Fatty Acids and Anionic Phospholipids Alter the Palmitoyl Coenzyme A Kinetics of Hepatic Monoacylglycerol Acyltransferase in Triton X-100 Mixed Micelles[†]

Rosalind A. Coleman,* P. Wang, and B. Ganesh Bhat

Departments of Nutrition and Pediatrics, University of North Carolina, Chapel Hill, North Carolina 27599-7400

Received January 29, 1996; Revised Manuscript Received May 15, 1996[⊗]

ABSTRACT: In order to gain a better understanding of the kinetics of activation and inhibition of hepatic monoacylglycerol acyltransferase (MGAT) (EC 2.3.1.22) by fatty acid, we examined the effect of fatty acid with respect to MGAT's long-chain acyl-CoA substrate in Triton X-100 mixed micelles. At concentrations between 2.5 and 5.3 mol %, oleic acid stimulated MGAT activity 2-fold, whereas oleic acid inhibited MGAT at concentrations higher than 7.5 mol %. The dependence on palmitoyl-CoA was highly cooperative with a Hill constant of greater than 2.4. When present at less than 3 mol %, oleic acid eliminated the lag in the dependence curve. When concentrations of oleic acid were higher than 3 mol %, Michaelis−Menton kinetics were observed with an apparent K_m value of about 54 μM for palmitoyl-CoA but with progressively decreasing V_{max} values. This effect was not observed with octanoic acid, suggesting that the medium-chain fatty acid is unable to associate stably with the mixed micelle and, thus, cannot substantially alter substrate affinity. When anionic phospholipids were tested, phosphatidic acid, lysophosphatidic acid, phosphatidylserine, and phosphatidylinositol eliminated some of the lag in activation by palmitoyl-CoA. At high molar concentrations of the anionic lipid activators, apparent K_m values ranged from 77 μM for phosphatidic acid to 196 μM for phosphatidylinositol. Zwitterionic phospholipids had no effect, nor did the non-phospholipid activators bovine serum albumin or *sn*-1,2-diacylglycerol. $CaCl_2$, but not neomycin or KCl, could overcome the inhibitory effect of oleic acid; thus, the inhibitory effect of fatty acid did not appear to occur by electrostatic interactions. These blockers did not change the effects observed with the anionic phospholipid activators or with the inhibitor, sphingosine. An altered K_m for palmitoyl-CoA in the presence of fatty acid or anionic phospholipid suggests that both long-chain fatty acids and phospholipid cofactors may induce a conformational change in MGAT, thereby altering the enzyme's affinity for its long-chain acyl-CoA substrate. These data further support the hypothesis that the synthesis of glycerolipids via the monoacylglycerol pathway may be highly regulated via a variety of lipid second messengers such as phosphatidic acid and diacylglycerol, as well as by the influx of fatty acids derived from high-fat diets, or from the hydrolysis of adipocyte triacylglycerol during fasting or diabetes.

Hepatic monoacylglycerol acyltransferase (EC 2.3.1.22) (MGAT)[1] is a developmentally regulated microsomal enzyme that catalyzes the stereospecific synthesis of *sn*-1,2-diacylglycerol from *sn*-2-monoacylglycerol and long-chain fatty acyl-CoA (Johnston, 1977; Coleman et al., 1986). We have hypothesized that the normal function of hepatic MGAT may be to aid in retaining essential fatty acids during developmental periods that are characterized by high rates of fatty acid oxidation because (1) MGAT activity is greatest with *sn*-2-monoacylglycerols that contain polyunsaturated acyl chains (Xia et al., 1993); (2) compared with the low hepatic activity found in adult animals, MGAT activity is as much as 700-fold higher in neonatal rats (Coleman & Haynes, 1984), 75-fold higher in fetal guinea pigs (Coleman et al., 1987), and 15-fold higher in developing chick embryo (Sansbury et al., 1989); (3) MGAT activity is high in hibernators such as marmots which must conserve essential fatty acids for reproductive needs in the spring (Xia et al., 1993); and (4) MGAT activity increases in streptozotocin-induced diabetic rats (Mostafa et al., 1993). MGAT may also play a more general role in regulating glycerolipid metabolism and second messenger function. MGAT's *sn*-2-monoacylglycerol substrate competitively inhibits glycerol-3-phosphate acyltransferase, the committed step of the alternate pathway of glycerolipid synthesis (Polheim et al., 1973; Coleman, 1988), and diacylglycerol kinase, a major route of diacylglycerol signal attenuation (Bishop et al., 1986). MGAT's product, *sn*-1,2-diacylglycerol, is not only an intermediate in triacylglycerol and phospholipid biosynthesis (Hjelmstad, 1991) but also an activator of protein kinase C. In addition to its role in liver, an MGAT isoenzyme forms the major pathway for chylomicra triacylglycerol synthesis in intestinal mucosa (Johnston, 1977).

One of the difficulties in trying to understand how the enzymes of triacylglycerol synthesis are regulated is that, in solution, these enzymes and their hydrophobic and amphiphathic substrates, inhibitors, and activators form non-

[†] This work was supported by United States Public Health Service Grant HD 19068 from the National Institutes of Health and by the Lineberger Cancer Center, Cancer Center Core Support Grant CA 16086.

* Corresponding author: Rosalind A. Coleman, M.D., Departments of Nutrition and Pediatrics, CB# 7400, University of North Carolina, Chapel Hill, NC 27599-7400. Phone: (919) 966-7213. Fax: (919) 966-7216.

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: MGAT, monoacylglycerol acyltransferase; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)-tetraacetic acid.

S0006-2960(96)00216-4 CCC: $12.00

homogeneous aggregates. The development of mixed micellar assays has allowed investigators to use surface silution kinetics to study enzyme−lipid interactions within a micellar structure that mimics the membrane bilayer (Carman et al., 1995). The mixed micellar technique is especially useful for MGAT, which is an integral membrane protein that must interact with its substrates within the plane of the endoplasmic reticulum bilayer. We have employed mixed micellar assays in order to comprehend MGAT's regulation more fully. In a single micelle, MGAT, its *sn*-2-monoacylglycerol and acyl-CoA substrates, and its lipid mediators can all be present, and molar concentrations of substrates and lipid mediators can be readily manipulated.

