Evidence That Cyclic AMP-induced Inhibition of Phosphatidylcholine Biosynthesis Is Caused by a Decrease in Cellular Diacylglycerol Levels in Cultured Rat Hepatocytes*

(Received for publication, April 26, 1991)

Haris Jamil, Amandip K. Ural†, and Dennis E. Vance§

From the Lipid and Lipoprotein Research Group and Department of Biochemistry, University of Alberta, Edmonton, AB T6G 2S2, Canada

The mechanism by which glucagon and cAMP analogues inhibit phosphatidylcholine biosynthesis was investigated in rat hepatocytes. The studies were facilitated by preparation of an antibody to a synthetic peptide (D-F-V-A-H-D-D-I-P-Y-S-S-A) corresponding to residues 164-176 of CTP:phosphocholine cytidylyltransferase. The antibody, which was purified by affinity chromatography, quantitatively immunoprecipitated cytidylyltransferase from rat liver cytosol. Various analogues of cAMP had no effect on the labeling of cytidylyltransferase with 32P in rat hepatocytes. Nor did the cAMP analogues have any effect on the distribution of cytidylyltransferase between cytosol and membranes. These results indicate that the supply of diacylglycerol does not limit phosphatidylcholine synthesis in hepatocytes treated with cAMP analogues. A decreased supply of diacylglycerol was considered as an alternative mechanism for inhibition of phosphatidylcholine biosynthesis. An approximately 30% decrease in diacylglycerol concentration was observed in hepatocytes treated with the cAMP analogues or glucagon, compared with controls. A similar decrease of phosphatidylcholine biosynthesis was observed. The cAMP-mediated decrease in diacylglycerol levels and inhibition of phosphatidylcholine biosynthesis were reversed by addition of 0.5-1.5 mM oleic acid to the treated hepatocytes. A correlation coefficient of 0.93 was calculated between the levels of diacylglycerol and the rate of phosphatidylcholine biosynthesis. In another approach, the diacylglycerol levels were increased by an inhibitor of diacylglycerol lipase (U-57908) which also reversed the cAMP effects on diacylglycerol levels and phosphatidylcholine biosynthesis. We conclude that the cAMP-mediated inhibition of phosphatidylcholine biosynthesis was not due to an effect on the phosphorylation of cytidylyltransferase. Instead, phosphatidylcholine biosynthesis appears to be inhibited due to a decreased level of diacylglycerol, a substrate for CDP-choline:1,2-diacylglycerol cholinephosphotransferase.

Phosphatidylcholine (PC) is the major phospholipid found in mammalian cells (1) and in rat liver the majority of PC (60-80%) appears to be synthesized via the CDP-choline pathway (2). CTP:phosphocholine cytidylyltransferase (CT), which can catalyze the rate-limiting step of the CDP-choline pathway (3), is located both in cytosol and microsomal membranes (1). In most cases the cytosolic form of CT appears to be an inactive reservoir which can be translocated reversibly to endoplasmic reticulum, where it becomes activated by interaction with phospholipids in the membrane (1). In several cell types an active, high molecular weight form of cytosolic CT has been found (4), but its role in control of PC biosynthesis remains to be established. The regulation of PC biosynthesis via CT translocation has been demonstrated in various cell types (5-10).

The mechanisms by which CT activity is regulated are not completely known. It has been postulated that CT may be regulated by a phosphorylation/dephosphorylation mechanism. Cyclic AMP analogues inhibit PC synthesis in cultured hepatocytes in short term incubations (11). The inhibition of PC biosynthesis is accompanied by partial inactivation of microsomal CT activity and redistribution of CT to cytosol (11). Okadaic acid, a potent protein phosphatase inhibitor, also inhibits PC synthesis in rat hepatocytes and increases cytosolic CT activity with a corresponding decrease in activity on the membrane fraction (12). Furthermore, in vitro experiments provided similar translocation patterns. For example, when a post-mitochondrial fraction from rat liver was incubated under phosphorylating conditions with either NaF or okadaic acid (13), CT activity in the microsomes was decreased with a corresponding increase in cytosolic activity. The translocation was reversed under dephosphorylating conditions by the addition of alkaline phosphatase (14). In a more direct approach serine residue(s) of pure rat liver CT were substrates for cAMP-dependent protein kinase (14). Hence, a plausible mechanism for inhibition of PC biosynthesis would be that the cAMP-dependent protein kinase phosphorylates CT, which decreases the binding affinity of the enzyme for the membranes and releases CT into the cytosol (1). Whether or not such a mechanism operates in intact cells has been an open question.

Glucagon, a hormone that elevates cAMP levels, has been shown to inhibit PC synthesis in hepatocytes without an effect on the subcellular distribution of CT (15). In other

*This work was supported by a grant from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Supported by a Graduate Assistantship award by the Department of Biochemistry, University of Alberta, Edmonton, Alberta.

§Medical Scientist of the Alberta Heritage Foundation for Medical Research.

