Sphingosine Inhibits Rat Hepatic Monoacylglycerol Acyltransferase in Triton X-100 Mixed Micelles and Isolated Hepatocytes†

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ABSTRACT: Hepatic monoacylglycerol acyltransferase (MGAT), a developmentally-regulated microsomal activity that catalyzes the synthesis of *sn*-1,2-diacylglycerol, is regulated by anionic phospholipids and *sn*-1,2-diacylglycerol in Triton X-100 mixed micelles. Sphingomyelin stimulated MGAT activity, whereas sphingosine, sphinganine, phytosphingosine, and stearylamine were inhibitors (IC_{50} of 9, 5.5, 5, and 6 mol %, respectively). Since ceramide and octylamine had relatively little effect, inhibition appears to require a free amino group and a long-chain hydrocarbon. Inhibition by sphingosine was competitive with respect to phosphatidic acid, phosphatidylinositol, or phosphatidylserine, suggesting that anionic phospholipids may activate MGAT at a specific site that is competitively blocked by sphingolipids. Both sphingosine and sphinganine inhibited MGAT activity in cultured hepatocytes from 10-day-old rats in a dose-dependent manner. Stimulation of MGAT activity by diacylglycerol was specific for *sn*-1,2-stereoisomers that contained two long fatty acyl chains. The diacylglycerol analogs phorbol 12-myristyl 13-acetate and ceramide had no effect. The highly cooperative activation of MGAT by *sn*-1,2-diacylglycerol was also inhibited by sphingosine. It is unlikely that activation of MGAT by low molar concentrations of anionic phospholipids is solely due to electrostatic interactions between the enzyme and negatively charged lipids because high ionic strength, neomycin, and Ca^{2+} had similar effects on enzyme activity irrespective of the presence or absence of phosphatidic acid. These data suggest that MGAT activity may be regulated physiologically by specific intermediates of glycerolipid metabolism and that, in neonatal rat liver, signal transduction may be linked to the synthesis of complex lipids via the monoacylglycerol pathway.

Monoacylglycerol acyltransferase (MGAT)[1] (EC 2.3.1.22) is a microsomal enzyme that catalyzes the stereospecific synthesis of *sn*-1,2-diacylglycerol from *sn*-2-monoacylglycerol and long-chain fatty acyl-CoAs (Johnston, 1977; Coleman et al., 1986). In rat liver, the specific activity of MGAT is as much as 700-fold higher during the neonatal period than in the adult (Coleman & Haynes, 1984). Although the physiological significance of this prominent monoacylglycerol pathway for glycerolipid synthesis in liver is not certain, we have provided conceptual and experimental evidence that MGAT aids in retaining essential fatty acids during physiological and pathological periods that are characterized by high rates of lipolysis (Xia et al., 1993; Mostafa et al., 1993; Emmison et al., 1995). In addition to its hypothesized role in the conservation of polyunsaturated fatty acids, MGAT may also serve to regulate overall hepatic glycerolipid synthesis. MGAT's product, *sn*-1,2-diacylglycerol, is an activator of protein kinase C as well as the substrate for the enzymes that synthesize triacylglycerol and the quantitatively most important phospholipids, phosphatidylcholine and phosphatidylethanolamine (Hjelmstad & Bell, 1991a). Further, MGAT's *sn*-2-monoacylglycerol substrate is a competitive inhibitor of glycerol-3-phosphate acyltransferase, the committed step in glycerolipid biosynthesis (Polheim et al., 1973; Coleman, 1988), and diacylglycerol kinase, a major attenuator of *sn*-1,2-diacylglycerol signals (Bishop et al., 1986). An increase in the concentration of cellular monoacylglycerol might, therefore, inhibit the glycerol 3-phosphate pathway of glycerolipid synthesis, and prolong the ability of *sn*-1,2-diacylglycerol to activate protein kinase C. In the intestine, MGAT provides the major pathway for the resynthesis of triacylglycerol and is critical for the assembly of chylomicra. Finally, MGAT may function to incorporate xenobiotic carboxylic acids into glycerolipids (Dodds, 1991). Although MGAT's function is critical for normal hepatic and intestinal lipid metabolism, its structure and regulation are poorly understood.

We have developed a mixed micellar assay to study the regulation of MGAT (Bhat et al., 1993, 1994). With this assay, one can alter concentrations of putative lipid modulators in a defined environment that obeys surface dilution kinetics (Hjelmstad & Bell, 1991a). Purified MGAT is activated by negatively charged phospholipids and *sn*-1,2-diacylglycerol, and is inhibited by sphingosine (Bhat et al., 1994). In order to delineate the mechanism of inhibition and to determine whether nonspecific ionic interactions might be involved, we used the mixed micellar assay to determine which structural aspects of diacylglycerol and sphingoid

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[1] Abbreviations: MGAT, monoacylglycerol acyltransferase; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)-tetraacetic acid; MEM, minimal essential media; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid.

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bases are required to modulate MGAT activity. We further tested the physiological relevance of these observations by determining whether MGAT can be activated or inhibited by specific lipids in hepatocytes or microsomal membranes. Our results suggest that MGAT may be regulated *in vivo* by changes in cellular concentrations of several intermediates of glycerolipid synthesis and that regulatory links may exist between signal transduction and the synthesis of complex lipids via the monoacylglycerol pathway.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of reagent grade. Phosphatidic acid (from egg lecithin), phosphatidylcholine (pig liver), phosphatidylserine (beef brain), sphingomyelin (beef brain), ceramide (beef brain), *sn*-1,2-diC18:1-glycerol, *sn*-1,2-diC18:1-glycerol ether, *sn*-2-monoC18:1-glycerol, stearylamine, phytosphingosine, and sphingosine (beef brain) were purchased from Serdary Research Laboratories, Inc. *sn*-1,2-Dioctanoin and *sn*-1-oleoyl-2-acetoylglycerol were from Avanti Polar Lipids. BSA (essentially fatty acid free), Triton X-100, DTT, EDTA, collagenase, leupeptin, phorbol 12-myristate 13-acetate, pepstatin, digitonin, sphinganine, phospholipase C (from *Clostridium perfringens*), and CM-Sepharose FF were from Sigma Chemical Co. QAE-Sephadex and hydroxylapatite (Bio-Gel HTP) were from Pharmacia and Bio-Rad, respectively. Phospholipase D (from *Streptomyces chromofusces*) was from Boehringer Mannheim. Tissue culture supplies were from Gibco-BRL.