Using Triton X-100/phospholipid mixed micelles, we have shown that MGAT activity is markedly stimulated by anionic phospholipids (Bhat et al., 1994). With phosphatidic acid, for example, activation was observed with a single molecule of phosphatidic acid per micelle and full activation (11-fold) with as few as six molecules of phosphatidic acid per micelle. These data suggest that anionic phospholipids stimulate MGAT activity by interacting with a specific site on the enzyme. To be an activator, the phospholipid needs at least one long-chain fatty acid, suggesting that the anionic head group must be anchored close to the micellar surface in order to interact with the enzyme. Further, *sn*-1,2-diacylglycerol, MGAT's product, activates the enzyme in a highly cooperative manner with a Hill number of 3.6. The *sn*-1,2-diacylglycerol effect is stereospecific, requires the presence of two long-chain fatty acyl groups (Bhat et al., 1995), and is additive with that of the anionic phospholipids (Bhat et al., 1994). MGAT is inhibited potently by sphingolipids that contain a free amino group and a long-chain hydrocarbon, and inhibition by sphingosine and sphinganine can also be demonstrated in cultured hepatocytes (Bhat et al., 1995). Because inhibition by sphingosine is competitive with respect to activation by anionic phospholipids, we hypothesize that the anionic phospholipids may activate at a specific site that is competitively blocked by sphingolipids.

Previous studies indicated that fatty acids had an unusual biphasic effect on MGAT in Triton X-100 mixed micelles (Bhat et al., 1994). Low concentrations of fatty acid stimulated MGAT activity, whereas high concentrations were inhibitory. This inhibition by oleic acid is noncompetitive or mixed with respect to the *sn*-2-monooleoylglycerol substrate. In order to gain a better understanding of the kinetics of activation and inhibition by fatty acid, we examined the effect of fatty acid with respect to MGAT's long-chain acyl-CoA substrate.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of reagent grade. Phosphatidic acid (from egg lecithin), phosphatidylcholine (pig liver), phosphatidylserine (beef brain), sphingomyelin (beef brain), *sn*-1,2-diC18:1-glycerol, *sn*-2-monoC18:1-glycerol, lysophosphatidic acid (from egg lecithin), and oleic acid were purchased from Serdary Research Laboratories, Inc. BSA, Triton X-100, DTT, EDTA, leupeptin, pepstatin, neomycin sulfate, and CM-Sepharose FF were from Sigma Chemical Co. QAE-Sephadex and hydroxylapatite (Bio-Gel HTP) were from Pharmacia and Bio-Rad, respectively.

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 Purina rat chow and water. Within 24 h after birth, each litter was culled to a maximum of 12 pups.

Partial Purification of MGAT. Between the eighth to eleventh 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 leupeptin and pepstatin, 0.5 mM benzamidine, 0.2% Triton X-100, and 0.2 M NaCl. Hydroxylapatite-purified MGAT specific activity was measured as described (Bhat et al., 1993). This enzyme preparation is free of phospholipids (Bhat et al., 1994).

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 0.2% 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. The concentration of each lipid in the mixed micelle, expressed as mol percent, was calculated by the equation 100[added lipid]/[total lipid] + [Trition X-100] (Carman et al., 1995). 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% Triton X-100 (3 mM micelle concentration),[2] 0−60 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). Palmitoyl-CoA concentrations are given as molar concentrations since this substrate is added as a water-soluble component to the assay, and we cannot be certain how much is associated with the micelles, particularly when albumin is present. Concentrations of palmitoyl-CoA higher than 60 μM were not used because inhibition was observed above 80 μM (see inset of Figure 2A). After a 10 min incubation, the products were extracted and analyzed (Bhat et al., 1993). 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, with 25 μM [^{3}H]palmitoyl-CoA and activating phospholipids present, measured initial rates.

Rat Liver Free Fatty Acid Analysis. Liver from four 9-day-old rats was quickly excised and cut into small pieces

[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.

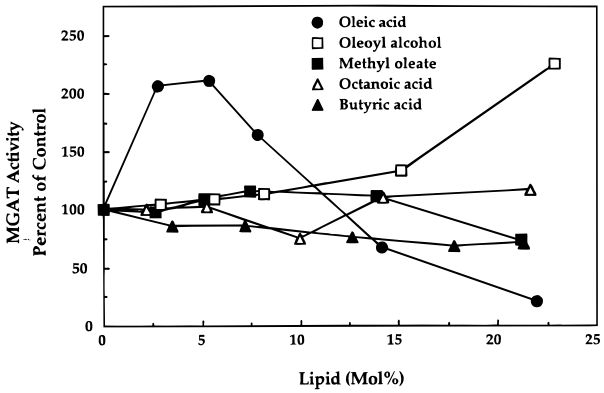

FIGURE 1: Effect of fatty acids and fatty acid analogs on MGAT activity in mixed micelles. MGAT activity was measured in Triton X-100 micelles (3 mM Triton X-100 and 150 μM *sn*-2-monooleoylglycerol) in the absence of activating phospholipids as described in Experimental Procedures. The individual concentrations of fatty acids or their analogs were added in Triton X-100. Control MGAT specific activity (100%) was 67 nmol min^{-1} mg^{-1}. Similar results were obtained using a different enzyme preparation.

using a scalpel. Liver lipids were extracted into chloroform (Bligh & Dyer, 1959). Phospholipids and free fatty acids were separated on Bond Elute aminopropyl-NH_2 columns (Kaluzny et al., 1985). Phospholipid was quantified by measuring inorganic phosphorus following perchloric acid digestion (Bartlett, 1959). The methyl esters of free fatty acids were quantified using a Perkin-Elmer AutoSystem gas chromatograph with a DB 225 capillary column by gas−liquid chromatography. The internal standard was C17:0.

Other Methods. Protein was measured with BSA as the standard (Lowry et al., 1951). *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).