The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CT, CTP:phosphocholine cytidylyltransferase; DG, diacylglycerol; CPTcAMP, 8-[4-chlorophenylthio]-adenosine-3':5'-monophosphate; bromo-cAMP, 8-bromoadenosine-3':5'-monophosphate; butyryl CAMP, N°, O°- dibutyryl adenosine-3':5'-monophosphate; EGTA, (ethylenebis(oxyethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate.
studies van Golde and colleagues (16) have reported that cellular DG levels decreased in hepatocytes treated with glucagon and at the same time PE biosynthesis was inhibited. They proposed that the inhibition of PE biosynthesis might have been due to a decrease in the supply of DG. Kolesnik (17) reported that exogenously added DG overcame cAMP-mediated inhibition of PC synthesis in GH3 pituitary cells. Furthermore, the supply of DG can regulate PC biosynthesis in permeabilized HeLa cells (18), and DG facilitates CT binding to membranes (6). Hence, a decrease in DG, as a result of elevated levels of cAMP, would be an equally plausible mechanism for inhibition of PC biosynthesis. Moreover, glucagon activates cAMP-dependent protein kinase and inhibits fatty acids synthesis via phosphorylation and inactivation of acetyl-CoA carboxylase in hepatocytes (19, 20). Therefore, a decrease in cellular fatty acids might occur with elevated levels of cAMP with release of CT from the membrane (5) and consequently decreased PC synthesis.

The experimental data summarized above led us to postulate two different mechanisms by which cAMP might inhibit PE biosynthesis: 1) direct phosphorylation and inactivation of CT by cAMP-dependent protein kinase and/or 2) decreased supply of DG, which could limit the rate of PC biosynthesis. Differentiation between these possibilities was facilitated by the preparation of an antibody that quantitatively precipitated this antibody. This technique was used to investigate the state of phosphorylation of CT in hepatocytes. Although CT is extensively phosphorylated, elevated levels of cAMP in cultured hepatocytes did not change the degree of phosphorylation of CT. Instead, the reduction of PC synthesis by cAMP appears to result from a decrease in cellular levels of DG which limits the cholinephosphotransferase reaction and thereby the rate of PC biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—A synthetic peptide fragment of CT between amino acid residues 164 and 176 (D-F-V-A-H-D-D-I-P-Y-S-S-A) according to Kalmer et al. (21) conjugated to bovine serum albumin and keyhole limpet hemocyanin was purchased from the Alberta Peptide Institute, University of Alberta, Edmonton, Alberta, Canada. The mole ratio of protein to peptide was 10:1. [methyl-3H]Choline chloride (15 Ci/mmol), cytidine 5'-diphospho[methyl-3H]choline, and [γ-32P]ATP were purchased from Amer sham International, United Kingdom. [methyl-3H]Choline (5–7 Ci/mmol) was synthesized enzymatically from [methyl-3H]choline and ATP with choline kinase as described (22). Cell culture medium. Hanks’s balanced salt solution, and fetal bovine serum were obtained from GIBCO. Primaria culture dishes (60 mm in diameter) were obtained from Becton, Dickinson & Co. (Oxnard, CA) and coated with collagen. Protein A-Sepharose CL-4B and CNBr-activated Sepharose 4B were obtained from Pharmacia-LKB Biotechnology AB, Uppsala, Sweden. The kit for measurement of free-fatty acid concentrations (NEFA C) was supplied by Wako Pure Chemical Industries, Ltd., Japan. All the other chemicals were obtained from Sigma.

**Preparation of Hepatocytes**—Hepatocytes were isolated from male Sprague-Dawley rats (150–180 g) by the collagenase perfusion technique and were plated (2.6 × 10^6 cells/dish) in a medium containing 17% fetal calf serum. The hepatocytes were incubated at 37 °C under an atmosphere of 95% air, 5% CO2 for 24 h prior to use.

**Pulse and Chase Studies**—The cultured hepatocytes were washed free of fetal calf serum and floating dead cells and incubated in serum-free medium which contained 28 μM choline. The hepatocytes were incubated at 37 °C for 24 h prior to use.

**Immunoprecipitation**—2P-Labeled Cytidylic transferase—Hepatocytes from a Sprague-Dawley rat were plated in a medium containing 17% serum. After 24 h, the medium was replaced with serum- and phosphate-free medium and incubated for 1 h. Cells were labeled in a phosphate-free medium containing 200 μCi/dish 32P (carrier-free) for 3 h. Subsequently, 25 μl of distilled water or 25 μl of 40 mM CAMP analogue solution was added to a final concentration of 10 μM, and cells were incubated for an additional 2 h. The incubation was terminated by washing the cell monolayer with 2 ml of ice-cold phosphate-buffered saline, and dishes were placed on an ice-cold metal tray. Cold digitonin buffer (0.8 ml/dish) containing 0.5 mM digitonin was added to samples (to precipitate CT) and mixed gently to bring the pH of the fractions to 7.0 as soon as collection of each fraction was completed (25). Fractions containing proteins were pooled, dialyzed against phosphate-buffered saline, and stored at −70 °C.