Animals. Animal use was approved by the Animal Care Committee of the University of North Carolina. Pregnant Sprague-Dawley rats from Zivic-Miller were housed in the University of North Carolina animal facility under controlled 12-h light cycles with free access to food and water. Within 24 h after birth, each litter was culled to a maximum of 12 pups.

Partial Purification of MGAT. On the 11th day after birth, rats were decapitated, and KCl-washed liver microsomes were prepared and stored at −70 °C (Bhat et al., 1993). Microsomes were solubilized with 0.3% Triton X-100, and MGAT was purified by sequential chromatography with QAE-Sephadex, CM-Sepharose (Fast Flow), and hydroxylapatite (Bhat et al., 1993). The partially purified enzyme preparation was stored in aliquots at −70 °C in a buffer containing 20% glycerol, 5% ethylene glycol, 20 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, 1 μg/mL each of leupeptin and pepstatin, 0.5 mM benzamidine, 0.2% Triton X-100, and 0.2 M NaCl. The enzyme preparation contained no phospholipid (Bhat et al., 1994), and no acyl-CoA synthase or diacylglycerol acyltransferase activities (Bhat et al., 1993). Hydroxylapatite-purified MGAT specific activity was measured as described (Bhat et al., 1993).

Preparation of Hepatocytes from 11-Day-Old Rats. Parenchymal hepatocytes from 10-day-old rats were isolated by collagenase perfusion (Emmison et al., 1995). All media contained 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamycin. Liver was perfused *in situ* with a hand-held syringe via the inferior vena cava in the retrograde direction at a flow rate of 2−4 mL/min. After perfusing Ca^{2+}-free buffer to remove the blood, the liver was perfused with 20 mL of digestion buffer containing 0.8 mg/mL collagenase. Hepatocytes were filtered through 50 μm nylon mesh, washed, and plated at about 5×10^4 cells/15 mm culture dish in a 24-well plate. Hepatocyte viability, assessed by Trypan blue exclusion, was >93%. After maintaining the cells in 1 mL of MEM containing 10% fetal bovine serum for at least 6 h, the medium was changed to serum-free MEM containing 10 nM dexamethasone and 10 mM nonessential amino acids.

Assay of MGAT in Mixed Micelles. Stock solutions (stored in chloroform or hexane) of the different lipids employed were dried under a stream of nitrogen in a glass tube and solubilized in Triton X-100 by vortex mixing and brief sonication in a water bath at room temperature. Required amounts of these lipids were then added to the reaction mixture. MGAT activity was assayed at 23 °C in a 0.2 mL reaction mixture that contained 100 mM Tris-HCl, pH 7.0, 0.5 mg/mL BSA, 150 μM *sn*-2-monoC18:1-glycerol, 0.22% (80−95 mol % depending on the amount of lipid added) Triton X-100 (3 mM micelle concentration),[2] 25 μM [^{3}H]-palmitoyl-CoA (115 Ci/mol), 0.25−0.5 μg of hydroxylapatite-purified protein, and the indicated concentrations of specific lipids (Bhat et al., 1994). After a 10-min incubation, the products were extracted into heptane, and an aliquot was counted (Bhat et al., 1994). The remaining portion of the heptane extract was concentrated in a Savant Speedvac concentrator and chromatographed with carrier lipids on a 10 cm silica gel G plate in heptane/isopropyl ether/acetic acid (60/40/4, v/v). Lipids were visualized by exposure to I_2 vapor, and the areas corresponding to diacylglycerol and triacylglycerol were scraped and counted. Because more than 97% of the labeled product was diacylglycerol, thin-layer chromatography was not routinely performed. All assays contained optimal amounts of *sn*-2-monoC18:1-glycerol and [^{3}H]palmitoyl-CoA and measured initial rates. For each study reported, data are shown for a representative experiment that was repeated 2−6 times using two to four different hydroxylapatite-purified MGAT preparations.

Assay of MGAT in Permeabilized Hepatocytes. Following treatment, hepatocytes in each well were gently washed with 1 mL of ice-cold phosphate-buffered saline. The cells were permeabilized with 0.15 mL of medium containing 300 μg/mL purified digitonin, 0.1 M Tris (pH 7), 0.3 M sucrose, 0.1 M KCl, 1 mM EDTA, and 16 mM NaF (Coleman, 1993). The final MGAT assay reaction mixture of 0.2 mL contained 2 mg/mL BSA, 50 μM *sn*-2-monoC18:1-glycerol in 10 μL of acetone, and 45 μM [^{3}H]palmitoyl-CoA. Activity with endogenous substrate was measured by adding 10 μL of acetone and omitting the *sn*-2-monoC18:1-glycerol. After a 5 min reaction at room temperature, the reaction products were extracted into heptane, and analyzed by thin-layer chromatography as described above. MGAT specific activity was calculated by subtracting half the cpm that appeared in triacylglycerol.

Assay of MGAT in Microsomes. In microsomes, MGAT activity was measured in a reaction mixture that contained 100 mM Tris-HCl, pH 7.0, 1 mg/mL BSA, 150 μg/mL phosphatidylcholine/phosphatidylserine (1/1, w/w), 25 μM [^{3}H]palmitoyl-CoA, 50 μM *sn*-2-monoC18:1-glycerol dispersed in 5 μL of acetone, and 1−3 μg of microsomal protein

[2] To calculate mole fractions, the aqueous monomeric concentration of Triton X-100 (0.3 mM) was subtracted from the total detergent concentration. Final concentrations include the Triton X-100 that is added together with the purified enzyme.

in a final volume of 0.2 mL. After incubation at 23 °C for 5 min, the reaction was terminated and the products were extracted and analyzed as described above.