RESULTS

Effect of Fatty Acids and Fatty Acid Derivatives on MGAT Activity. Because we had previously found that oleic acid has a biphasic effect on MGAT activity (Bhat et al., 1994), we examined the effect of fatty acids and acyl derivatives on partially purified rat hepatic MGAT activity (Figure 1). At concentrations between 2.5 and 5.3 mol %, oleic acid stimulated MGAT activity 2-fold, whereas oleic acid inhibited MGAT at concentrations higher than 7.5 mol %. At 24 mol % oleic acid, MGAT activity was inhibited 90%. Virtually identical results were obtained with linoleic acid, linolenic acid, and arachidonic acid and with sodium oleate (data not shown). Oleoyl alcohol and methyl oleate did not show this biphasic effect on MGAT activity. At concentrations lower than 15 mol %, methyl oleate, oleoyl alcohol, octanoic acid, and butyric acid had little or no effect on MGAT activity. Very high concentrations of oleoyl alcohol (22.5 mol %) activated MGAT activity 2.4-fold; however, high molar concentrations of lipids may alter the micellar structure (Lichtenberg, 1983; Robson, 1983). These results suggested that the biphasic effect on MGAT activity was specific for long-chain fatty acids and that the effect required a free carboxyl group. The long-chain hydrocarbon is probably required to anchor the lipid molecule in the Triton X-100 micelle.

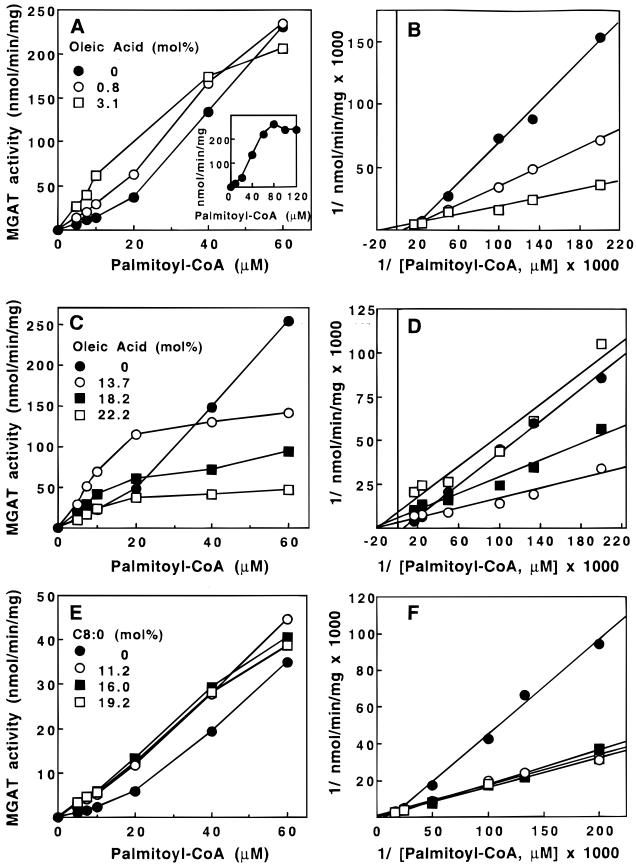

FIGURE 2: Effect of fatty acids on MGAT's dependence on palmitoyl-CoA. (A) Dependence on palmitoyl-CoA in the presence of low molar concentrations of oleic acid and (B) double-reciprocal plot of these data. The inset shows the dependence on palmitoyl-CoA from 0 to 120 μM. (C) Dependence on palmitoyl-CoA in the presence of high molar concentrations of oleic acid and (D) double-reciprocal plot of these data. (E) Dependence on palmitoyl-CoA in the presence of high molar concentrations of octanoic acid and (F) double-reciprocal plot of these data. MGAT activity was measured in Triton X-100 micelles with varying concentrations of palmitoyl-CoA in the absence of activating phospholipids as described in Experimental Procedures. Activity was measured in the absence or presence of concentrations of oleic acid as indicated. The double-reciprocal plots were analyzed by computer-assisted least-squares analysis, and regression coefficients were greater than 0.95. Panels A−D are representative of four independent studies using two different enzyme preparations.

In order to characterize the ability of fatty acid to activate or inhibit MGAT, we examined the effect of oleic acid on the kinetics of the enzyme's activity with respect to its dependence on palmitoyl-CoA. In mixed micelles, in the absence of activating anionic phospholipids, the dependence on palmitoyl-CoA was highly cooperative with a calculated Hill constant of greater than 2.4[3] (Figure 2A,B). When present at 3.1 mol %, oleic acid eliminated the lag in the dependence curve and reduced the calculated Hill constant to 1.0. Because microsomal MGAT activity from sparrow fat showed a similar in the palmitoyl-CoA dependence that was eliminated in the presence of 2−10 mM concentrations of several divalent cations (Mostafa, 1993), we tested the

[3] Because concentrations of palmitoyl-CoA higher than 80 μM inhibited MGAT activity, the calculated Hill constant is a minimum value.

Table 1: Kinetic Constants for MGAT with Different Lipid Activators[a]

lipid[b]	mol %	concentration at which maximal activation is observed (mol %)[c]	K_m (app)[d]	V_{max} (app)[d]
C18:1	3.1−22.2	2.5−5	54 ± 9	270 ± 134
C8:0	11.3−19.2		>330	>2000
PA	0.4−14.8	5−10	88 ± 19	2398 ± 1367
LPA	3.5−12.7	3−6	111 ± 46	1831 ± 717
PS	7.5−15.3	5−15	146[e]	2480[e]
PI	8.5−12.5	12−16	196[f]	3506[f]
SM	4.1−11.3	10−20	nc	nc
PC	7.5−19.5	13−18	nc	nc

[a] The kinetic values were calculated from the data shown in Figures 2−4. [b] The abbreviations are as follows: C18:1, oleic acid; C8:0, octanoic acid; PA, phosphatidic acid; LPA, lysophosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; PC, phosphatidylcholine; nc, not calculable. [c] From Bhat (1994). [d] Means ± S derived from data shown in the figures. In each case, three or four determinations were used. [e] Only one of the determinations was calculable. [f] Average of two determinations that varied by less than 5%.

effects of 2.5 and 10 mM $MgCl_2$, $CaCl_2$, $BaCl_2$, $ZnCl_2$, and $MnCl_2$ on MGAT activity with low concentrations (5 and 10 μM) of palmitoyl-CoA. None of these divalent cations altered the palmitoyl-CoA dependence; 10 mM $ZnCl_2$ or $MnCl_2$ inhibited MGAT activity (data not shown).

