The mechanism of inhibition of phosphatidylcholine biosynthesis by okadaic acid was investigated in suspension cultures of isolated rat hepatocytes. Cells were pulsed with \([\text{methyl-}^3\text{H}]\text{choline}\) and chased in the absence or presence of 1 \(\mu\text{M}\) okadaic acid for up to 120 min. Phosphatidylcholine biosynthesis was inhibited after 15 min of chase. To see if okadaic acid altered the degree of phosphorylation of cytidylyltransferase (CT), hepatocytes were incubated with \[^{32}\text{P}\]\text{ATP}\), and chased in the absence or presence of okadaic acid. Okadaic acid caused a rapid (within 15 min) increase in the phosphorylation state of the cytosolic enzyme. Two-dimensional peptide map analysis revealed an increase in the phosphorylation of several peptides in okadaic acid-treated hepatocytes compared with controls. After 15 min of incubation of hepatocytes with okadaic acid, membrane CT activity was decreased and a corresponding increase in cytosolic CT activity was observed. In hepatocytes incubated with okadaic acid and oleate a correlation between membrane CT activity, diacylglycerol level, and phosphatidylcholine biosynthesis was observed. These data suggest that the concentration of diacylglycerol is responsible for the increase in the amount of CT that is phosphorylated and subsequently phosphatidylcholine biosynthesis in oleate-treated cells. We postulate that the okadaic acid-induced decrease in phosphatidylcholine biosynthesis is due to an increase in the phosphorylation state of CT which promotes a translocation of CT activity from the membranes to the cytosol.

CTP:phosphocholine cytidylyltransferase (CT)\(^1\) can catalyze the rate-limiting step for phosphatidylcholine (PC) biosynthesis via the CDP-choline pathway (1–3). The enzyme is localized in both the membrane and soluble fractions of rat liver homogenates. An important regulatory mechanism for the control of CT activity and PC biosynthesis is the translocation of the enzyme between the membrane (active form) and cytosolic (inactive form) compartments. Many such instances have been documented in the literature (1–3).

An increase in the phosphorylation state of hepatocytes is associated with an attenuation of PC biosynthesis (1–3). Okadaic acid is a complex polyketol produced by marine dinoflagellates and is a potent and specific inhibitor of protein phosphatases 1 and 2A (4). This compound is recognized as an excellent probe for studying cellular processes that are regulated by phosphorylation (5). We have demonstrated that incubation of the 10,000 \(\times\) g supernatant from a rat liver homogenate with okadaic acid inhibited the time-dependent association of cytosolic CT with the microsomal membranes (6). Recently, we have demonstrated that this compound inhibited PC biosynthesis and altered the subcellular distribution of CT in isolated rat hepatocytes (7). These studies provided indirect evidence that CT was translocated from membranes to the soluble fraction under phosphorylating conditions and implied an increased phosphorylation state of CT in intact rat hepatocytes. However, we were not in a position at that time to determine the mechanism of the inhibition of PC biosynthesis by okadaic acid or directly demonstrate CT phosphorylation in intact hepatocytes since we did not have an antibody to CT.

In the present study we now demonstrate that the decrease in PC biosynthesis observed in hepatocytes incubated with okadaic acid coincides directly with an increased phosphorylation of cytosolic CT. In addition, peptide maps of phosphorylated cytosolic CT revealed that the phosphorylation state of several peptides was markedly increased in the presence of okadaic acid. It therefore appears that the okadaic acid-mediated inhibition of PC biosynthesis is due to translocation of CT activity from membranes to cytosol caused by increased phosphorylation of CT. We also demonstrate that the increased biosynthesis of PC observed in hepatocytes incubated with oleate is due to an increase in membrane CT activity caused by an increase in cellular DG concentration.