**Inhibition of Phosphatidylcholine Synthesis by cAMP**
Preparation of Samples for Measurement of Cytidylyltransferase Activity—Two different methods, digitonin permeabilization and homogenization and centrifugation, were used to prepare cytosolic and membrane samples from CAMp analogue-treated hepatocytes. In digitonin permeabilization, the cytosolic proteins were released from hepatocytes as described earlier for immunoprecipitation (see also Ref. 26). Hepatocytes were incubated with digitonin for 20 min, and the digitonin solution was transferred to plastic microcentrifuge tubes, and the cell debris in the digitonin sample was removed by centrifugation at 14,000 rpm for 2 min in a microcentrifuge. After the digitonin treatment the ghosts of the cells were collected in 1 ml of buffer R (10 mM Tris-HCl, pH 7.4, 0.25 % sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 2.0 mM EDTA, 1.0 mM EGTA, and 0.025 % NaN3) and sonicated for 7 × 1 s (27). In the homogenization method, one dish of cells was harvested with 1 ml of buffer R (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2.0 mM EDTA, 2.0 mM dithiothreitol, 50 mM NaF, 1.0 mM EGTA, and 0.025 % NaN3) and homogenized with 50 strokes in a tight fitting 7-ml Potter-Elvehjem homogenizer at 0 °C (29). The homogenate was centrifuged at 800 × g for 5 min to remove unbroken cells and nuclei and the supernatant centrifuged at 10,000 × g for 10 min. The cytosol was obtained after centrifugation of the 10,000 × g supernatant at 125,000 × g for 60 min, and the resulting microsomal pellet was resuspended in 0.5 ml of buffer R.

Cytidylyltranserase Activity Assay—CT activity was determined essentially as described earlier (26, 28). Phospho|methyl-3H|choline was used as a substrate, and the reaction was stopped by immersion of the assay tubes in boiling water for 1 min. [methyl-3H|CDP-choline was separated from phosphocholine on plastic-backed silica gel G 60 thin-layer chromatography plates (10 × 20 cm) developed in methanol, 0.5% NaCl, 28% NH4OH (10:10:1, v/v/v). Cytidylyltranserase Activity Assay—Cholinephosphotransferase activity in permeabilized hepatocytes was measured (18) using endogenous diacylglycerol and excess CDP-choline. Hepatocytes were incubated at 37 °C on a slow rocking platform in 0.8 ml of medium containing 0.05 mg/ml digitonin, 5 mM EGTA, 200 μM CDP|methyl-3H|choline (0.26 nCi/nmol). After 5 min, the medium was removed and cells were harvested with 2.0 ml methanol/water (1:1) and sonicated. Duplicate aliquots were removed for protein determination. Lipids in the remaining methanol/water solution were extracted by the method of Bligh and Dyer (29). The lipid-containing chloroform extract was dried under air and radioactivity was determined.

Measurement of CDP-choline in Hepatocytes—The concentration of CDP-choline in hepatocytes was measured by a modified method of Vance et al. (30). Hepatocytes were prepared and treated with 0.5 mM CPT-cAMP as described earlier. After 2 h of incubation with 0.5 mM CPT-cAMP, the medium was recovered from two 60-mm dishes and the cells were harvested with 2.0 ml of methanol/water (1:1) and sonicated. Duplicate 50-μl aliquots were recovered for protein determination. Extraction of the remaining methanol/water solution was performed by the method of Bligh and Dyer (29). The organic phase was discarded, and aqueous phase containing metabolites were dried under Nz. The residue in the aqueous phase was redissolved in 1 ml of distilled water, added to 1 ml of Norit A solution (10 g/ml/100 ml H2O), and vortexed. The Norit A was washed three times with 1 ml of distilled water and seven times with 1.0 ml of 2% ethanol. CDP-choline was extracted from Norit A with 40% ethanol containing 1% NH4OH (3 × 1 ml). CDP-choline was separated from choline and CDP-ethanolamine on a fast protein liquid chromatography Mono Q column using 20 mM piperazine, pH 10.8. The CDP-choline fraction was lyophilized and digested with 2.5 units of alkaline phosphatase and 0.2 units of Creola adamanteus phosphodiesterase in 1 ml of H2O for 3 h at 37 °C. The reaction was stopped by boiling each tube for 2 min, lyophilized, and extracted three times with 1 ml of 70% ethanol. The ethanol solution was evaporated to dryness under Nz, and the amount of choline was determined by quantitative conversion of choline to phosphocholine as described in (30).

Chemical Analysis Phospholipids were quantitated by phosphorus assay (31). Protein was determined according to (32) with bovine serum albumin as a standard. Free fatty acid concentrations were measured using the NEFA C kit (27). Statistical analysis was performed using Student’s t test.

RESULTS

Characterization of Cytidylyltransferase Antibody—An antibody against CT was required to investigate the regulation of CT activity by phosphorylation in intact cells in the presence of elevated levels of cAMP. Previously we have attempted to produce antibodies to CT using purified enzyme injected in rabbits and chickens. The production of CT antibody in rabbits was unsuccessful, but we obtained low titer antibodies in the chickens. However, antibody raised in chickens did not precipitate CT from either crude cell extracts or from partially purified enzyme preparations (26). Therefore, we decided to use a synthetic peptide, generated from the known amino acid sequence of cytidylyltransferase between amino acid residues 164–176 (21) to raise an antibody which will be referred as “CT-peptide antibody.”

