Ontogeny of hepatic *sn*-1,2-diacylglycerol content and protein kinase C activity in the neonatal rat: lack of concordance

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Altered activity of protein kinase C has commonly been related to activator-induced changes in cellular sn-*1,2-diacylglycerol (1,2-DAG) concentration. In neonatal liver 1,2-DAG can be synthesized by the developmentally expressed monoacylglycerol acyltransferase (MGAT) activity (EC 2.3.1.22). Rat liver homogenates were examined on selected days after birth to determine whether the high MGAT activity present in neonatal rat liver was associated with high 1,2-DAG concentrations and altered protein kinase C activity and location. Although MGAT specific activity peaked between days 5 and 12, 1,2-DAG concentrations declined 63% between days 1 and 10, and the activity and membrane location of protein kinase C activity remained unchanged. Liver triacylglycerol content changed little during this time period, but the phospholipid and ceramide content of liver increased about 60 and 100%, respectively. Thus, changes in cell membrane 1,2-DAG content may not always be associated with changes in protein kinase C activity because multiple factors (including 1,2-DAG, fatty acids, and sphingosine) modulate the activity of this enzyme. Glycerolipid synthesis is likely to be the primary fate of the 1,2-DAG synthesized by the monoacylglycerol pathway.*

Keywords: protein kinase C; diacylglycerol; ceramide; acyltransferase; ontogeny

Introduction

Cellular diacylglycerol has two distinct functions. Diacylglycerol is a central intermediate in the synthesis of triacylglycerol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid[1]; it also functions as a second messenger in its role as a physiological activator of protein kinase C.[2] Only the *sn*-1,2-stereoisomer of diacylglycerol is active as a second messenger. 1,2-DAG can be formed after phospholipase hydrolysis of membrane phospholipids in response to an extracellular signal[2] or via the sequential acylation of glycerol-3-phosphate.[1] The metabolism of diacylglycerol is more complex in neonatal than in adult liver and most other tissues because a second synthetic pathway is present. This alternate route of diacylglycerol biosynthesis is defined by the presence of monoacylglycerol acyltransferase (MGAT) (EC 2.3.1.22), a microsomal enzyme whose specific activity is as much as 700-fold higher in neonatal rat liver than is observed in the adult[3] and that stereospecifically acylates the *sn*-1 position of *sn*-2-monoacylglycerols.[4] Peak MGAT activity occurs between postnatal days 5 and 12 and then declines 87% by day 21.[3] In in vitro studies, the specific activity of MGAT is about 200-fold higher than the activity of the committed step of the alternate glycerol-3-phosphate pathway.[5] Although the relative activities of enzymes measured in vitro may not accurately reflect their in vivo activities, the monoacylglycerol pathway is likely to constitute a major route for the de novo synthesis of diacylglycerol in the neonate.

The primary function of the 1,2-DAG product of MGAT has not been fully established. We have provided evidence that in the neonatal liver MGAT may function physiologically to ensure the reacylation of those monoacylglycerols that contain essential fatty

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acids.[6] We wondered whether the very high neonatal MGAT activity might also result in increased 1,2-DAG concentrations that would activate protein kinase C.

Activated protein kinase C is believed to be associated primarily with 1,2-DAG in the plasma membrane.[2] Although MGAT activity, like the other major activities of triacylglycerol and phospholipid biosynthesis, is located in the endoplasmic reticulum,[3] the 1,2-DAG that is synthesized in this membrane should be able to move to the plasma membrane. Diacylglycerols can readily traverse membranes[7,8] and have been shown to move rapidly between different cellular membranes.[9] After being activated by 1,2-DAG, membrane-associated protein kinase C has wide-ranging effects, including the enhancement of cell division and differentiation.[2] Unlike the quiescent hepatocytes of adult liver, both cell division and differentiation are prominent in neonatal hepatocytes.[10] We hypothesized that the high peak of MGAT in neonatal rat liver might be associated with altered 1,2-DAG levels and with changes in the translocation of protein kinase C to membrane. To test this hypothesis, we measured rat hepatic 1,2-DAG concentration and protein kinase C activity on selected days during the first 4 weeks of life.