**MATERIALS AND METHODS**

\[\text{methyl-}^3\text{H}]\text{choline}, \text{CDP-[methyl-}^4\text{C}]\text{choline}, \[^{32}\text{P}\]\text{ATP}, and \[^{32}\text{P}\]\text{ATP}, were obtained from Amersham Corp. Phospho[\text{methyl-}^3\text{H}]\text{choline} was enzymatically synthesized from \[\text{methyl-}^3\text{H}]\text{choline}\) (8). Albumin (A-7030) was obtained from Sigma. Silica Gel 60 plates were obtained from Merck. WAKO non-esterified free fatty acid kit was obtained from BDH. All other biochemicals were of analytical grade and were purchased from either Sigma or Fisher Scientific (Edmonton, Canada). Okadaic acid was a generous gift of Dr. Yasamu Tsukitani, Fujisawa Pharmaceutical Co., Tokyo or Dr. Charles Holmes, University of Alberta. Male Sprague-Dawley rats (150–225 g) were used throughout the study. Rats were maintained on Purina rat chow and tap water, ad libitum, in a temperature- and light-controlled room.

**Incubation and Harvesting of Hepatocytes**—Hepatocytes were iso-
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Okadaic acid causes a reduction in phosphatidylcholine biosynthesis in isolated rat hepatocytes—Suspension cultures of hepatocytes were incubated with [methyl-3H]choline (6 μCi/flask) for 30 min. Subsequently, the radioactivity was chased in the absence or presence of 1 μM okadaic acid for up to 120 min. Biosynthesis of labeled PC increased with time in control incubations (Fig. 1). In the presence of okadaic acid, PC biosynthesis was not inhibited between 0 and 15 min, but was inhibited by 24% (p < 0.05) between 15 and 30 min and 50% (p < 0.05) between 30 and 60 min of incubation. Okadaic acid promotes translocation of CT activity from membranes to cytosol—In parallel experiments, the activity of CT was determined in cell membranes and detergent-released cytosolic fractions. Okadaic acid treatment of hepatocytes caused a rapid (within 15 min) decrease (30%, p < 0.05) in cell ghost CT activity compared with untreated cells (Fig. 24) and a concomitant increase in cytosolic activity (Fig.

RESULTS

Okadaic Acid Causes a Reduction in Phosphatidylcholine Biosynthesis in Isolated Rat Hepatocytes—Suspension cultures of hepatocytes were incubated with [methyl-3H]choline (6 μCi/flask) for 30 min. Subsequently, the radioactivity was chased in the absence or presence of 1 μM okadaic acid for up to 120 min. Biosynthesis of labeled PC increased with time in control incubations (Fig. 1). In the presence of okadaic acid, PC biosynthesis was not inhibited between 0 and 15 min, but was inhibited by 24% (p < 0.05) between 15 and 30 min and 50% (p < 0.05) between 30 and 60 min of incubation. Okadaic acid promotes translocation of CT activity from membranes to cytosol—In parallel experiments, the activity of CT was determined in cell membranes and detergent-released cytosolic fractions. Okadaic acid treatment of hepatocytes caused a rapid (within 15 min) decrease (30%, p < 0.05) in cell ghost CT activity compared with untreated cells (Fig. 24) and a concomitant increase in cytosolic activity (Fig.
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**FIG. 1. Effect of okadaic acid on phosphatidylcholine biosynthesis.** Hepatocytes were incubated for 30 min with [methyl-3H]choline (5 μCi/flask), and subsequently the radioactivity was chased in the absence or presence of 1 μM okadaic acid for up to 120 min and the radioactivity in PC determined. Closed symbols, control; open symbols, okadaic acid treated. Each point represents the mean ± S.D. of three experiments.

2B). The decrease in membrane CT activity and the increase in cytosolic CT activity were maintained throughout the incubation with okadaic acid.

Okadaic Acid Increases the Phosphorylation State of Cytosolic Cytidylyltransferase in Intact Rat Hepatocytes—To determine whether or not okadaic acid affected the phosphorylation state of CT, hepatocytes were incubated with 32P, for 1 h, and then the 32P, was chased in the absence or presence of 1 μM okadaic acid for various times. Subsequently, the cells were permeabilized with digitonin, CT in the resulting cell membranes and in released cytosol was immunoprecipitated with antibody to CT, and the CT was analyzed by SDS-polyacrylamide gel electrophoresis. As seen in Fig. 3A, okadaic acid caused a rapid (within 15 min) increase in the phosphorylation state of cytosolic CT compared with cells incubated without okadaic acid. Greater than 95% of the radioactivity was associated with the 42-kDa band. Analysis of cell membrane CT revealed minor phosphorylation of the 42-kDa band (Fig. 3B). A densitometric scan of the phosphorylated proteins from the autoradiogram of Fig. 3A revealed that the enhanced phosphorylation of CT was essentially complete within 15 min of treatment with okadaic acid (Fig. 4). Thus, okadaic acid caused a rapid increase in the phosphorylation of cytosolic CT.

