prescott, S. M., and McIntrye, T. M. (1989). *J. Biol. Chem.* **264**, 21652–21659.
Rider, L. G., Dougherty, R. W., and Niedel, J. E. (1988). *J. Immunol.* **140**, 200–207.
Robertson, D. G., DiGirolamo, M., Merrill, A. H., Jr., and Lambeth, J. D. (1989). *J. Biol. Chem.* **264**, 6773–6779.
Roisen, F. J., Bartfeld, H., Nagele, R., and Yorke, G. (1981). *Science* **214**, 577–578.
Saito, M., Terui, Y., and Nojiri, H. (1985). *Biochem. Biophys. Res. Commun.* **132**, 223–231.
Sakane, F., Yamada, K., and Kanoh, H. (1989). *FEBS Let.* **255**, 409–413.

Sanchez, A., Alonso, M. T., and Collazos, J. M. (1988). *Biochim. Biophys. Acta* **938**, 497–500.
Siffert, W., and Akkerman, J. W. N. (1988). *J. Biol. Chem.* **263**, 4223–4227.
Slife, C. W., Wang, E., Hunter, R., Wang, S., Burgess, C., Liotta, D. C., and Merrill, A. H., Jr. (1989). *J. Biol. Chem.* **264**, 10371–10377.
Smal, J., and De Meyts, P. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 4705–4709.
Soliven, B., Szuchet, S., Arnason, B. G. W., and Nelson, D. J. (1988). *J. Membr. Biol.* **105**, 177–186.
Sonderfeld, S., Conzelmann, E., Schwarzmann, G., Burg, J., Hinrichs, U., and Sandoff, K. (1985). *Eur. J. Biochem.* **149**, 247–255.
Spence, M. W., Reed, S., and Cook, H. W. (1986). *Biochem. Cell Biol.* **64**, 400–404.
Spiegel, S. (1989a). *J. Biol. Chem.* **264**, 6766–6772.
Spiegel, S. (1989b). *J. Biol. Chem.* **264**, 16512–16517.
Spiegel, S., and Fishman, P. H. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 141–145.
Spiegel, S., and Panagiotopoulos, C. (1988). *Exp. Cell Res.* **177**, 414–427.
Spiegel, S., Fishman, P. H., and Weber, R. J. (1985). *Science* **230**, 1285–1287.
Srinivas, L., Gindhart, T. D., and Colburn, N. H. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 4988–4991.
Stevens, V. L., Winton, E. F., Smith, E. E., Owens, N. E., Kinkade, J. M., Jr., and Merrill, A. H., Jr. (1989). *Cancer Res.* **49**, 3229–3234.
Stevens, V. L., Nimkar, S., Jamison, W. C., Liotta, D. C., and Merrill, A. H., Jr. (1990a). *Biochim. Biophys. Acta* **1051**, 37–45.
Stevens, V. L., Owens, N. E., Winton, E. F., Kinkade, J. M., Jr., and Merrill, A. H., Jr. (1990b). *Cancer Res.* **50**, 2222–2226.
Stoffel, W., and Grol, M. (1974). *Chem. Phys. Lipids* **13**, 373–388.
Sweeley, C. C., ed. (1980). *Cell Surface Glycolipids*, American Chemical Society, Washington, D.C.
Sweeley, C. C. (1985). In *Biochemistry of Lipids and Membranes* (Vance, D. E., and Vance, J. E., eds.), Benjamin/Cummings, Menlow Park, California, pp. 361–403.
Trinchera, M., Wiesmann, U., Pitto, M., Acquotti, D., and Ghidoni, R. (1988). *Biochem. J.* **252**, 375–379.
Tsuji, S., Nakajima, U., Sasaki, T., and Nagai, Y. (1985). *J. Biochem.* **97**, 969–972.
Tsuji, S., Yamashita, T., Tanaka, M., and Nagai, Y. (1988a). *J. Neurochem.* **50**, 414–423.
Tsuji, S., Yamashita, T., and Nagai, Y. (1988b). *J. Biochem.* **104**, 498–503.
Unsicker, K., and Wiegandt, H. (1988). *Exp. Cell Res.* **178**, 377–389.
Usuki, S., Hoops, P., and Sweeley, C. C. (1988a). *J. Biol. Chem.* **263**, 10595–10599.
Usuki, S., Lyu, S.-C., and Sweeley, C. C. (1988b). *J. Biol. Chem.* **263**, 6847–6853.
Vance, D. E. (1988). In *Biochemistry* (Zubay, Z., ed.), Macmillan, New York, Chapter 22.
Van Veldhoven, P. P., Bishop, W. R., and Bell, R. M. (1989). *Anal. Biochem.* **183**, 177–189.
Voet, D., and Voet, J. G. (1990). *Biochemistry*, Wiley, New York, pp. 669–674.
Wang, E., and Merrill, A. H., Jr. (1986). *J. Biol. Chem.* **261**, 3674–3769.
Wedegaertner, P. B., and Gill, G. N. (1989). *J. Biol. Chem.* **264**, 11346–11353.
Wertz, P. W., and Downing, D. T. (1989). *Biochim. Biophys. Acta* **1002**, 213–217.
Wiedmer, T., Ando, B., and Sims, P. J. (1987). *J. Biol. Chem.* **262**, 13674–13681.
Wilson, E., Olcott, M. C., Bell, R. M., Merrill, A. H., Jr., and Lambeth, J. D. (1986). *J. Biol. Chem.* **261**, 12616–12623.
Wilson, E., Rice, W. G., Kinkade, R. M., Jr., Merrill, A. H., Jr., Arnold, R. R., and Lambeth, J. D. (1987). *Arch. Biochem. Biophys.* **259**, 204–214.
Wilson, E., Wang, E., Mullins, R. E., Liotta, D. C., Lambeth, J. D., and Merrill, A. H., Jr. (1988). *J. Biol. Chem.* **263**, 9304–9309.
Winicov, I., and Gershengorn, M. C. (1988). *J. Biol. Chem.* **263**, 12179–12182.
Ziesel, S. H., Char, D., and Sheard, N. F. (1986). *J. Nutr.* **116**, 50–58.
Zhang, H., Buckley, N. E., Gibson, K., and Spiegel, S. (1990). *J. Biol. Chem.* **265**, 76–81.