Antibody was raised in rabbits using the synthetic peptide fragment conjugated to the highly antigenic protein, keyhole limpet hemocyanin. Antiserum was passed through a protein A-Sepharose 4B column to separate IgG from other serum proteins. CT-peptide antibody from the whole IgG fraction was purified on an affinity column prepared by linking CT-peptide-bovine serum albumin conjugate to CNBr-activated Sepharose 4B. The results showed that the IgG fraction contained 3% CT-peptide antibody. The purified CT-peptide antibody was tested for its capacity to precipitate quantitatively CT from cytosol. Rat liver cytosol containing 1.9 milliunits of CT activity was incubated with various amounts of CT-peptide antibody, and immune complexes were precipitated with protein A-Sepharose beads. CT activity was measured in the supernatant. As shown in Fig. 1, 20 μg of CT-peptide antibody precipitated 94% of the CT activity from cytosol. However, immunoglobulin fraction U (antibody not adsorbed to the affinity column) did not precipitate CT from cytosol (Fig. 1, inset). In a separate experiment rat liver cytosol was incubated with various amounts of CT-peptide antibody for 2 h to investigate its ability to inhibit CT activity. CT activity was measured after addition of antibody but without adding protein A-Sepharose beads. CT-peptide antibody inhibited rat liver cytosolic activity (1.9 milliunits) in a dose-dependent manner to 1.33 milliunits (30% inhibition) in the presence of 20 μg of antibody. No inhibition of CT activity was observed in the presence of IgG fraction U. These results support the hypothesis that CT plays a role in the regulation of phosphatidylcholine synthesis by cAMP.
showed that CT-peptide antibody can partially inhibit, as well as precipitate, CT from cytosol.

**Phosphorylation of CT in the Presence of cAMP analogues**—Some reports suggest that phosphorylation of CT may be involved in the inhibition of PC biosynthesis in the presence of cAMP analogues (1). In order to investigate the putative role of phosphorylation of CT, hepatocytes were prelabeled with $^{32}$P, for 3 h and then incubated in the absence or presence of one of CPT-cAMP, dibutyryl cAMP, or bromo-cAMP. CT was immunoprecipitated from digitonin released (cytosolic) and cell ghosts (membranes) and analyzed by 7–15% gradient SDS-polyacrylamide gel electrophoresis. Fig. 2 shows the autoradiogram of the gel. Most of the radioactive label close to the 43,000-dalton marker where CT migrates showed that CT-peptide antibody can partially inhibit, as well as precipitate, CT from cytosol. Moreover, two-dimensional peptide maps of CT were identical from hepatocytes incubated with or without CPT-cAMP showed an increase in CT activity in digitonin-released samples and a decrease in CT activity in membrane samples after the treatment of hepatocytes with CAMP analogues. These results suggested that cAMP promoted the release of CT from membrane to cytosol. However, when hepatocytes were incubated in the presence of 100 nM glucagon, a hormone that raises cAMP levels in hepatocytes, no change in CT activity in microsomes or cytosol was detected (15). Thus, the results were not conclusive as to whether or not increases in cAMP levels have any effect on the subcellular distribution of CT.

We investigated further the effect of CAMP analogues on subcellular distribution of CT by using two different methods for preparation of soluble and membrane samples (see “Experimental Procedures”). Hepatocytes were incubated with or without CAMP analogues for up to 2 h. After various times hepatocytes were either permeabilized with digitonin or homogenized. CT activity was measured (Table I). Our results from both approach did not show any significant difference in CT activity in soluble and membrane fractions after the treatment of hepatocytes with CAMP analogues. Therefore, the subcellular distribution of CT does not change with changes in cellular cAMP levels.

CT activity in soluble and membrane fractions was also measured in choline- and methionine-depleted hepatocytes. Hepatocytes were cultured for 4 h and subsequently incubated in choline- and methionine-free medium for 24 h. This treatment resulted in a change of the subcellular distribution of CT (Table I) because of translocation of CT from cytosol to membranes (25). Incubation of these hepatocytes with 0.5 mM CPT-cAMP showed an increase in CT activity in digitonin-released samples and a decrease in CT activity in membrane cell ghosts. Therefore, the subcellular distribution of CT changes with changes in cellular cAMP levels only when there is an excess of CT on the membrane.

The effect of CPT-cAMP on the CDP-choline pool was also investigated. The concentration of CDP-choline was meas-
The effect of 0.5 mM CPTcAMP on the subcellular distribution of cytidylyltransferase activity

Rat liver hepatocytes were cultured for 24 h in a medium containing 17% fetal calf serum (A) or first cultured in a choline- and methionine-deficient medium (+17% fetal calf serum) for 4 h and subsequently incubated in the same medium without serum (B). After 24 h, the medium was replaced and hepatocytes were incubated with or without cyclic AMP analogues as described in Methods. At various time intervals cytosolic protein and membrane samples were prepared by using digitonin to release the cytosolic protein or homogenization in a glass homogenizer as described in Methods. Cytidylyltransferase activity was measured as described in Methods. Each value is mean ± S.D. of four determinations from two separate experiments. Values were calculated using Student's t test.