Materials and methods

Lipid extraction and measurements

Timed-pregnant Sprague-Dawley rats were obtained from Zivic Miller (Raleigh, NC USA) and fed Purina Rodent Chow (Purina Mills, Richmond, IN). After birth, rat litters were culled to 12 pups and maintained at 12 pups per litter throughout the study. Pups were weaned on day 20 by being removed from the dams. On selected days, rat pups were decapitated and their livers were rapidly removed and homogenized with 3 mL buffer/g liver. The buffer contained 20 mmol/L Tris-HCl, pH 8.0, 2 mmol/L $MgCl_2$, 0.5 mmol/L EGTA, 2 mmol/L EDTA, 0.25 M sucrose, 0.1% aprotinin, 20 μmol/L leupeptin, 0.1 mmol/L n-tosyl-l-phenylalanine chloromethyl ketone, 5 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride (all obtained from Sigma, St. Louis, MO USA). Homogenates were immediately frozen in liquid N_2 and stored at −80° C.

Total lipids were extracted from liver homogenate.[11] Neutral lipids and phospholipids were separated on Bond Elut aminopropyl columns (Analytichem International, Harbor City, CA USA).[12] The phospholipids were quantified by phosphate analysis[13] using pH 7.0 standard buffer from Fisher Scientific (Pittsburgh, PA USA) as the standard. The triacylglycerol content was determined colorimetrically[14] using glycerol as a standard.

Preparation of sn-*1,2-dioleoylglycerol*

The 1,2-DAG standard was prepared from *sn*-1,2-dioleoylglycerol-3-phosphocholine (Avanti Polar Lipids, Inc, Alabaster, AL USA) by modifying a previously described method.[15] Briefly, 50 mg of the phosphatidylcholine was dried under a stream of nitrogen and then suspended by sonication in 4 mL of 30 mmol/L sodium barbital buffer, pH 7.4 containing 0.12 M NaCl and 1 mmol/L $ZnSO_4$. After adding phospholipase C (approximately 1 U/μmol phosphatidylcholine), the reaction mixture was incubated at 37° C for 15 minutes. The reaction mixture was extracted three times with 3 mL of diethyl ether. The combined ether extracts were evaporated under N_2 and the white residue was extracted twice with 3 mL of heptane. The combined heptane extracts were evaporated under N_2 and dissolved in $CHCl_3$. *sn*-1,2-Dioleoylglycerol was separated from 1,3-dioleoylglycerol by thin layer chromatography on silica gel G plates impregnated with 3% sodium borate (Analtech, Newark, DE USA). The solvent system was $CHCl_3$/acetone/methanol/acetic acid (90:5:2:0.5; vol/vol). After the *sn*-1,2-dioleoylglycerol was eluted from the silica gel and quantified colorimetrically[16] using triolein as the standard, it was stored under N_2 at −20° C and used within 1 month after synthesis.

Measurement of sn-*1,2-diacylglycerol and ceramide*

To determine the content of 1,2-DAG and ceramide, total lipids were freshly extracted from liver homogenates (corresponding to 7.5 mg liver tissue) and assayed within 24 hr. 1,2-DAG and ceramide were quantified using *E. coli* 1,2-DAG kinase (Lipidex Inc, Westfield, NJ USA).[17] The lipid extract was evaporated under N_2 and sonicated in a bath sonicator for 15 sec with 20 μL of solubilization buffer containing 5 mmol/L cardiolipin (Sigma), 1 mmol/L DETAPAC, pH 7.0 (Sigma), and 7.5% β-octylglucopyranoside (Sigma). The extract was then incubated at 23° C for 15 min. The reaction was started by adding 100 μL of assay buffer containing 50 mmol/L imidazole, pH 6.6, 50 mmol/L NaCl, 12.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L [^{32}P]ATP (0.5 μCi), 0.1 mmol/L DETAPAC, 0.31 mg/mL dithiothreitol, and 5 μg 1,2-DAG kinase, and incubated at 23° C for 35 min. To stop the reaction, 3 mL of $CHCl_3$/methanol (1:2; vol/vol) was added and mixed well. Then 1.7 mL of 1% $HClO_4$ and 1 mL of $CHCl_3$ were added to separate the phases. After aspirating the aqueous layer, the organic layer was washed twice with 2 mL of 1% $HClO_4$/methanol (7:1; vol/vol). The organic phase was dried in a Speed Vac concentrator (Savant, Farmingdale, NY USA) and resuspended in 50 μL of $CHCl_3$/methanol (4:1; vol/vol). Thirty μL of the resuspendant was spotted on a Si250-PA, 19C thin layer plate (J.T. Baker, Phillipsburg, NJ USA) and developed in $CHCl_3$/pyridine/88% formic acid (60:30:7; vol/vol). The radioactivity present in phosphatidic acid and in ceramide-1-P on the thin layer plate was measured on a System 200 Imaging Scanner (Bioscan, Inc, Washington, DC USA). The freshly synthesized 1,2-DAG standard was assayed under identical conditions. The recovery of the 1,2-DAG standard through the lipid extraction procedure was 95 to 100%. The standard curve was assayed in duplicate, and samples were assayed in triplicate. Ceramide was measured simultaneously by the same procedure; ceramide is a substrate of *E. coli* 1,2-DAG kinase, and the ceramide 1-phosphate product is well separated from phosphatidic acid on thin layer chromatography.[17] The R_f for phosphatidic acid was 0.71 and the Rf for ceramide 1-phosphate was 0.54.