Preparation and Treatment of Microsomes with Phospholipases C and D. KCl-washed rat liver microsomes from 10-day-old rat pups were prepared by differential centrifugation and resuspended in medium I (0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-Cl, pH 7.4). Microsomes (1 mg of protein) were incubated for 15 min at 37 °C in the absence or presence of 0.01−5 units of phospholipase C or 0.025−0.5 unit of phospholipase D in 1.0 mL of 50 mM Tris-HCl, pH 8.0, containing 1 mg of BSA and 0.1 mM $CaCl_2$. Phospholipase action was stopped by adding 0.5 mL of ice-cold 10 mM EGTA in 50 mM Tris-HCl, pH 7.4. The treated microsomes were repelleted at 105000*g* for 1 h at 4 °C. The resulting pellets were each resuspended in medium I before assay for MGAT activity by the microsomal method described above.

Other Methods. Protein was measured with BSA as the standard (Lowry et al., 1951). Hepatocyte total cellular protein was measured directly in 4−6 wells of each 24-well tissue culture dish (Coleman, 1993). *sn*-1,2-DiC18:1-glycerol was separated from its racemic isomer *sn*-1,3-diC18:1-glycerol on 20 cm 3% borate silica gel G plates chromatographed with chloroform/acetone/methanol/acetic acid (90/5/2/0.5; v/v). After being scraped from the plate, the *sn*-1,2-diacylglycerol was quantified colorimetrically (Fletcher, 1968). [^{3}H]Palmitoyl-CoA was synthesized enzymatically (Merrill et al., 1982). To quantify phosphatidic acid, chloroform-extracted samples were chromatographed on silica gel G thin-layer plates in $CHCl_3$/methanol/H_2O (65/25/4; v/v). Phosphatidic acid was scraped from the plate and extracted into $CHCl_3$, and inorganic phosphorus was measured after digestion with perchloric acid (Bartlett, 1959).

RESULTS

Effect of Sphingoid Bases on MGAT Activity. Using a Triton X-100 mixed micellar assay to determine whether specific structural features are required for the inhibition of MGAT by sphingosine, we examined the effect of related sphingolipids and sphingoid bases (Figure 1). Like the zwitterionic phospholipids, phosphatidylcholine and phosphatidylethanolamine (Bhat et al., 1994), sphingomyelin was a moderate stimulator of MGAT activity; at 15 mol % sphingomyelin, MGAT activity increased up to 2.8-fold. In contrast, anionic phospholipids stimulate MGAT activity as much as 11-fold at concentrations as low as 4.2 mol % (Bhat et al., 1994). Inhibition by sphingosine, sphinganine, and phytosphingosine was dose-dependent with 50% inhibition observed at 9, 5.5, and 5 mol %, respectively (Figure 1). These sphingoid bases are unlikely to inhibit MGAT by a nonspecific detergent effect (Hannun & Bell, 1987), since lysophospholipids, which have strong detergent properties, either stimulated MGAT or had no effect (Bhat et al., 1994). Inhibition by stearylamine, an 18-carbon primary amine, was dose-dependent (IC_{50} of 6 mol %) and similar to inhibition by sphinganine and phytosphingosine. However, octylamine, an 8-carbon primary amine, and ceramide, which lacks a free amino group, did not inhibit MGAT activity. These results suggest that inhibition requires a free amino group and a long-chain hydrocarbon. The primary hydroxyl group on the sphingoid base did not appear to be critical for inhibition.

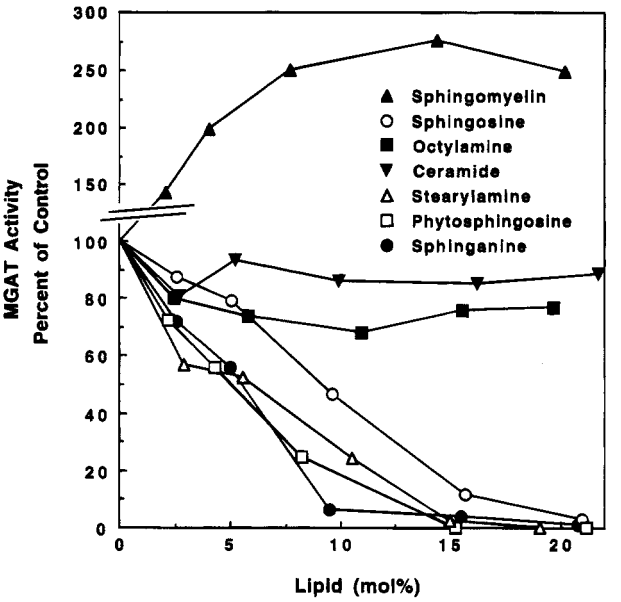

FIGURE 1: Effect of sphingoid bases and sphingolipids on MGAT activity in Triton X-100 mixed micelles. MGAT activity was measured in Triton X-100 micelles (3 mM Triton X-100 and 150 μM *sn*-2-monooleoylglycerol) in the absence of activating phospholipids as described under Experimental Procedures. The indicated concentrations of sphingolipids or sphingoid bases were added. Control MGAT specific activity (100%) was 67 nmol min^{-1} mg^{-1}.

To determine whether sphingosine interacted with a possible anionic activation site, we examined the kinetics of inhibition with respect to phosphatidic acid, phosphatidylinositol, and phosphatidylserine (Figure 2). Double-reciprocal plots indicated that sphingosine behaved like a competitive inhibitor of anionic phospholipid-stimulated activation with an apparent K_i of less than 0.7 mol % for phosphatidic acid (Figure 2A), of 1.23 mol % for phosphatidylserine (Figure 2B), and of 1.28 mol % for phosphatidylinositol (Figure 2C).