<table>
<thead>
<tr>
<th>Incubation time and additive</th>
<th>Digitonin released method</th>
<th>Homogenization method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Digitonin released sample</td>
<td>Cell ghost</td>
</tr>
<tr>
<td></td>
<td>milliunits/mg protein</td>
<td>milliunits/mg protein</td>
</tr>
<tr>
<td>A. Hepatocytes cultured in normal medium for 24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h (none)</td>
<td>2.36 ± 0.15</td>
<td>0.71 ± 0.23</td>
</tr>
<tr>
<td>1 h</td>
<td>2.16 ± 0.05</td>
<td>0.62 ± 0.1</td>
</tr>
<tr>
<td>+0.5 mM CPTcAMP</td>
<td>2.28 ± 0.09</td>
<td>0.67 ± 0.1</td>
</tr>
<tr>
<td>+0.5 mM dibutyryl cAMP</td>
<td>2.47 ± 0.1</td>
<td>0.46 ± 0.07</td>
</tr>
<tr>
<td>+0.5 mM bromo-cAMP</td>
<td>2.32 ± 0.08</td>
<td>0.35 ± 0.17</td>
</tr>
<tr>
<td>B. Hepatocytes cultured and incubated in choline- and methionine-deficient medium for 24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h (none)</td>
<td>1.13 ± 0.08</td>
<td>2.02 ± 0.19</td>
</tr>
<tr>
<td>1 h</td>
<td>1.06 ± 0.09</td>
<td>2.01 ± 0.09</td>
</tr>
<tr>
<td>+0.5 mM CPTcAMP</td>
<td>1.37 ± 0.09</td>
<td>1.82 ± 0.2</td>
</tr>
<tr>
<td>(p = 0.005)</td>
<td>(p = &lt;0.005)</td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td>1.21 ± 0.06</td>
<td>2.04 ± 0.07</td>
</tr>
<tr>
<td>+0.5 mM CPTcAMP</td>
<td>1.58 ± 0.04</td>
<td>1.88 ± 0.03</td>
</tr>
<tr>
<td>(p = &lt;0.0005)</td>
<td>(p = &lt;0.025)</td>
<td></td>
</tr>
</tbody>
</table>

To investigate the effect of 0.5 mM CPTcAMP on cellular DG levels in hepatocytes, we measured cellular DG concentrations in a sensitive assay using DG kinase and [32P]ATP (33, 34). Experimental conditions were the same as described for the pulse-chase experiment under "Experimental Procedures." Hepatocytes were incubated under the same conditions as described for the pulse-chase experiment under "Experimental Procedures." Hepatocytes were incubated in the presence or absence of 0.5 mM CPTcAMP for various time intervals, lipids were extracted, and DG concentration was determined (33, 34). The results showed a significant decrease in cellular DG levels in the presence of 0.5 mM CPTcAMP (Fig. 3). The cellular DG levels were decreased by 24, 36, and 28% of control in the presence of 0.5 mM CPTcAMP for 1, 2, and 3 h, respectively (Fig. 3A).

DG concentration was also measured in microsomal fraction from cells after incubation of hepatocytes with 0.5 mM CPTcAMP for 2 h as described under "Experimental Procedures." The values obtained were 0.99 ± 0.2 nmol/mg protein for CPTcAMP-treated hepatocytes and 1.2 ± 0.3 (n = 4, p = N.S.) for control cells. The results showed that the incubation of hepatocytes with CPTcAMP does not affect the concentration of the CDP-choline pool for PC synthesis.

Decrease in 1,2-Diacylglycerol Levels in the Presence of 0.5 mM CPTcAMP—Although cAMP analogues did not alter the state of phosphorylation (Fig. 2), subcellular distribution of CT (Table I), or the supply of CDP-choline, the incorporation of [methyl-14C]choline into PC was reduced when hepatocytes were incubated in the presence of cAMP analogues. Therefore, some other factor(s) must be responsible for cAMP-induced inhibition of PC biosynthesis by the CDP-choline pathway.

In order to investigate the effect of 0.5 mM CPTcAMP on cellular DG levels in hepatocytes, we measured cellular DG concentrations by a sensitive assay using DG kinase and [32P]ATP (33, 34). Experimental conditions were the same as described for the pulse-chase experiment under "Experimental Procedures." Hepatocytes were incubated in the presence or absence of 0.5 mM CPTcAMP for various time intervals, lipids were extracted, and DG concentration was determined (33, 34). The results showed a significant decrease in cellular DG levels in the presence of 0.5 mM CPTcAMP (Fig. 3). The cellular DG levels were decreased by 24, 36, and 28% of control in the presence of 0.5 mM CPTcAMP for 1, 2, and 3 h, respectively (Fig. 3A).

DG concentration was also measured in microsomal fraction from cells after incubation of hepatocytes with 0.5 mM CPTcAMP. DG concentration was decreased from 6.78 ± 0.4 nmol/mg protein (22%) to 4.98 ± 0.37 (27%; p < 0.025) and from 6.4 ± 0.66 to 5.01 ± 0.4 nmol/mg protein (22%; p < 0.025) of control after 1 and 2 h, respectively. This decrease was similar to that observed for cellular DG levels in whole cells.

To investigate the decrease in DG concentration was responsible for the decrease in PC biosynthesis, we utilized...
an alternative method to assess DG levels at the site of PC biosynthesis. Therefore, cholinephosphotransferase activity was assayed under conditions where the amount of DG would be limiting (5-min assay with excess CDP-choline and no exogenous DG). Thus, hepatocytes were treated with 0.5 mM CPTcAMP, and cholinephosphotransferase activity was measured in the membranes from digitonin-permeabilized hepatocytes. Cholinephosphotransferase activity was decreased by 31, 38, and 39% in cells treated with 0.5 mM CPTcAMP for 1, 2, and 3 h, respectively, compared with controls (Fig. 3B). This decrease in cholinephosphotransferase activity is consistent with the decrease in cellular DG mass shown in Fig. 3A. Thus, a decrease in supply of DG may be responsible for the inhibition of PC biosynthesis observed in the presence of elevated levels of cAMP.