Protein kinase C assay

Liver homogenate was separated into cytosol and total particulate fractions by ultracentrifugation at 100,000*g* for 60 min. To partially purify protein kinase C, each fraction was solubilized with 1% Nonidet-P40 (Sigma) and 1% CHAPSO (Boehringer Mannheim, Indianapolis, IN USA) and sonicated for five pulses (225R, 30% duty cycle, output = 3, Heat Systems-Ultrasonics Inc, Plainview, NY USA) followed by a 60-min incubation on ice. Then 600 μL (0.07g/mL) of DEAE-52 cellulose (Whatman, Maidstone, UK) suspension was added to 1.5 mL microcentrifuge tubes and washed four times with 600 μL washing buffer containing 20 mmol/L Tris-HCl, pH 7.5, 2

mmol/L EDTA, and 0.5 mmol/L EGTA. The sonicated sample was applied to DEAE-52 cellulose. After washing twice with 600 μL of washing buffer, protein kinase C was eluted with 300 μL then 150 μL of elution buffer (washing buffer plus 0.15 M NaCl) and the eluates were combined. Protein concentration was assayed by the method of Lowry et al. using bovine serum albumin as the standard.[18] Protein kinase C activity was assayed by the phosphorylation of histone III-S (Sigma).[19] Nonspecific protein phosphorylation was measured by replacing the protein kinase C activators, phosphatidylserine, phorbol 12-myristate 13-acetate (Sigma), and Ca^{++}, with 2.5 mmol/L EGTA in 20 mmol/L Tris, pH 7.5. Blanks without enzyme were employed for each condition. All samples were assayed in triplicate in the presence and absence of activators. The reaction mixture was incubated at 32° C for 7 min and stopped by adding 500 μL of 20% trichloroacetic acid and 200 μg of bovine serum albumin. A 30-min incubation on ice allowed complete protein precipitation. Precipitated protein was filtered with a Skatron semiautomatic cell harvester (Skatron Instruments, Sterling, VA) with 12 cells and washed with 7.5% trichloroacetic acid. The filter was counted with a Bioscan System 200 Imaging Scanner (Bioscan, Inc, Washington, DC).

Monoacylglycerol acyltransferase assay

MGAT was measured as previously described using 50 μmol/L *sn*-,2-monooleoylglycerol and 25 μmol/L [^{3}H]palmitoyl-CoA except that $MgCl_2$ was omitted from the assay.[3]

Data analysis

The data were subjected to one-way analysis of variance followed by Scheffé's test.[20]