Activation of MGAT by Diacylglycerol and Effect of Sphingosine. Stimulation of MGAT by diacylglycerol requires the *sn*-1,2-stereoisomer and ester rather than alkyl bonds (Bhat et al., 1993, 1994). To determine whether water-soluble diacylglycerols can activate MGAT as they can protein kinase C (Bell & Burns, 1991), we investigated the effect of *sn*-1,2-diacylglycerols with different acyl-chain lengths (Figure 3). *sn*-1,2-DiC8:0-glycerol had very little effect on MGAT activity, and *sn*-1-oleoyl-2-acetoylglycerol had no effect, suggesting that activation would require diacylglycerol to interact with MGAT within the micelle, and that water-soluble diacylglycerols were unable to interact. We also tested phorbol ester which interacts with protein kinase C at its diacylglycerol binding site (Neidel et al., 1983), and ceramide which is a competitive inhibitor of diacylglycerol kinase (Younes et al., 1992). Although both are functional diacylglycerol analogs, neither ceramide nor phorbol myristyl acetate stimulated MGAT activity. Thus, stimulation of MGAT activity specifically required a diacylglycerol that contains long fatty acyl chains at the *sn*-1 and *sn*-2 positions and can partition into the micelle. These results suggest that the MGAT−diacylglycerol interaction is highly specific, but that it differs significantly from the interaction of diacylglycerol with protein kinase C or diacylglycerol kinase.

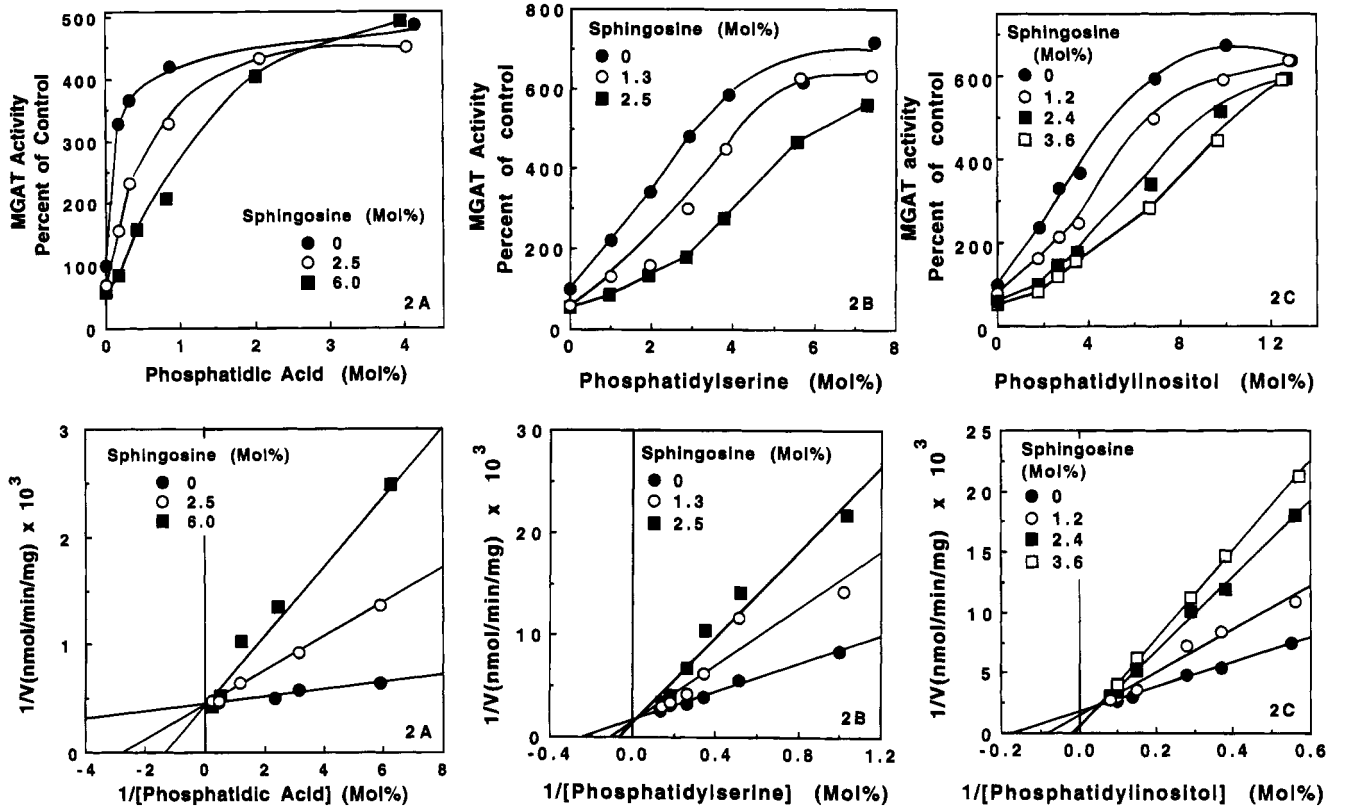

FIGURE 2: Double-reciprocal plots showing inhibition of phospholipid-activated MGAT activity by the indicated concentrations of sphingosine. Activities were determined in the same experiment using the same enzyme preparation in Triton X-100 mixed micelles containing varying amounts of (A) phosphatidic acid, (B) phosphatidylinositol, or (C) phosphatidylserine. Similar results were obtained using two different enzyme preparations. The apparent K_i values were calculated by secondary plot of the slopes of the lines as a function of sphingosine concentrations. K_i values with respect to phosphatidic acid, phosphatidylserine, and phosphatidylinositol activation were 0.7, 1.23, and 1.28 mol %, respectively. Data were analyzed by computer-assisted least-squares analysis, and regression coefficients were greater than 0.94.

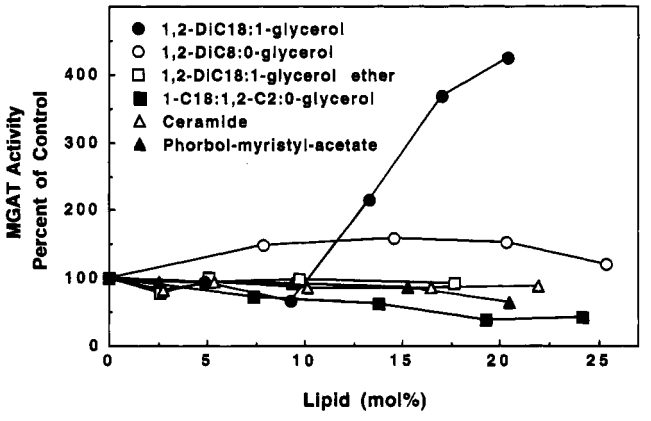

FIGURE 3: Effect of diradylglycerols, ceramide, and phorbol 12-myristyl 13-acetate on monoacylglycerol acyltransferase activity in Triton X-100 mixed micelles. The concentration of lipid modulators in the MGAT reaction mixture was varied as indicated. Control specific activities measured under Triton X-100 micellar assay conditions in the absence of added activating lipid ranged from 49 to 93 nmol min^{-1} mg^{-1}.