In order to determine whether the concentration of fatty acid in liver membranes was normally in the range that would activate MGAT, we measured fatty acid content in liver from 9-day-old rats. Free fatty acid content was 32.6 ± 3.1 nmol/(μmol of phospholipid) (n = 4).[4] This is about 3 mol %. For the endoplasmic reticulum, which contains little cholesterol or sphingolipid, 3 mol % is probably a reasonable estimate and is within the range of maximal activation by fatty acid.

We previously showed that high concentrations of fatty acid (7.5−11.7 mol %) inhibited MGAT activity in the presence of an activating amount of phospholipid (Bhat et al., 1994). The inhibition appeared to be noncompetitive or mixed with respect to *sn*-2-monooleoylglycerol, suggesting that oleic acid might interact with a site that participates in both substrate binding and catalysis. When concentrations of oleic acid were higher than 3 mol %, Michaelis−Menton kinetics were observed with an apparent K_m value of about 54 μM for palmitoyl-CoA but with progressively decreasing V_{max} values (Figure 2C,D).

To determine whether the effect on palmitoyl-CoA kinetics was specific for long-chain fatty acids, a similar study was performed using octanoic acid (Figure 2E,F). Although octanoic acid increased MGAT activity at low concentrations of palmitoyl-CoA, even at 16 and 19 mol % octanoic acid, the apparent K_m value was high and inconsistent (Table 1), suggesting that the medium-chain fatty acid is unable to associate stably with the mixed micelle and, thus, cannot substantially alter substrate affinity. Because MGAT is an integral membrane protein and can interact with lipids throughout the entire length of their acyl chains, it may require an acyl chain length longer than eight carbons. Shorter-chain lipids are similarly unable to activate diacylglycerol kinase (Walsh et al., 1986). Alternatively, the increased water solubility of octanoic acid could allow greater movement of the medium-chain fatty acid on and off the micelle, thereby decreasing its ability to interact with MGAT.

Effect of Anionic Phospholipids. The sigmoidal shape of the dependence on palmitoyl-CoA is similar to that of allosteric enzymes that are regulated by specific effectors and suggested that the fatty acid might be substituting for an activating palmitoyl-CoA binding site. But because anionic phospholipids are excellent activators of MGAT in mixed micelles, we wondered whether they would have an effect similar to that of fatty acids on the kinetics of MGAT activity with respect to palmitoyl-CoA. Phospholipids were tested both at low concentrations, at concentrations that stimulate MGAT maximally, and at high concentrations that begin to inhibit MGAT activity (Bhat et al., 1994). We did not test phospholipids at total micellar lipid concentrations of greater than 18 mol % because higher concentrations may alter Triton X-100 micellar structure and size (Litchenberg, 1983; Robson, 1983). The most potent activator of MGAT activity is phosphatidic acid, which activates enzyme activity 11-fold at 4.2 mol % (Bhat et al., 1994). A decreasing order of activation is observed with lysophosphatidic acid, phosphatidylserine, cardiolipin, and phosphatidylinositol. When the effects of these phospholipids were tested on MGAT's dependence on palmitoyl-CoA, phosphatidic acid (Figure 3A,B), lysophosphatidic acid (Figure 3C,D), phosphatidylserine (Figure 3E,F), and phosphatidylinositol (Figure 3G,H) eliminated some of the lag in activation by palmitoyl-CoA. At high molar concentrations of the anionic lipid activators, the apparent K_m values ranged from 80 μM for phosphatidic acid to 200 μM for phosphatidylinositol (Table 1). The apparent K_m values were lowest with the strongest phospholipid activators (Table 1). The apparent V_{max} values were similarly variable, and like the corresponding study of oleic acid, high concentrations of the anionic phospholipids generally decreased the apparent V_{max} value.

Effect of Zwitterionic Phospholipids on MGAT Kinetics. Even at high molar concentrations, zwitterionic phospholipids are poor activators of MGAT in Triton X-100/phospholipid mixed micelles (Bhat et al., 1994). The addition of phosphatidylcholine at 7.5−19.5 mol % or sphingomyelin at 4−11.3 mol % (Figure 4) had little effect on the kinetics of the dependence of MGAT on palmitoyl-CoA.

Effect of Other Activators on MGAT Kinetics. Bovine serum albumin and *sn*-1,2-diacylglycerol are both non-phospholipid activators of MGAT in mixed micelles. Each was tested in order to determine whether it might alter the kinetics of MGAT with palmitoyl-CoA. In the presence of a fully activating concentration of a mixture of phosphatidylcholine and phosphatidylserine (1:1, w/w), bovine serum albumin did not stimulate further (Figure 5). *sn*-1,2-Diacylglycerol is a potent activator of MGAT at concentrations between 10 and 20 mol %. Although *sn*-1,2-dioleoylglycerol stimulated MGAT activity 2.3-fold in the presence of 60 μM palmitoyl-CoA, sigmoidal curves were retained (Figure 6).

Does Fatty Acid Inhibit MGAT via Ionic Effects? We previously considered whether MGAT activation by anionic phospholipids might be facilitated by electrostatic interactions. Evidence for ionic interactions can be obtained by using the blocking agents KCl, neomycin, or Ca^{2+}. KCl

[4] Free fatty acid content can also be presented as 0.48 ± 0.04 μmol/(g liver wet weight) or as 132.3 μg/(g liver wet weight).