Results and discussion

MGAT activity and sn-*1,2-diacylglycerol concentrations*

To determine whether peak expression of MGAT activity between days 5 and 12 is accompanied by an increase in 1,2-DAG, livers were analyzed on days 1, 3, 7, 10, 20, and 30 after birth. Confirming previous results,[1] MGAT specific activity in homogenates from these samples peaked between days 7 and 10 (45.5 ± 6.6 nmol/min/mg protein on day 7) compared with the activity on day 30 (1.2 ± 0.2 nmol/min/mg protein) *(Figure 1)*. The peak specific activities from liver homogenates are about 42% less than those reported in liver total particulate preparations.[3] Despite the high MGAT activity during the first 10 days after birth, hepatic 1,2-DAG concentration decreased 63% ($P < 0.001$) from 18.8 ± 4.2 to 7.0 ± 1.5 pmol/nmol phospholipid during the same period, and then was unchanged through day 30 *(Figure 2)*. We report 1,2-DAG as a function of cellular phospholipid content because 1,2-DAG is located in cell membranes and because the activation and translocation of protein kinase C depend on the amount of 1,2-DAG in membrane. During the same time period, the phospholipid content of neonatal liver increased about 50% (see below), consistent with postnatal increases in intracellular membranes and organelles.[10,21] This increase in cellular phospholipid was not sufficient to explain the decline in 1,2-DAG content; when the data were analyzed as nmol of 1,2-DAG/mg liver, there was still a 47% decrease in 1,2-DAG concentration at day 10 ($P < 0.01$) (*Figure 2*, inset). The 1,2-DAG content reported per mg of liver at 20 days is similar to that previously reported from liver of 3-week-old rats.[22]

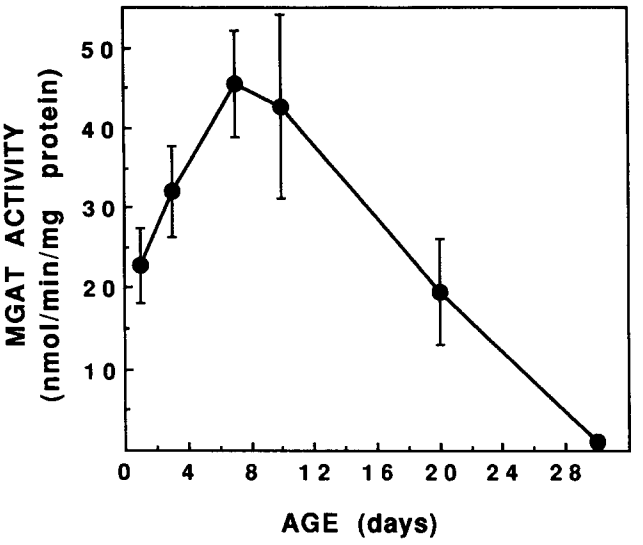

Figure 1 Ontogeny of monoacylglycerol acyltransferase in activity homogenates from postnatal rat liver. MGAT was assayed as described under Materials and methods. Each value represents the mean ± SD from nine livers on day 1, and six livers on each of the subsequent days.

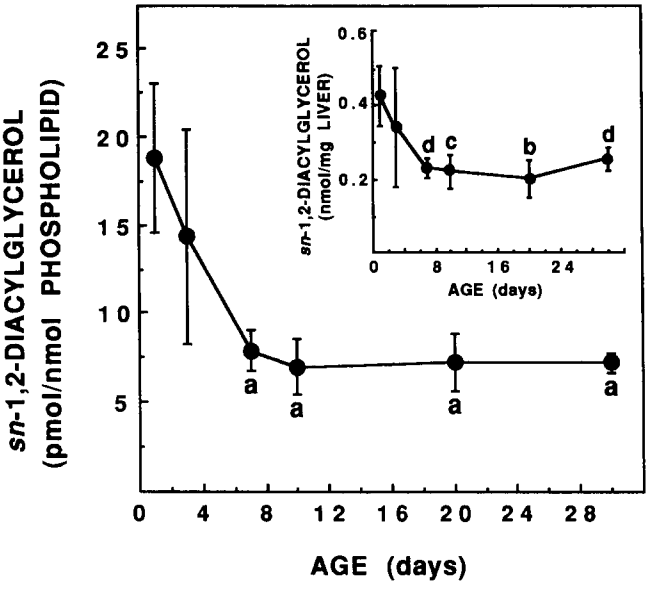

Figure 2 Ontogeny of *sn*-1,2-diacylglycerol content in developing rat liver. 1,2-DAG content (pmol/nmol phospholipid) was quantified as described under Materials and methods. Each value represents the mean ± SD from nine livers on day 1, and six livers on each of the subsequent days. Each liver homogenate was measured in triplicate. The inset represents the same measurements plotted as nmol/mg liver. Statistical significance compared to day 1: a, $P < 0.001$; b, $P < 0.005$; c, $P < 0.01$; d, $P < 0.05$.