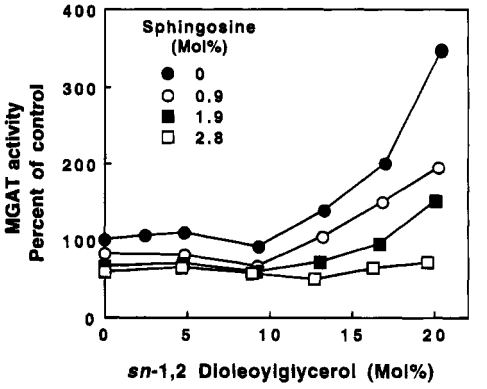

FIGURE 4: Inhibition of diacylglycerol-activated MGAT activity by the indicated concentrations of sphingosine. Activities were determined in the same experiment using the same enzyme preparation in Triton X-100 mixed micelles containing varying amounts of *sn*-1,2-dioleoylglycerol. Similar results were obtained using two different enzyme preparations.

To determine whether sphingosine would inhibit the ability of *sn*-1,2-diacylglycerol to activate MGAT, we examined the kinetics of inhibition (Figure 4). A Triton X-100 concentration of 3 mM was used for all micellar assay experiments. The total diacylglycerol concentration in the mixed micelles did not exceed 20 mol %, in order to ensure that the structure of the mixed micelles was similar to the structure of micelles composed of Triton X-100 alone, and to maintain a constant micellar size (Litchenberg et al., 1983; Robson & Dennis, 1983). No MGAT activation is observed when the *sn*-1,2-diacylglycerol concentration is less than 10 mol %, suggesting that the mechanism of activation by *sn*-1,2-diacylglycerol differs from that of the anionic phospholipids which activate at low molar concentrations. When phospholipid is absent, stimulation by *sn*-1,2-dioleoylglycerol is highly cooperative with a Hill constant of 3.8, and maximal stimulation requires more than 25 molecules of *sn*-1,2-diacylglycerol per micelle (Bhat et al., 1994). Inhibition by sphingosine was dose-dependent with 50% inhibition of maximal activation observed at about 2 mol % sphingosine (data not shown).

Increasing concentrations of sphingosine up to 2.75 mol % completely inhibited the activation observed with 20 mol % *sn*-1,2-dioleoylglycerol (Figure 4).

Does Phosphatidic Acid Simulate MGAT Activity via Ionic Effects? In Triton X-100/phospholipid mixed micelles, anionic phospholipids are potent activators of MGAT activity (Bhat et al., 1994). Stimulation requires a negatively charged phospholipid head group and at least one long fatty acyl chain, but the fatty acyl chain species is not critical. Since MGAT's isoelectric point is 9.6 (Bhat et al., 1993), the enzyme has a positive charge at pH 7.0 in the micellar assay. At this pH, an electrostatic attraction might be possible between the positively charged enzyme and the negatively charged anionic phospholipid. Such ionic interactions have been inferred for sterol carrier protein-2 (Butko et al., 1990) and CTP:phosphocholine cytidylyltransferase (Cornell, 1991). To determine whether ionic interactions play a role in the ability of anionic phospholipids to stimulate MGAT activity, the surface charges of phosphatidic acid, the most potent anionic activator, and/or MGAT were screened by adding KCl, neomycin, or $CaCl_2$ to the assay in the presence or absence of phosphatidic acid. We reasoned that if activation by phosphatidic acid occurred by ionic interactions, the enzyme activity would be inhibited only when phosphatidic acid was present, and that no effect would be observed when the phospholipid activator was absent.

KCl acts to screen surface charges and could affect either phosphatidic acid or MGAT itself. Adding KCl to the enzyme reaction mixture inhibited enzyme activity 60% at 1 M KCl when phosphatidic acid was present and about 80% when the enzyme activity was measured in the absence of phosphatidic acid (Figure 5A). Thus, little difference was observed that depended solely on the presence of the anionic phospholipid.

The polyamine antibiotic neomycin binds to negatively charged phospholipids and could thereby act to prevent ionic interactions between phosphatidic acid and MGAT (Palmer, 1981). In a study of sterol carrier proteins, the response to neomycin demonstrated that electrostatic interactions were responsible for the stimulation of intermembrane cholesterol transfer (Butko et al., 1990). Since MGAT activity in both the presence and absence of phosphatidic acid was affected very little by increasing concentrations of neomycin at up to a 10 molar excess compared with phosphatidic acid (Figure 5B), ionic interactions seemed unlikely.

We tested the ability of Ca^{2+} to inhibit MGAT activity because Ca^{2+} might block ionic interactions in one of two ways. It could form divalent metal ion bridges between MGAT and the negatively charged phosphatidic acid, or it might screen negatively-charged lipids within the Triton X-100 mixed micelle. However, the addition of $CaCl_2$ caused a decrease in MGAT activity that was almost identical in both the presence and absence of phosphatidic acid (Figure 5C).

Taken as a whole, these results, with three different compounds that alter ionic interactions, suggest that the ability of phosphatidic acid to stimulate MGAT activity is highly lipid-specific and is not based merely on electrostatic interactions between MGAT and the negatively charged phospholipid.

Modulation of MGAT by Lipid Mediators in Rat Hepatocytes and Microsomes. To determine whether lipid mediators that alter MGAT activity in Triton X-100 mixed micellar assays would have similar effects physiologically, we attempted to change the lipid environment of MGAT in cultured hepatocytes. Hepatocytes expressing high MGAT activity were isolated from 10-day-old rats and treated by directly adding diacylglycerol, phosphatidic acid, sphingosine, or sphinganine. Immediately following each of these treatments, the hepatocytes were permeabilized, and MGAT activity was measured.