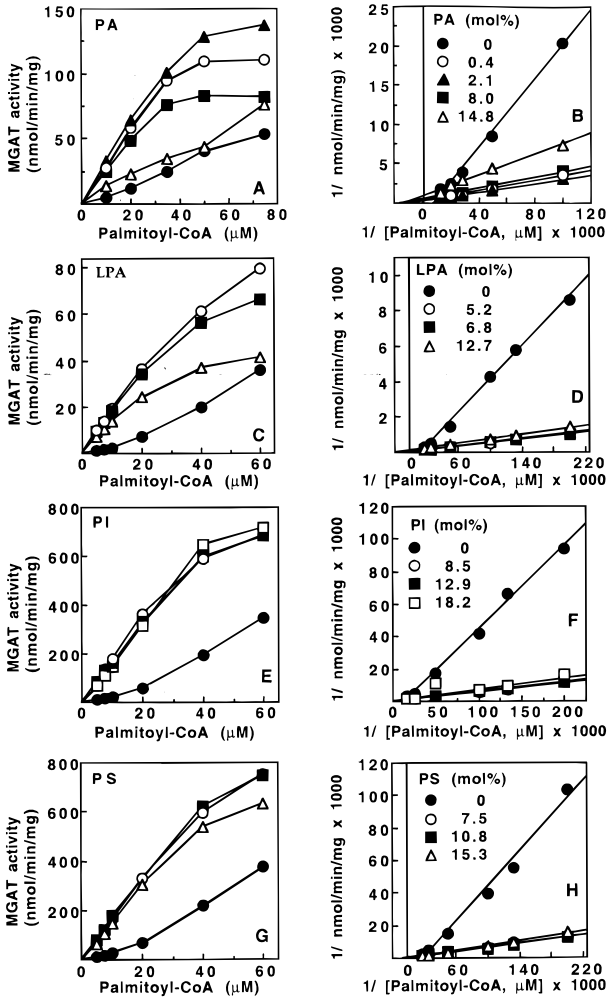

FIGURE 3: Effect of anionic phospholipids on the dependence on palmitoyl-CoA. MGAT activity was measured in Triton X-100 micelles with varying concentrations of palmitoyl-CoA in the absence or presence of concentrations of (A and B) phosphatidic acid, (C and D) lysophosphatidic acid, (E and F) phosphatidylinositol, or (G and H) phosphatidylserine as indicated. Panels B, D, F, and H show the double-reciprocal plots of these data. The double-reciprocal plots were analyzed by computer-assisted least-squares analysis, and regression coefficients were greater than 0.97. Panels A−D, G, and H are representative of two to three independent studies using two different enzyme preparations.

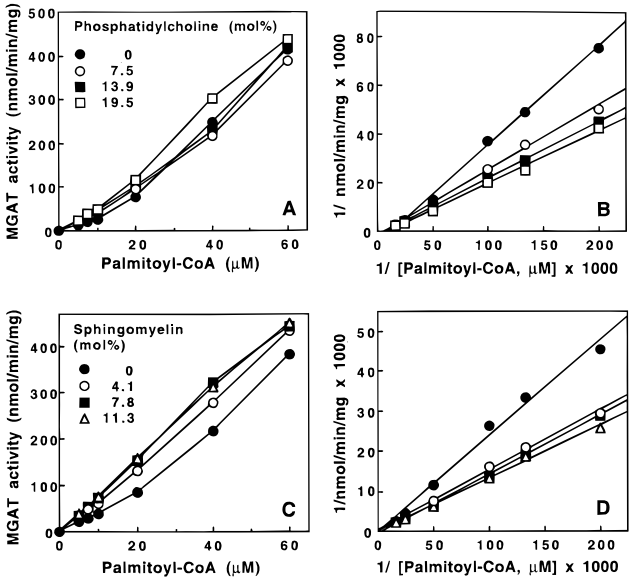

FIGURE 4: Effect of phosphatidylcholine and sphingomyelin on the dependence on palmitoyl-CoA. MGAT activity was measured in Triton X-100 micelles with varying concentrations of palmitoyl-CoA in the absence or presence of different concentrations of (A and B) phosphatidylcholine or (C and D) sphingomyelin as indicated. Panels B and D show the double-reciprocal plots of these data. The double-reciprocal plots were analyzed by computer-assisted least-squares analysis, and regression coefficients were greater than 0.98.

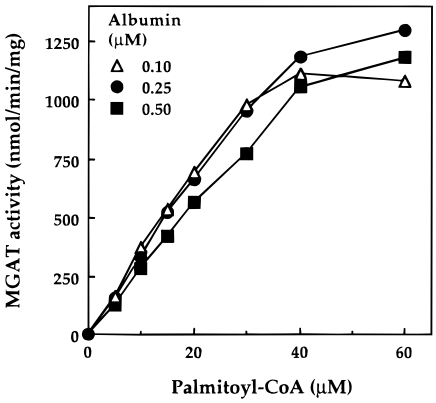

FIGURE 5: Effect of bovine serum albumin on the dependence on palmitoyl-CoA. MGAT activity was measured in Triton X-100 micelles with varying concentrations of palmitoyl-CoA in the presence of activating 8.33 mol % phosphatidylcholine/phosphatidylserine (1:1, w/w) as described in Experimental Procedures. Activity was measured in the absence or presence of the indicated concentrations of bovine serum albumin.

screens surface charges, neomycin prevents ionic interactions by binding to negatively charged phospholipids (Palmer, 1981), and Ca^{2+} can form divalent metal ion bridges between enzymes and negatively charged phospholipids and can screen negatively charged phospholipids. Because neither KCl, neomycin, nor $CaCl_2$ is able to block the MGAT activation attributable to phospholipids, we concluded that phospholipid activation does not occur via electrostatic interactions (Bhat et al., 1995).