Protein kinase C

Protein kinase C activities in cytosolic and membrane fractions were measured after partial purification by DEAE-52 cellulose adsorption. It is believed that membrane binding is essential for activation of protein kinase C.[2] Thus, the translocation of protein kinase C from a

cytosolic to a membrane fraction indicates that the kinase has been activated. Neither the membrane-bound nor the cytosolic protein kinase C activity changed during the neonatal period, indicating a lack of net change in activation and translocation *(Figure 3)*. Because membrane 1,2-DAG content decreased 63% during this time, one would have expected a concomitant change in protein kinase C activity, unless the 1,2-DAG of postnatal liver remained in a compartment that is inaccessible to protein kinase C. Alternatively, the effects of changes in 1,2-DAG may have been offset by changes in other modulators of protein kinase C, such as sphingosine or free fatty acids.[2]

In adult rat liver, the predominant isoforms of protein kinase C are α, δ, ζ,[23,24] and, perhaps, β.[25] Neonatal hepatic protein kinase C activity and isoforms have not been fully studied. Although two reports are available, one does not present data from the first 3 weeks after birth,[26] and the other measures only the cytosolic activity, which probably reflects the pool of unactivated enzyme.[27] Our values for protein kinase C specific activity are more than ten-fold higher than these measurements by other laboratories that did not partially purify the enzyme.[26,27] Because protein kinase C is purified 11-fold by the DEAE-52 cellulose column chromatography,[19] it is likely that the differences in specific activity are due to the relative purity of the protein kinase C preparations. Not only is protein kinase C activity low in crude cellular extracts, but additionally the unpurified extracts contain unknown amounts of Ca^{2+} that may modify protein kinase C activity.

Protein kinase C is activated physiologically by acute increases in cellular 1,2-DAG. These increases arise by phospholipase-mediated hydrolysis of membrane phosphatidylinositol and phosphatidylcholine.[2] Few studies

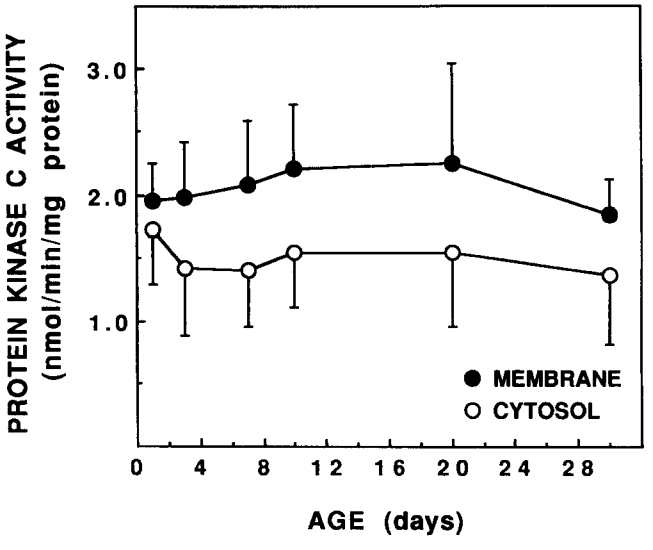

Figure 3 Ontogeny of protein kinase C activity in developing rat liver. Cytosolic and membrane-associated protein kinase C activities were measured as described under Materials and methods. Each value represents the mean ± SD from nine livers on day 1, and six livers on each of the subsequent days. Each liver sample was assayed in triplicate.