Inhibition of PC Biosynthesis by Glucagon—In order to investigate further the role of a decrease in cellular DG in the cAMP-mediated inhibition of PC biosynthesis, the effect of glucagon was studied. In a pulse-chase experiment the incorporation of [3H]choline into PC was measured in the absence or presence of 100 nM glucagon (Fig. 4A). The incorporation of label into PC was reduced to 85, 75, and 71% of the control values after 0.5, 1, and 2 h, respectively (Fig. 4B). This decrease in DG concentrations in the presence of glucagon was confirmed by measuring the cholinephosphotransferase activity using endogenous DG and excess amounts of CDP-choline. When hepatocytes were incubated with 100 nM glucagon for 2 h, cholinephosphotransferase activity was decreased from 0.99 ± 0.03 nmol/min/mg to 0.81 ± 0.01 nmol/min/mg (18%, p < 0.0005). This decrease in cholinephosphotransferase activity is comparable with the 24% decrease in cellular DG mass (Fig. 4B). These results lend additional support to the hypothesis that cAMP inhibits PC biosynthesis by causing a decrease in cellular DG levels.

Oleic Acid Reverses the CPTcAMP-induced Inhibition of PC Synthesis—Inhibition of PC biosynthesis by cAMP analogue was abolished when hepatocytes were simultaneously incubated with 3 mM oleic acid. Furthermore, an increase in DG levels was reported when hepatocytes were incubated with fatty acid (36).

We investigated the reversal of cAMP-induced inhibition of PC biosynthesis in the presence of oleic acid. Hepatocytes were incubated in the absence or presence of 0.5 mM CPTcAMP with various concentrations of oleic acid for 1 h as shown in Table II. Incorporation of [3H]choline into PC, free fatty acid concentrations, and cellular DG levels (Table II) were measured in the same samples. CPTcAMP inhibited [3H]choline incorporation into PC, and under the same conditions cellular DG levels were also decreased. However, the inhibition of PC synthesis by CPTcAMP was abolished when 0.5 mM oleic acid was added and DG levels returned to normal. In the presence of 1.0 or 1.5 mM oleic acid (+ CPTcAMP) the incorporation of [3H]choline into PC was higher than that of control with concomitant increases in cellular free fatty acid and DG levels (Table II). Thus, incubation of hepatocytes with oleic acid abolishes the CPTcAMP-induced inhibition of PC biosynthesis and is accompanied by increases in cellular fatty acid and DG levels.

The data of Table II were plotted, and a positive correlation (r² = 0.93) was found between the cellular DG concentrations and [3H]choline incorporation into PC. In addition, our results showed a strong correlation between cellular free fatty acid concentrations and [3H]choline incorporation into PC (r² = 0.98).

Activation and Reversal of cAMP-induced Inhibition of PC Biosynthesis by a DG Lipase Inhibitor—U-57908 is a specific inhibitor of DG lipase (38). In cultured pituitary cells basal levels of DG were significantly increased after incubation with this inhibitor (39).

Since the decrease in PC biosynthesis by CPTcAMP correlated with both DG and fatty acid levels, we dissociated these factors by use of U-57908. Hepatocytes were prepared as described under "Experimental Procedures," pulsed for 1 h with [3H]choline, and chased with or without a fixed concentration of the DG lipase inhibitor (10 μM) for up to 2 h (Fig. 5A). In addition, U-57908 was added to the chase medium at various concentrations for 2 h (Fig. 5B). The incorporation of [3H]choline into PC was increased in a dose- and time-dependent manner in the presence of DG lipase inhibitor. An increase in the cellular DG concentrations from 2.46 ± 0.8 to 3.04 ± 0.16 nmol/mg was also detected after the treatment of hepatocytes with 10 μM DG lipase inhibitor for 2 h (Table III). As we have seen earlier and in Table III, treatment of hepatocytes with 0.5 mM CPTcAMP resulted in reduction of [3H]choline incorporation into PC with a decrease in cellular DG levels. However, when 10 μM DG lipase inhibitor was added in the presence of 0.5 mM CPTcAMP, there was no significant difference in cellular DG levels and incorporation of [3H]choline into PC in comparison to cells incubated without CAMP or lipase inhibitor. Thus, the accumulation of DG in hepatocytes in the presence of DG lipase inhibitor counteracted the inhibitory effect of CPTcAMP on PC synthesis when both compounds were added together. Free fatty acid concentrations were also measured when hepatocytes were incubated in the absence or presence of the DG lipase inhibitor. Cellular free fatty acid concentrations were not altered in the presence of 10 μM DG lipase inhibitor for up to 2 h (data not shown). These results showed that DG lipase treatment abolishes the CPTcAMP-induced inhibition of PC biosynthesis by an increase in supply of DG.