In order to determine whether the effects of fatty acid on MGAT activity occurred by means of electrostatic interactions, we tested the ability of KCl, neomycin, and Ca^{2+} to block the ability of low concentrations of oleic acid to activate MGAT and the ability of high concentrations of oleic acid to inhibit MGAT activity. Although salts can also induce phase changes in phospholipid vesicles (Papahadjopoulos et al., 1977), micelles do not generally undergo phase changes. Partially purified MGAT in mixed micelles was incubated with no addition or with activating (2.5 mol %) or inhibiting (20.5 mol %) amounts of oleic acid in the presence of various concentrations of $CaCl_2$ (Figure 7A). Both with no fatty acid present and with an activating amount of fatty acid, MGAT activity decreased about 40% in the presence of 5 mM $CaCl_2$. However, $CaCl_2$ was able to block the ability of 20.5 mol % oleic acid to inhibit MGAT activity. Although these data suggested that inhibition of MGAT activity might occur via ionic interactions, similar studies using KCl and neomycin did not confirm this interpretation. KCl at concentrations up to 1 M inhibited MGAT in both the presence and the absence of oleic acid, and neomycin at concentrations up to 2 mM had little effect (Figure 7B,C). A more likely possibility is that the presence of $CaCl_2$ relieved inhibition by precipitating excess oleic acid as a calcium soap.

Because we could not attribute the effect of $CaCl_2$ to its ability to block electrostatic interactions, we wondered

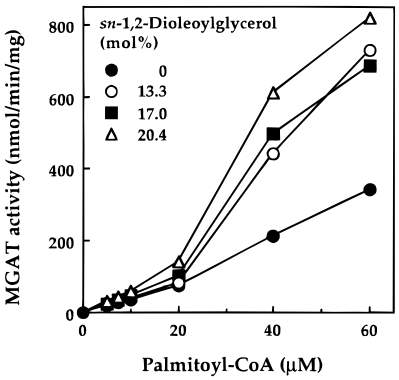

FIGURE 6: Effect of *sn*-1,2-dioleoylglycerol on the dependence on palmitoyl-CoA. MGAT activity was measured in Triton X-100 micelles with varying concentrations of palmitoyl-CoA in the absence of activating phospholipids as described in Experimental Procedures. Activity was measured in the absence or presence of concentrations of *sn*-1,2-dioleoylglycerol as indicated.

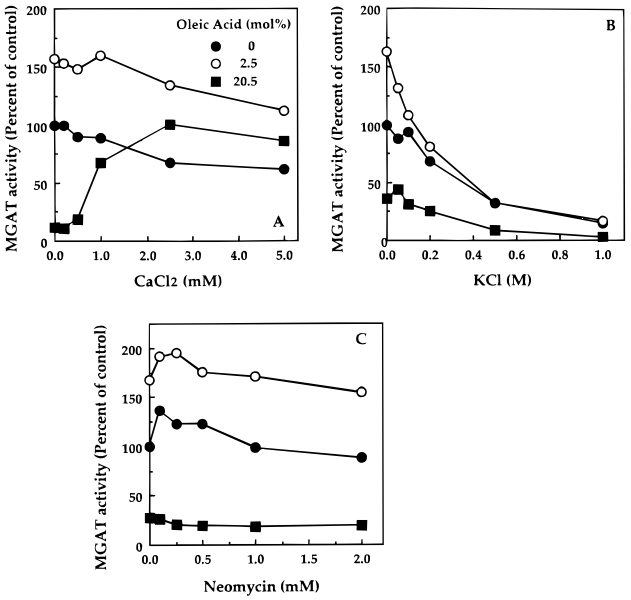

FIGURE 7: Effect of blocking ionic interactions on the stimulation and inhibition of MGAT by oleic acid. MGAT activity was measured in Triton X-100 micelles with 25 μM palmitoyl-CoA in the absence of activating phospholipids as described in Experimental Procedures. The effect of (A) $CaCl_2$, (B) KCl, and (C) neomycin on MGAT activity was measured in the absence or the presence of 0, 2.5, or 20.5 mol % oleic acid as indicated. This figure is representative of two independent studies using two different enzyme preparations.

whether calcium might play a specific role in MGAT inhibition. $CaCl_2$ did not, however, have any effect on the ability of sphingosine to inhibit MGAT activity (Figure 8).

DISCUSSION

Intrinsic membrane-bound enzymes of glycerolipid synthesis interact with their hydrophobic or amphipathic substrates at the surface or within the plane of the membrane. In the pathway of triacylglycerol biosynthesis, one must envisage fatty acids, acyl-CoAs, lysophosphatidate, phosphatidate, monoacylglycerol, and diacylglycerol anchored to the membrane by their acyl chains. How do the enzymes that use these substrates interact with their substrates within the forest of phospholipid acyl chains that comprise the membrane? How do lipid mediators interact with enzymes within the bulk membrane phospholipid? Although mixed

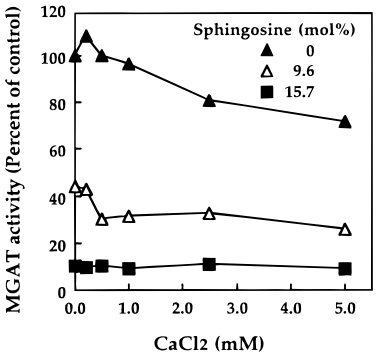

FIGURE 8: Effect of $CaCl_2$ on the inhibition of MGAT by sphingosine. MGAT activity was measured in Triton X-100 micelles with 25 μM palmitoyl-CoA in the absence of activating phospholipids as described in Experimental Procedures. The effect of $CaCl_2$ on MGAT activity was measured in the absence or the presence of two different concentrations of sphingosine as indicated.

micellar assays have facilitated the analysis of enzyme kinetics regarding substrate specificity and the regulation of activity by lipid activators or inhibitors (Carman et al., 1995), many important questions remain unanswered.

MGAT is among the first of the acyltransferases to have been purified sufficiently to be studied in mixed micelles. We previously reported that MGAT is activated by anionic phospholipids which increase both the apparent V_{max} and the apparent K_m with respect to *sn*-1-monooleoylglycerol (Bhat et al., 1994). The enzyme is also activated by its product *sn*-1,2-diacylglycerol but not by *sn*-1,3-diacylglycerol or by the ether analogs of diacylglycerol (Bhat et al., 1994). We have also reported that the ability of sphingolipids to inhibit MGAT activity requires a free amino group and a long-chain hydrocarbon, that inhibition by sphingosine is competitive with respect to activation by phosphatidic acid, phosphatidylinositol, or phosphatidylserine, and that sphingosine and sphinganine can inhibit MGAT activity in cultured hepatocytes from neonatal rats (Bhat et al., 1995). These studies suggested that the anionic phospholipids might activate MGAT at a site that is competitively blocked by sphinolipids. Activation by phospholipids does not result from ionic interactions because Ca^{2+}, neomycin, and KCl have similar effects on the enzyme activity whether phosphatidic acid, the most potent activator, is present (Bhat et al., 1995).