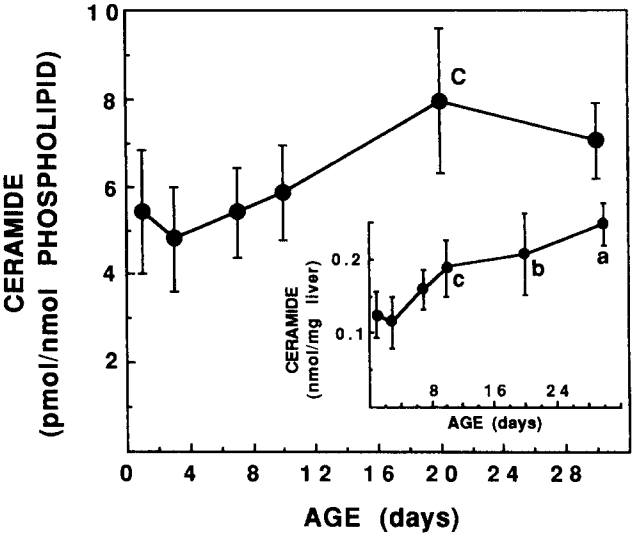

Figure 4 Ontogeny of ceramide content in developing rat liver. Ceramide content was quantified as described under Materials and methods. Each value represents the mean ± SD from nine livers on day 1, and six livers on each of the subsequent days. Each liver sample was assayed in triplicate. The inset represents the same measurements plotted as nmol/mg liver. Statistical significance compared to day 1: a, $P < 0.001$; b, $P < 0.005$; c, $P < 0.05$.

have examined simultaneously both the cellular concentration of diacylglycerol and the activity and subcellular location of protein kinase C. In postnatal liver we were surprised to find a lack of concordance between the cellular 1,2-DAG concentration and protein kinase C activity and location. The changes we observed in cellular 1,2-DAG concentrations were equal to those that have been associated with profound alterations in protein kinase C-mediated events.[28] Lack of concordance between cellular 1,2-DAG concentration and protein kinase C activation has previously been reported in Rat-6 PKC-3 fibroblasts that overexpress the β1 isozyme of PKC[29] and in α-thrombin-stimulated fibroblasts.[30]

Ceramide concentrations

Interest in sphingolipids has grown recently because ceramide and its metabolite sphingosine modulate cellular functions,[31] including cell differentiation,[32] inhibition of mammalian diacylglycerol kinase,[33] inhibition of protein kinase C,[34] and release of intracellular Ca^{++}.[35] In neonatal rats, the cellular ceramide concentration increased from 5.4 ± 1.4 to 7.9 ± 1.6 pmol/nmol phospholipid between days 1 and 20 after birth, but only reached statistical significance on day 20 ($P < 0.01$) *(Figure 4)*. The ceramide concentration, as expressed per mg of liver, doubled by day 30; the rate of increase was greatest between days 3 and 10 (63% increase) (*Figure 4* inset). Because the ceramide concentrations correlated highly with hepatic phospholipid concentrations (positive correlation coefficient was 0.74 [data not shown]), the increase in ceramide appeared to be predominantly a function of membrane growth. The ceramide concentration observed at 20 days is similar to that reported by others for liver from 3-week-old rats.[22]

Hepatic triacylglycerol and phospholipid content

The monoacylglycerol substrate of MGAT may originate from incomplete lysosomal hydrolysis of the triacylglycerol that enters in chylomicra remnants. Our previous study showed that *sn*-2-monoacylglycerol is the major hydrolytic product (69%) of 1,2-DAG after hydrolysis by lysosomal lipase.[14] Because neonatal rats have a significant chylomicronemia,[36] the monoacylglycerol substrate is unlikely to be limiting. What then is the fate of the diacylglycerol synthesized by MGAT activity? Triacylglycerol and phospholipid were measured in postnatal liver samples to determine whether there were net increases in these potential diacylglycerol products.

Although the liver triacylglycerol content decreased 40% between postnatal days 1 and 30 *(Figure 5)*, these changes were not statistically significant because of the large variation in triacylglycerol among the pups within each group. Others have reported a 30% decrease in rat liver triacylglycerol observed between postnatal days 1 and 20.[37,38] In contrast, a single report describes an extremely high hepatic triacylglycerol content within 1–2 days after birth (30 μg/mg liver), followed by a 90% decrease during the first 10 days after birth.[36] We cannot explain why these investigators found liver triacylglycerol content to be 3.6-fold higher than we and others observed. It is important to note that neonatal fatty liver can result from malnutrition or iron deficiency.[38] We found no evidence for a net gain in hepatic triacylglycerol per mg of liver during the neonatal period, although the neonatal rat secretes hepatic very low density lipoprotein relatively poorly.[39]