**DISCUSSION**

**Evidence That cAMP Inhibits PC Biosynthesis by Decreasing the Supply of DG**—At the start of these investigations two hypotheses were considered as explanations for the decrease in PC biosynthesis observed in hepatocytes incubated with cAMP analogues or glucagon (11, 15). The results clearly demonstrate that direct phosphorylation of CT is not in-
TABLE II

Oleic acid reverses the CPT cAMP-induced inhibition of PC biosynthesis

Hepatocytes were pulse-labeled with 10 μCi/dish [methyl-3H]choline (28 μM choline) for 1 h, and radioactivity was subsequently chased for 1 h under the indicated conditions. Chase medium contained 10 mg of bovine serum albumin. Lipids were extracted from the cells, and radioactivity in PC was measured. Cellular free fatty acid and DG concentrations were measured in the same samples. This experiment was repeated and similar results were obtained.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Phosphatidylcholine</th>
<th>Free fatty acid</th>
<th>1,2-Diacylglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/dish</td>
<td>% of control</td>
<td>nmol/mg protein</td>
</tr>
<tr>
<td>None</td>
<td>34,850</td>
<td>100</td>
<td>9.13</td>
</tr>
<tr>
<td>0.5 mM CPTcAMP</td>
<td>11,303</td>
<td>32</td>
<td>7.95</td>
</tr>
<tr>
<td>0.5 mM CPTcAMP + 0.5 mM oleic acid</td>
<td>35,080</td>
<td>109</td>
<td>10.62</td>
</tr>
<tr>
<td>0.5 mM CPTcAMP + 1.0 mM oleic acid</td>
<td>109,387</td>
<td>314</td>
<td>15.39</td>
</tr>
<tr>
<td>0.5 mM CPTcAMP + 1.5 mM oleic acid</td>
<td>162,397</td>
<td>466</td>
<td>18.33</td>
</tr>
</tbody>
</table>

The experiment was repeated twice with similar results.

The evidence that the supply of DG limits PC biosynthesis in CAMP analogue- or glucagon-treated hepatocytes is compelling. 1) The levels of DG decreased in CAMP analogue- or glucagon-treated hepatocytes to an extent that was similar to the decrease in the rate of PC biosynthesis. 2) The CAMP-mediated inhibition of PC biosynthesis was reversed by the addition of oleic acid which increased the DG levels. There was an excellent positive correlation between the levels of DG and the rate of PC biosynthesis in the CAMP- and oleate-treated cells. 3) The CAMP-mediated inhibition of PC biosynthesis was also reversed when the hepatocytes were incubated with a DG lipase inhibitor, U-57908, which concomitantly raised the levels of DG in the cells. Because the nature of the evidence in support of this hypothesis is correlative, it is possible that the change in DG levels is not causing the decrease rate of PC biosynthesis. However, no other reasonable explanations are currently available.

TABLE III

Reversal of CPTcAMP-induced inhibition of PC biosynthesis in the presence of the DG lipase inhibitor (DGLI)

Hepatocytes were pulse-labeled with 10 μCi/dish [methyl-3H]choline for 1 h as described in Table II. Subsequently the medium was replaced with chase medium with or without CPTcAMP and lipase inhibitor as indicated. After 2 h, cells were harvested and lipids were extracted. Radioactivity in PC was measured, as were the cellular DG concentrations. Each value is the mean ± S.D. of three measurements. This experiment was repeated, and similar results were obtained. p values in parentheses were calculated using Student's t test. NS, not significant.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Phosphatidylcholine</th>
<th>1,2-Diacylglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/dish</td>
<td>% of control</td>
</tr>
<tr>
<td>None</td>
<td>71,972 ± 7,103</td>
<td>100</td>
</tr>
<tr>
<td>0.5 mM CPTcAMP</td>
<td>34,941 ± 3,721</td>
<td>48</td>
</tr>
<tr>
<td>(p &lt; 0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM DGLI</td>
<td>103,875 ± 5,642</td>
<td>144</td>
</tr>
<tr>
<td>(p &lt; 0.005)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mM CPTcAMP + 10 μM DGLI</td>
<td>58,672 ± 10,871</td>
<td>82</td>
</tr>
<tr>
<td>(NS)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
It is likely that the mechanism for the decrease in DG is related to the inhibition of acetyl-CoA carboxylase and fatty acid synthesis by cAMP (19, 20). This hypothesis is also supported by the reversal of the cAMP-mediated inhibition by the addition of oleic acid. Although it is well established that fatty acids activate CT and PC biosynthesis (5), a small decrease in the level of fatty acids did not alter the distribution of CT in the cAMP-treated hepatocytes. cAMP also releases phosphatidate phosphohydrolase (which produces DG from phosphatidic acid) from hepatocyte membranes and thereby decreases its activity (40). Thus, the lower level of DG could be due to this effect. However, an accumulation of labeled phosphatidic acid was not observed in the present study.

Two approaches were used to estimate the concentration of DG in hepatocytes. The first was mass measurements of total cellular DG. The second was based on the cholinephosphotransferase reaction. This enzyme, located chiefly in endoplasmic reticulum, catalyzes the last step in the CDP-choline pathway. The enzyme activity should reflect the DG level within its local environment. Previously we had shown that this enzyme-catalyzed reaction could be limited in permeabilized HeLa cells by the supply of DG in the presence of excess CDP-choline (18). A similar approach was used in the present study with permeabilized hepatocytes and the time of the assay was limited to 5 min so that the reaction would be linear with time. The results were similar to the mass measurements. Both assays indicated an approximate 30-40% decrease in DG concentration after cAMP-analogue or glucagon treatment.

Our conclusions are in accord with the recent report by Tijburg et al. (16). They showed an inhibition of PE biosynthesis in hepatocytes treated with glucagon and a decreased level of DG. They were unable to detect an effect on the ethanolaminephosphatidylcholine cytidylyltransferase reaction in their studies and concluded that the level of DG was limiting the rate of PE biosynthesis.