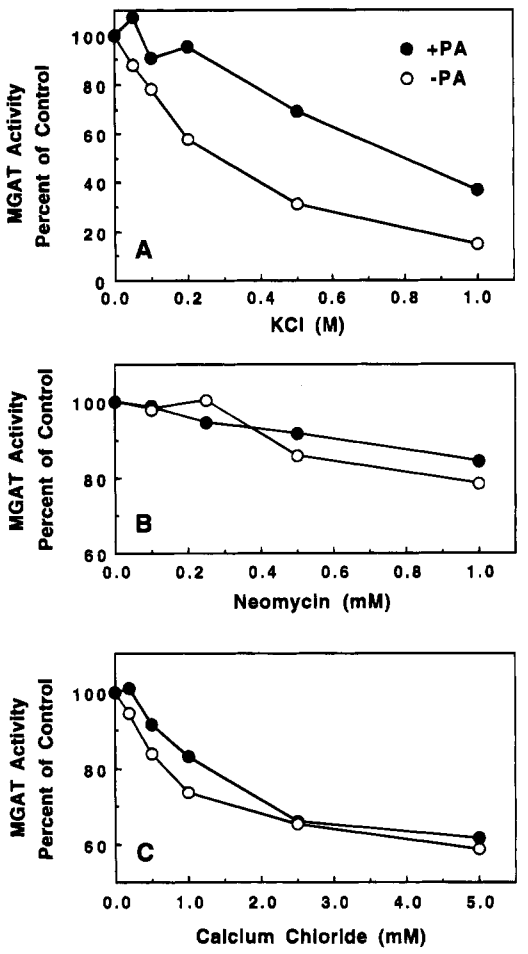

FIGURE 5: Effect of (A) KCl, (B) neomycin, and (C) calcium chloride on MGAT activity in the absence and presence of phosphatidic acid in Triton X-100 mixed micelles. Enzyme assay conditions are as described under Experimental Procedures except that KCl, neomycin, or $CaCl_2$ was included in the reaction mixture as indicated. Control MGAT activity (100%) measured in the absence of phosphatidic acid was 56.8 + 4.6 nmol min^{-1} mg^{-1} (n = 3). Control enzyme activity (100%) measured in the presence of 4 mol % phosphatidic acid was 302.4 ± 50.2 nmol min^{-1} mg^{-1} (n = 3).

Adding 10−200 μM sphingosine or sphinganine to the culture medium inhibited MGAT activity in a dose-dependent manner (Figure 6). Sphingosine inhibited MGAT activity 31% at 10 μM and 71% at 50 μM. As was also observed in the mixed micellar assay (Figure 1), sphinganine was a more potent inhibitor than sphingosine, inhibiting MGAT activity 49% at 10 μM and 95% at 50 μM. Thus, sphingosine and sphinganine inhibited MGAT activity similarly both in mixed micelles and in intact cultured rat hepatocytes. Other studies have shown enzyme inhibition by sphingosine at the same or higher concentrations. Sphingosine at 25 mol % inhibits rat liver CTP:phosphocholine cytidylyltransferase by 50% in phosphatidylcholine vesicles (Sohal & Cornell, 1990), and

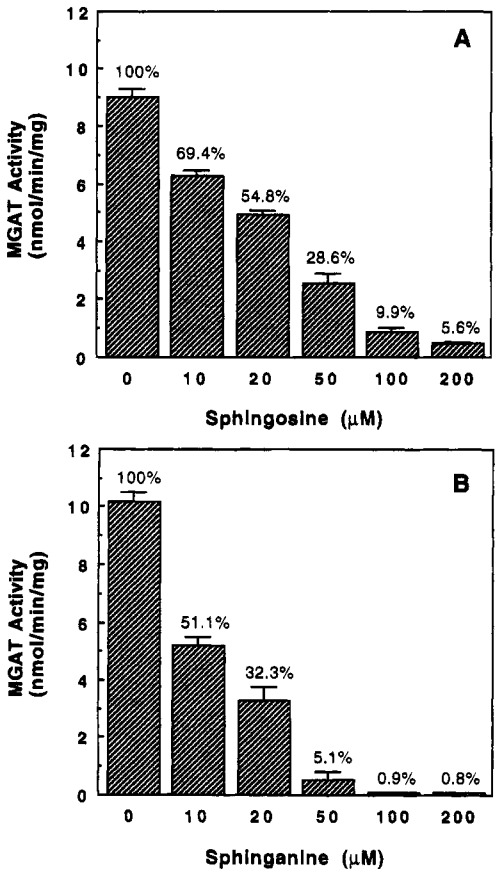

FIGURE 6: Concentration dependence of sphingosine (A) and sphinganine (B) inhibition of MGAT activity in cultured rat hepatocytes. Hepatocytes were isolated from 10-day-old rats and cultured as described under Experimental Procedures. After an overnight incubation in serum-free media, cells (5×10^4/well) were treated with the indicated concentrations of sphingosine or sphinganine added in ethanol. The final concentration of ethanol in the medium was 0.5%. After a 3 h incubation, cells were washed and permeabilized with digitonin, and MGAT activity was measured as described under Experimental Procedures. Each bar represents the mean + SEM for six wells. Similar results were obtained with three independently-isolated rat hepatocyte preparations.