The data presented in this study showing that MGAT dependence on palmitoyl-CoA was highly cooperative allow us to infer that the amount of acyl-CoA available to the enzyme could play a major role in determining MGAT activity *in vivo*. Hepatic concentrations of long-chain acyl-CoA have been reported to be in the range of 15−45 nmol/(g wet wt) [reviewed in Bretcher (1983)] or 94.3 nmol/(g dry liver weight) in fed rats (Corkey, 1988) with a 2.3−4-fold increase after fasting (Corkey, 1988; Bretcher, 1983; Tardi, 1992). These data allow a calculation of acyl-CoA of 0.19−0.76 mol % in membranes.[5]

The amount of nonesterified fatty acid in the endoplasmic reticulum is not known. We found 32.6 nmol of fatty acid per micromole of phospholipid in liver from 9-day-old rats (approximately 3 mol %). Like the acyl-CoAs, cytosolic fatty acid is probably bound to fatty acid binding protein, and the partitioning between cytosol and membranes is unknown, although most of the fatty acid is probably in membranes. Additionally, nonesterified long-chain fatty acid may not be evenly distributed among all membranes.

Unless a specific mechanism exists to bring substrates directly to the enzyme, MGAT would normally be functioning in microsomes that contain very low amounts of its monoacylglycerol (Xia et al., 1993) and acyl-CoA substrates. At the low acyl-CoA concentrations that are likely to exist in the endoplasmic reticulum, relatively small changes in the membrane content of long-chain fatty acid could alter palmitoyl-CoA kinetics dramatically. At 0.8 and 3.1 mol % oleic acid (25 and 100 μM if assumed to be dispersed throughout the entire assay solution and not restricted to the micelle), MGAT activity in the presence of 10 μM palmitoyl-CoA increased 2- and 4-fold, respectively. Thus, one can envisage a situation in which a rapid influx of fatty acid into hepatocytes might enhance the incorporation of existing acyl-CoAs into glycerolipids, thereby releasing more free CoA required by acyl-CoA synthase to activate the additional fatty acids.

The phospholipid microenvironment of the endoplasmic reticulum could play an additional critical role in regulating MGAT activity. Several specific phospholipids lower MGAT's apparent K_m for palmitoyl-CoA. Phosphatidic acid, lysophosphatidic acid, phosphatidylserine, and phosphatidylinositol altered palmitoyl-CoA kinetics such that they could be subjected to Michaelis−Menton analysis. Even then, the apparent K_m values obtained were more than 10-fold higher than the range of probable acyl-CoA concentrations in microsomal membranes (Table 1). At very low acyl-CoA concentrations, however, small amounts of each of these anionic phospholipids would be able to dramatically increase MGAT activity.

An altered K_m for palmitoyl-CoA in the presence of fatty acid and anionic phospholipids suggests that both long-chain acyl-CoAs and lipid cofactors may induce a conformational change in MGAT, thereby altering the enzyme's affinity for its substrate. Altered substrate kinetics have been reported in other mixed micellar studies of membrane-associated enzymes. In Triton X-100 micelles, EGF-mediated phosphorylation of phospholipase C-γ1 allosterically modifies the enzyme (Wahl, 1992). The control enzyme shows sigmoidal kinetics with respect to its phosphatidylinositol 4,5-bisphosphate substrate, but after activation by EGF, the enzyme exhibits Michaelis−Menton kinetics. Similarly, the activity of *Escherichia coli sn*-1,2-diacylglycerol kinase is altered by lipid cofactors; both its substrate *sn*-1,2-diacylglycerol and the nonsubstrate *sn*-1,3-diacylglycerol alter the enzyme's dependence on *sn*-1,2-diacylglycerol (Walsh & Bell, 1986). These investigators concluded that activation occurred after the lipids had changed the enzyme's conformation (Walsh, 1986). Several groups have shown that specific lipids can alter the affinity of protein kinase C for its activators. For example, arachidonic acid allosterically enhances the affinity of PKCα for *sn*-1,2-diacylglycerol (MacEwan et al., 1993), *sn*-1,2-diacylglycerol reduces the mole percent of phosphatidylserine needed for maximal activation (Hannun et al., 1985), and oleate acts as a cofactor in allowing diacylglycerol to activate protein kinase C when phospholipid cofactors are absent (Touney et al., 1990). Thus, a number of enzymes that require lipid cofactors have similar properties, suggesting that similar kinds of lipid−protein interactions may occur.

Our previous studies of MGAT in Triton X-100/phospholipid mixed micelles have strongly suggested that this microsomal enzyme can be regulated by its phospholipid microenvironment and by lipid second messengers such as phosphatidic acid, *sn*-1,2-diacylglycerol, and sphingoid bases (Bhat et al., 1994, 1995). In the present study, we show that the apparent K_m of MGAT for its palmitoyl-CoA substrate can be altered dramatically by small changes in the concentration of long-chain fatty acids or specific anionic phospholipids, including phosphatidic acid, a putative intracellular second messenger (Lee & Bell, 1989; Hannun & Bell, 1987; Bell & Burns, 1991). These data further support the hypothesis that the synthesis of glycerolipids via the monoacylglycerol pathway may be highly regulated in neonatal animals (Coleman & Haynes, 1984), in diabetes (Mostafa et al., 1993), and in hibernators (Xia et al., 1993). Regulation could occur via a variety of lipid second messengers as well as by the influx of fatty acids derived from high-fat diets or from fatty acids derived from the hydrolysis of adipocyte triacylglycerol during fasting or diabetes.