Several interactions between DG and cAMP have recently been reported. Treatment of GH3 cells with dibutyryl cAMP or cholera toxin inhibited PC biosynthesis, which was reversed by incubation of the cells with dioctanoylglycerol (17). If the cells were first stimulated by phorbol esters, the resulting enhanced biosynthesis of DG could be reversed with cholera toxin (17). Formyl-methionyl-leucyl-phenylalanine stimulated the formation of DG from PC via activation of a phospholipase D. This was blocked by agents that elevate the level of cAMP (41). Pretreatment of A431 cells with forskolin (which increases cAMP levels) blocked the epidermal growth factor-induced production of DG (42). The present studies and those of Tijburg et al. (16) add another dimension to the interaction between cAMP and DG by showing that cAMP can affect the levels of DG in cultured hepatocytes in the absence of an induction of DG formation by an agonist.

Regulation of PC and PE biosynthesis by supply of DG provides a facile and rapid mechanism in which metabolic energy can be conserved. Thus, when cAMP levels rise, fatty acid biosynthesis would be curtailed and the supply of DG and PC biosynthesis would drop accordingly. It is noteworthy that the decrease in PC and PE (11, 16) biosynthesis is not dramatic, probably because the cell needs to maintain a minimal level of phospholipid synthesis for membrane integrity.

*cAMP and Phosphorylation of CT.*—It is clear from the studies presented herein, concurrent studies (43), and previous work (44) that CT does contain phosphorylated residues. The function of multiple phosphorylations of CT is unknown. It is apparent from our studies that treatment of hepatocytes with cAMP analogues had no effect on the phosphorylation of CT. Similarly, phorbol ester activation of CT and PC biosynthesis (43, 44) is not related to phosphorylation of the enzyme. Moreover, pure cytidylyltransferase is not a substrate for pure protein kinase C in vitro even though six potential sites for phosphorylation of CT have been identified (21). Similarly, acetyl-CoA carboxylase has a unique protein kinase C site (Ser-95) that was not phosphorylated in isolated adipocytes after phorbol ester treatment (45). The state of phosphorylation of CT was also apparently unchanged in hepatocytes derived from choline-deficient rats in which CT was translocated to membranes and PC biosynthesis decreased (27).

The lack of an effect on phosphorylation of CT in cAMP-treated hepatocytes was surprising in view of our previous studies which showed that serine residues on CT could be phosphorylated by cAMP-dependent protein kinase (14). In those studies the maximum incorporation of 32P into CT was 0.2 mol/mol of enzyme and this phosphorylation now appears to be nonspecific. There is another report in which in vitro phosphorylation of an enzyme (e.g., acetyl-CoA carboxylase) did not appear to coincide with studies on intact cells (46). Considerable caution needs to be used in the interpretation of in vitro phosphorylation studies. The presence of phosphorylated high molecular weight proteins associated with immunoprecipitated CT is not explained. The identity of the kinases that phosphorylate CT and what the function of this phosphorylation is will be important topics for future research.

The inhibition of PC biosynthesis by glucagon and cAMP analogues has been consistently reported (1, 11, 15, 46). In contrast, the effect of cAMP analogues and glucagon on the translocation of CT from membranes to cytosol has varied according to the model system involved. In previous studies a translocation of CT from microsomes to cytosol was observed in CPT-cAMP-treated hepatocytes derived from female Wistar rats (11). When these same hepatocytes were treated with glucagon PC biosynthesis was inhibited without an effect on CT distribution between cytosol and microsomes (15). In the current experiments, hepatocytes from male Sprague-Dawley rats were used and various cAMP analogues had no effect on CT translocation. Although the reasons for these variations remain problematic, the cAMP effect on PC biosynthesis does not appear to involve an effect on the activity of CT.

Regulation of PC Biosynthesis by the Supply of DG and CDP-choline—The present studies provide the first clear evidence that DG, as well as CDP-choline, can limit the rate of PC biosynthesis. Although the possibility that DG could limit PC biosynthesis was first demonstrated in permeabilized HeLa cells (18), this had not been demonstrated previously in a physiologically relevant model system.

There is evidence that DG can also function to stimulate PC biosynthesis not only by the supply of substrate for the cholinephosphotransferase reaction but by activation of CT. Several studies have indicated that DG has the capacity to enhance the binding of CT to membranes (6, 37, 47). DG activation of PC biosynthesis and CT translocation has now been demonstrated in HeLa cells activated with phorbol esters (43). In this model system it appears that the biosynthesis of CDP-choline is limiting the rate of PC biosynthesis.

We conclude that both the supply of DG and CDP-choline can limit PC biosynthesis. Under conditions where metabolic energy needs to be conserved, the supply of DG could limit PC biosynthesis. On the other hand, we envision other circumstances such as choline deficiency where the supply of CDP-choline would be limiting PC biosynthesis (26).

---

lation at the level of supply of CDP-choline allows for a selective effect on PC biosynthesis, whereas effects on DG supply would also alter PE and triacylglycerol biosynthesis.

Acknowledgments—We thank Dr. David Severson for the gift of DG lipase inhibitor and Dr. Antonio Gomez-Muñoz for providing us with glucagon. We thank Drs. Grant M. Hatch, Jean Vance, and Tomoko Nishimaki-Mogami for helpful discussions. We are grateful to Sandra Ungarian for excellent technical assistance.

REFERENCES

35. Deleted in proof