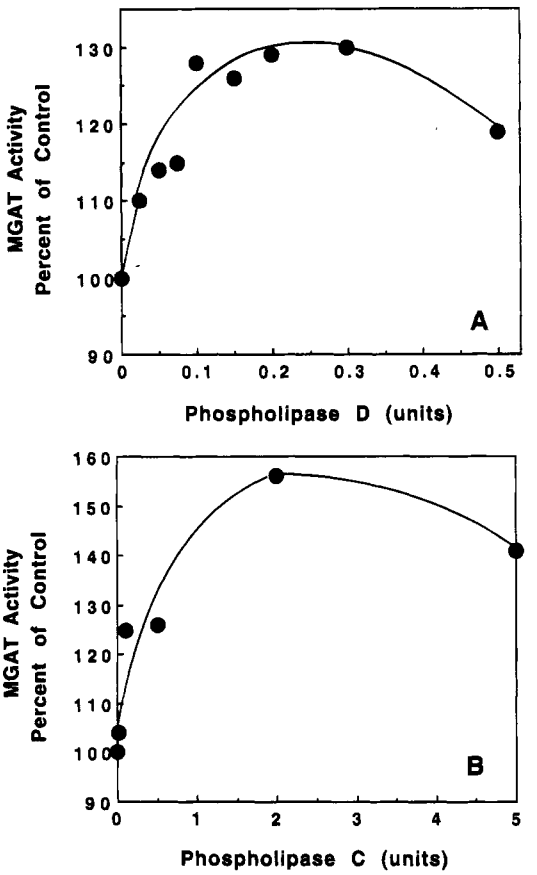

FIGURE 7: Effect of exogenous (A) phospholipase D and (B) phospholipase C treatment on MGAT activity in rat hepatic microsomes. Microsomal membranes (1 mg of protein) were incubated for 15 min at 37 °C in the absence or presence of 0.01−5 units of phospholipase D (A) or 0.025−0.5 unit of phospholipase C (B) as described under Experimental Procedures. Microsomal membranes were repelleted by centrifugation at 105000*g* for 1 h after stopping phospholipase C or D treatment with ice-cold 10 mM EGTA. Microsomal MGAT activity in the resulting pellet was measured as described under Experimental Procedures. Values are the average of duplicate measurements.

sphingosine at 5 mol % inhibits protein kinase C by 50% in Triton X-100 mixed micelles (Merrill et al., 1989).

To generate *sn*-1,2-diacylglycerol or phosphatidic acid *in situ*, cultured hepatocytes were incubated for 30 min with 0.01−5 units of phospholipases C or D. No effect on MGAT activity was observed (data not shown). Since treating [^{3}H]-oleate-labeled hepatocytes with 2−10 units of phospholipase C increased radiolabeled diacylglycerol 25−82% (data not shown), the lack of MGAT stimulation by the phospholipases may be due to the inability of the diacylglycerol and phosphatidic acid produced in the plasma membrane to translocate to the endoplasmic reticulum where MGAT resides. Supporting this hypothesis, no change in MGAT activity was observed after incubation of hepatocytes with *sn*-1,2-diC18:1-glycerol (10 and 100 μM), *sn*-1,2-diC8:0-glycerol (10−50 μM), phosphatidic acid (10−100 μM), or diC8:0-phosphatidic acid (10−50 μM), all added in ethanol. This lack of effect suggests that *sn*-1,2-diacylglycerol and phosphatidic acid either added directly to hepatocytes or produced by phospholipase treatment did not reach the endoplasmic reticulum in amounts sufficient to stimulate MGAT or that MGAT was already fully activated in its endogenous microsomal environment.

In order to distinguish between these two possibilities, we tested the ability of phospholipases C and D to stimulate microsomal MGAT activity by directly generating diacylglycerol and phosphatidic acid in microsomes (Figure 7). A preliminary experiment showed that treatment for 15 min with phospholipase C (5 units) increased diacylglycerol in microsomes from 45 to 253 nmol/mg of microsomal protein, and that phospholipase D (5 units) increased phosphatidic acid from 1.7 to 54 nmol/mg of microsomal protein. As little as 0.025 unit of phospholipase D/mg of microsomal protein stimulated MGAT activity (Figure 7A). Maximum stimulation of 30% was observed with 0.1−0.3 unit of phospholipase D. Adding phospholipase C at 0.1 unit/mg of protein stimulated MGAT activity 25%, and maximal stimulation of 1.55-fold was attained with phospholipase C at 2 units/mg of microsomal protein (Figure 7B). The extent of stimulation in microsomes was considerably less than was observed with phosphatidic acid or *sn*-1,2-diacylglycerol in Triton X-100 mixed micelles (Figures 2A, 3), probably because MGAT was already activated by endogenous microsomal phospholipids. In the Triton X-100 mixed micellar assay, the added lipids are the only ones present.

DISCUSSION

In order to understand the regulation of energy metabolism, the synthesis of phospholipids destined for incorporation into membranes, lipoproteins, and bile, and the production of cellular lipid second messengers, study of the microsomal enzymes of glycerolipid synthesis is critical. Most of the enzymes of glycerolipid synthesis are intrinsic membrane proteins that use amphipathic and/or hydrophobic substrates and require lipid cofactors for activity. Although in some cases a nonspecific hydrophobic environment may be able to meet the cofactor requirement, other proteins are highly selective for particular lipid species (Merrill & Schroeder, 1993; Yeagle, 1989), perhaps because these lipids are allosteric regulators (Hjelmstad & Bell, 1991a). In most cases, we do not fully understand the mechanisms by which specific lipids regulate enzyme activity.

Molecular analysis of the regulation of membrane-bound enzymes has been limited because of the difficulty in assessing the effects of phospholipids, cofactors, and lipophilic substrates in states that are physically undefined and nonhomogeneous. Reconstituting membrane-bound proteins in mixed micelles facilitates the study of interactions between lipid cofactors and enzymes. Kinetic properties of both integral and peripheral membrane proteins can be determined in detergent micelles that have been supplemented with limiting amounts of specific lipid modulators. Triton X-100 mixed micelles, for example, have been used to identify the stoichiometry and specificity of lipids that modulate protein kinase C (Hannun et al., 1986a), choline and ethanolamine phosphotransferases (Hjelmstad & Bell, 1991b), phospholipase A_2 (Deems et al., 1975), rat hepatic lipase (Thuren et al., 1990), and yeast phosphatidylserine synthase (Bae-Lee & Carman, 1990).

Using a Triton X-100/phospholipid mixed micellar assay, we have shown that MGAT activity is activated by *sn*-1,2-diacylglycerol and by anionic phospholipids, and is inhibited by sphingosine (Bhat et al., 1994). In the present study, we used the mixed micellar assay to determine which structural features of diacylglycerol and sphingoid bases were critical for activation or inhibition, respectively. Our results suggest that these lipids regulate MGAT by means of specific structural features. Activation of MGAT activity by diacylglycerol required an *sn*-1,2-stereoisomer with two long fatty acyl chains. Activation was minimal with water-soluble diacylglycerols, and did not occur with the ether analogs of *sn*-1,2-diacylglycerol, or with the structurally similar compounds ceramide and phorbol 12-myristate 13-acetate that inhibit and activate, respectively, protein kinase C (Bell & Burns, 1991). To inhibit MGAT activity, sphingoid bases required a free amino group and a long-chain hydrocarbon. These structural features are similar to those required for the sphingoid base inhibition of protein kinase C (Merrill et al., 1989; Hannun et al., 1986b), phosphatidate phosphatase (Wu et al., 1993), and CTP:phosphocholine cytidylyltransferase (Sohal & Cornell, 1990).