In contrast, the hepatic phospholipid content/mg liver increased about 40% between postnatal days 3 and 10 *(Figure 6)*. The rate of increase then slowed so that between day 10 and day 30 phospholipid increased an additional 10%. In addition, total liver weight increased 20-fold between days 1 and 30, necessitating a very large net synthesis of phospholipid. These changes in phospholipid content correlate inversely with the hepatic 1,2-DAG content, suggesting that a major fate of diacylglycerol in neonatal rat is its utilization as a precursor for cellular phospholipids.

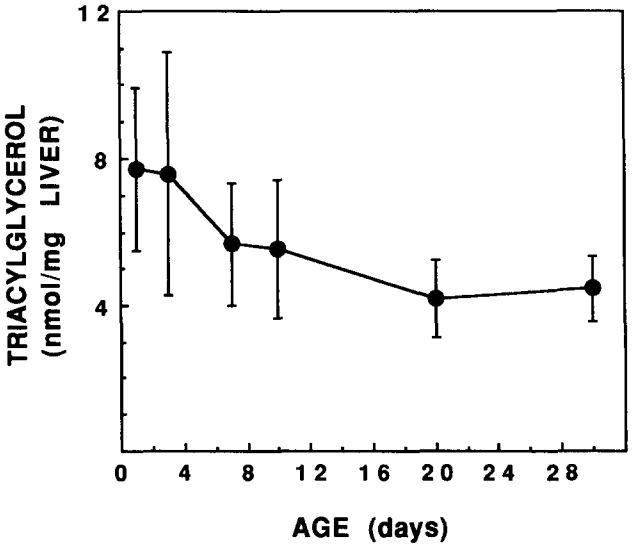

Figure 5 Ontogeny of triacylglycerol content in developing rat liver. Triacylglycerol content was quantified from the neutral lipid fraction as described under Materials and methods. Each value represents the mean ± SD from nine livers on day 1, and six livers on each of the subsequent days. Each liver sample was assayed in duplicate.

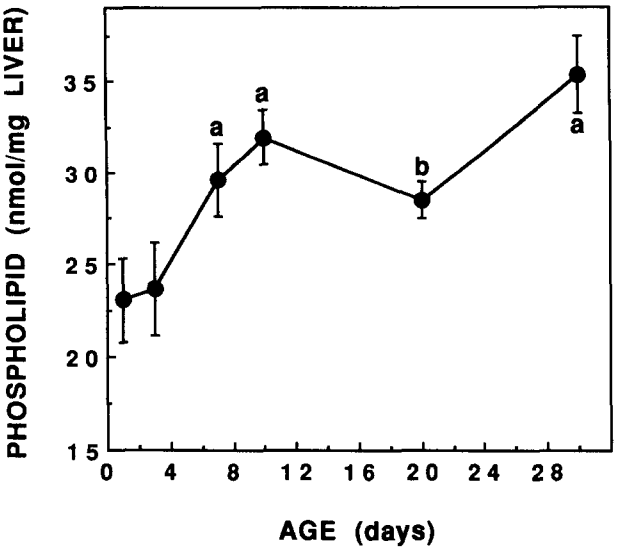

Figure 6 Ontogeny of phospholipid content (nmol/mg liver) in developing rat liver. Phospholipid content was quantified from the total lipid extract as described under Materials and methods. Each value represents the mean ± SD from nine livers on day 1, and six livers on each of the subsequent days. Each liver sample was assayed in duplicate. Statistical significance compared with day 1: a, $P < 0.001$; b, $P < 0.005$.

Thus, we conclude that any 1,2-DAG that is synthesized by high postnatal MGAT activity appears to be utilized promptly, probably for phospholipid and triacylglycerol synthesis. Although the 1,2-DAG concentration decreases in hepatic membranes, this 60% decrease is not associated with an alteration in the activity or location of protein kinase C. These data suggest that either 1,2-DAG does not always move freely between membranes or that other modulators of protein kinase C play a prominent counter-regulatory role in developing liver.

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