[5] One assumes similar increases with increased flux of fatty acids into the liver from the high fat diet of the neonate. If we assume that total lipid is 4.5% of dry weight (Russel, 1989), then the content is 94.3 nmol of acyl-CoA per 45 mg of total lipid. Assuming that 81% of the lipid is phospholipid, then each gram of dry weight contains 36.45 mg of phospholipid. Distrubuted throughout all liver membranes, this comes out to 0.19 mol % acyl-CoA in the fed state and as much as 0.76 mol % in the fasting state. However, one cannot assume that acyl-CoAs are distributed in all membranes or homogeneously within any single membrane. No acyl-CoA is detected in cell cytosol, and it is likely that virtually all acyl-CoA is associated with cell membranes (Bretcher, 1983). Cytosolic acyl-CoA binding protein may alter the partitioning of acyl-CoAs between cytosol and specific membranes or membrane regions (Rasmussen, 1994), or specific factors may increase acyl-CoA concentrations in a region of the endoplasmic reticulum that is rich in acyltransferases. Further, the concentrations of both fatty acid and acyl-CoAs are likely to be higher in neonatal rat liver because of the larger influx of milk lipid, of which 70% of the calories are derived from triacylglycerol (Jones & Rolph, 1985; Girard et al., 1992).

ACKNOWLEDGMENT

We thank Dr. Patricia A. Gallagher for quantifying rat liver free fatty acids.

REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.
Bell, R. M., & Burns, D. J. (1991) *J. Biol. Chem. 266*, 4661−4664.
Bhat, B. G., Bardes, E. S.-G., & Coleman, R. A. (1993) *Arch. Biochem. Biophys. 300*, 663−669.
Bhat, B. G., Wang, P., & Coleman, R. A. (1994) *J. Biol. Chem. 269*, 13172−13178.
Bhat, B. G., Wang, P., & Coleman, R. A. (1995) *Biochemistry 34*, 11237−11244.
Bishop, W. R., Ganong, B. R., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 6993−7000.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.
Brecher, P. (1983) *Mol. Cell. Biochem. 57*, 3−15.
Carman, G. M., Deems, R. A., & Dennis, E. A. (1995) *J. Biol. Chem. 270*, 1−4.
Coleman, R. A. (1988) *Biochim. Biophys. Acta 963*, 367−374.
Coleman, R. A., & Haynes, E. B. (1984) *J. Biol. Chem. 228*, 915−922.
Coleman, R. A., Walsh, J. P., Millington, D. S., & Maltby, D. A. (1986) *J. Lipid Res. 27*, 158−165.
Coleman, R. A., Haynes, E. B., & Coats, C. D. (1987) *J. Lipid Res. 28*, 320−325.
Corkey, B. E. (1988) *Methods Enzymol. 166*, 55−69.
Emmison, N., Gallagher, P., & Coleman, R. A. (1995) *Am. J. Physiol. 269*, R80−R86.

Fletcher, M. J. (1968) *Clin. Chim. Acta 22*, 393−397.
Girard, J., Ferré, P., Pégorier, J.-P., & Duée, P.-H. (1992) *Physiol. Rev. 72*, 507−562.
Hannun, Y. A., & Bell, R. M. (1987) *Science 235*, 670−674.
Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1985) *J. Biol. Chem. 261*, 7184−7190.
Hjelmstad, R. H., & Bell, R. M. (1991) *Biochemistry 30*, 1731−1740.
Johnston, J. J. (1977) in *Lipid Metabolism in Mammals* (Snyder, F., Ed.) pp 151−187, Plenum, New York.
Jones, C. T., & Rolph, T. P. (1985) *Physiol. Rev. 65*, 357−430.
Kaluzny, M. A., Duncan, L. A., Merritt, M. V., & Epps, D. E. (1985) *J. Lipid Res. 26*, 135−140.
Korn, E. D. (1969) *Annu. Rev. Biochem. 38*, 263−288.
Lee, M.-H., & Bell, R. M. (1989) *J. Biol. Chem. 264*, 14797−14805.
Litchenberg, D., Robson, R. J., & Dennis, E. A. (1983) *Biochim. Biophys. Acta 737*, 285−304.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
MacEwan, D. J., Mitchell, R., Johnson, M. S., Thomson, F. J., Lutz, E. M., Clegg, R. A., & Connor, K. (1993) *Eur. J. Pharmacol. 246*, 9−18.
Merrill, A. H., Jr., Gidwitz, S., & Bell, R. M. (1982) *J. Lipid Res. 9*, 786−791.
Mostafa, N., Bhat, B. G., & Coleman, R. A. (1993) *Biochim. Biophys. Acta 1169*, 189−195.
Palmer, F. B. St. C. (1981) *J. Lipid Res. 22*, 1296−1300.
Papahjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K.; Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579−598.
Polheim, D., David, J. S. K., Schultz, F. M., Wylie, M. B., & Johnston, J. M. (1973) *J. Lipid Res. 14*, 415−421.
Rasmussen, J. T., Faergeman, N. J., Kristiansen, K., & Knudsen, J. (1994) *Biochem. J. 299*, 165−170.
Robson, R. J., & Dennis, E. A. (1983) *Acc. Chem. Res. 16*, 251−258.
Russel, J. (1989) *J. Lipid Res. 30*, 877.
Sansbury, K., Millington, D. S., & Coleman, R. A. (1989) *J. Lipid Res. 30*, 1251−1258.
Tardi, P. G., Mukherjee, J. J., & Choy, P. C. (1992) *Lipids 27*, 65−67.
Touny, S. E., Khan, W., & Hannun, Y. (1990) *J. Biol. Chem. 265*, 16437−16442.
Wahl, M. I., Jones, G. A., Nishibe, S., Rhee, S. G., & Carpenter, G. (1992) *J. Biol. Chem. 267*, 10447−10456.
Walsh, J. P., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 15062−15069.
Xia, T., Mostafa, N., Bhat, B. G., Florant, G. L., & Coleman, R. A. (1993) *Am. J. Physiol. 265*, R414−R419.

BI9602167