Sphingosine was a competitive inhibitor with respect to activation by the anionic phospholipids phosphatidic acid, phosphatidylinositol, or phosphatidylserine. Our previous studies showed that as little as 0.4 mol % phosphatidic acid can activate MGAT 5-fold and that full activation requires only about six molecules/micelle (Bhat et al., 1994). The hypothesis that anionic phospholipids interact with MGAT at a specific binding site is supported by the present finding that sphingosine appears to compete for this site and that the apparent K_i values are very low. The alternative possibility that sphingosine changes MGAT's configuration, secondarily altering the anionic phospholipid site, cannot yet be addressed since molecular tools are lacking. In contrast, inhibition by sphingosine with respect to MGAT's substrate *sn*-2-monoC18:1-glycerol is noncompetitive or mixed with an apparent K_i of 18.3 mol % (Bhat et al., 1994). Sphingosine has been shown to be a competitive inhibitor of the activation of CTP:phosphocholine cytidylyltransferase by phosphatidylcholine/oleic acid vesicles, but, in this case, competition required concentrations of sphingosine as high as 25 mol % for 50% inhibition and was believed to result from sphingolipid-induced alterations in charge interactions between enzyme and membrane (Sohal & Cornell, 1990).

Two aspects of our previous studies (Bhat et al., 1994) supported the possibility that electrostatic interactions might play a role in the ability of anionic phospholipids to stimulate MGAT activity: (a) anionic phospholipid activation increases with increasing head-group charge; and (b) zwitterionic phospholipids are very weak activators compared to anionic phospholipids. However, our present studies strongly suggest that electrostatic interactions between the positively charged enzyme and the negatively charged phospholipid do not play a role, because MGAT activity was similarly affected both in the absence and in the presence of phosphatidic acid by high ionic strength, the presence of a divalent cation, or the addition of the polycationic antibiotic neomycin. Thus, these additions did not block a theoretical electrostatic interaction between MGAT and the anionic phospholipids. It seems unlikely that important electrostatic interactions would occur in the nonaqueous phase where they could not be blocked by $CaCl_2$, KCl, or neomycin, because the phospholipid head-group is exposed on the micelle's surface. In similarly designed experiments with liposomes, KCl and neomycin blocked the ability of anionic phospholipids to stimulate the activity of sterol carrier protein-2 (Butko et al., 1990), indicating that the phospholipid head-group was accessible to the water-soluble KCl and neomycin. Also supporting the lack of ionic interaction in modulating MGAT activity are previous studies showing that oleic acid, a lipid that has a net negative charge at neutral pH (Kamp & Hamilton, 1992), inhibits MGAT (Bhat et al., 1994). Thus, the activation of MGAT activity by anionic phospholipids such as phosphatidic acid appears to be selective for the phospholipid head-group (Bhat et al., 1994) and unrelated to nonspecific ionic factors.

In order to determine whether the lipid modulators of MGAT identified in Triton X-100 mixed micelles might be relevant physiologically, we tested their effects on MGAT in intact hepatocytes and isolated microsomal membranes. Both cell and microsomal studies support the mixed micelle observations. Sphingosine and sphinganine, similar to their effect in the mixed micellar assays, inhibited MGAT activity in hepatocytes. Further, MGAT activity was stimulated by treating hepatic microsomes with phospholipases that increased the content of either diacylglycerol or phosphatidic acid. Since hepatocytes incubated with phospholipases C or D did not show increased MGAT activity, the results in microsomes suggest that MGAT activation requires an increase of diacylglycerol or phosphatidic acid in the endoplasmic reticulum. Diacylglycerol and phosphatidic acid

generated in the plasma membrane may not participate in MGAT stimulation. Additionally, these studies suggest that plasma membrane diacylglycerol and phosphatidic acid either do not translocate to the endoplasmic reticulum in significant amounts or are rapidly converted to products that are unable to stimulate MGAT. Since fluorescent analogs of phosphatidic acid and diacylglycerol added in liposomes to cultured cells are rapidly converted into complex glycerolipids, the latter hypothesis seems more likely (Pagano, 1990).

Our studies strongly suggest that MGAT activity can be directly regulated by signal-mediated increases in phosphatidic acid, diacylglycerol, and sphingoid bases if the microsomal content of these bioactive lipid second messengers increases. Ceramide, released from sphingolipids by agonist-mediated stimulation of sphingomyelinase, may be hydrolyzed to sphingosine by the action of ceramidase (Merrill & Jones, 1990; Hannun, 1994). Sphingosine, thus generated, may act to modulate MGAT, as well as other target enzymes such as protein kinase C (Hannun & Bell, 1987; Merrill et al., 1993; Pushkareva et al., 1993) and CTP:phosphocholine cytidylyltransferase (Sohal & Cornell, 1990). Although activation of phospholipases C or D in response to agonists or hormones can release diacylglycerol or phosphatidic acid from membrane phospholipids, our results suggest that these lipid messengers may not reach the endoplasmic reticulum in amounts sufficient to regulate MGAT activity or that they may be rapidly metabolized to inactive compounds. Regulation of MGAT by diacylglycerol or phosphatidic acid may require that these lipids be generated locally in order to alter the rate of synthesis of glycerolipids via the monoacylglycerol pathway. The fact that several of these lipid mediators modulate the activities of protein kinase C (Bell & Burns, 1991) and the regulatory enzyme of phosphatidylcholine synthesis, CTP:phosphocholine cytidylyltransferase (Cornell, 1991; Sohal & Cornell, 1990), suggests that signal transduction and the synthesis of complex lipids via the monoacylglycerol pathway may be linked. The signal-activated pathways that produce each of the lipid mediators, the roles of altered concentrations of lipid modulators, and their regulation of hepatic MGAT activity remain to be studied in neonatal animals.

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