Peptide map analysis of the phosphorylated cytosolic CT was performed to determine if the phosphorylation state of one or more peptides was altered. Hepatocytes were incubated with [32P]P, for 60 min and subsequently chased in the absence or presence of okadaic acid for 60 min. The cells were permeabilized with digitonin and CT in the released cytosol immunoprecipitated. The CT was incubated with trypsin, and the resulting peptides were separated by electrophoresis and ascending chromatography. The resulting autoradiograph of the two-dimensional peptide map revealed that the phosphorylation state of several peptides (1, 2, 6, 8, and possibly 3 and 7) was increased in the presence of okadaic acid (Fig. 5B) compared with control (Fig. 5A). We also performed electrophoresis under acidic conditions prior to chromatography (15) and observed a similar increase in the phosphorylation state of several phosphopeptides in okadaic acid-treated hepatocytes compared with control (data not shown). However, under these conditions some peptides remained at the origin.

Okadaic Acid Inhibits 1,2-Diacylglycerol Biosynthesis in Isolated Rat Hepatocytes—8-(4-Chlorophenylthio)-adenosine 3',5'-cyclic monophosphate causes a reduction in PC biosynthesis in cultured hepatocytes by decreasing the intracellular level of DG but not altering the phosphorylation state of CT (12). Thus, a decrease in the DG concentration in addition to the increased phosphorylation of CT in suspension cultures of hepatocytes might have been responsible for the decreased PC biosynthesis. Hepatocytes were incubated in the absence or presence of 1 μM okadaic acid for up to 120 min and the DG concentration measured (12). Incubation of isolated rat hepatocytes in the presence of okadaic acid rapidly blocked the observed increase in DG in control hepatocytes (Fig. 6A). The addition of okadaic acid to cultures which had previously been incubated for 60 min in the absence of okadaic acid prevented a further increase in DG level (Fig. 6A). Since the endoplasmic reticulum is the major site for PC biosynthesis in hepatocytes (16), the DG content of microsomes was determined. Okadaic acid prevented an increase in microsomal DG content (Fig. 6B) that paralleled the increase in cellular DG (Fig. 6A). In another approach the DG levels at the site of PC biosynthesis were monitored using an alternative method. Hepatocytes were incubated in the absence or presence of okadaic acid, or okadaic acid with ± mM oleate. Cholinephosphotransferase activity was measured in the membranes of digitonin-permeabilized cells under conditions where the DG concentration in the membrane would limit the reaction. As seen in Fig. 6C cholinephosphotransferase activity was decreased by 32% (p < 0.05). This decrease was similar to the percent inhibition of phosphatidylcholine biosynthesis at this time of incubation (Fig. 1).

If the concentrations of DG were limiting PC biosynthesis when incubations ± okadaic acid were initiated (Figs. 1 and 6A), an increase in DG in control cells (Fig. 6A) should be
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FIG. 3. Phosphorylation of cytidylyltransferase in hepatocytes incubated with okadaic acid. Hepatocytes were incubated with $^{32}$P, for 60 min, and subsequently the radioactivity was chased for up to 120 min in the absence or presence of okadaic acid. Radioactivity in the immunoprecipitated CT was examined by SDS-polyacrylamide gel electrophoresis. A representative autoradiogram of three experiments is presented. Panel A, released cytosolic CT; panel B, membrane CT.

reflected by an increase in PC biosynthesis. This was not observed (Fig. 1). Instead the rate of PC biosynthesis in control hepatocytes remained constant during the first 60 min and gradually declined (probably due to a decrease in the specific activities of phosphocholine). Since there was no change in the concentration of DG in okadaic acid-treated cells (Fig. 6A), yet there was a decline in PC biosynthesis after 15 min (Fig. 1), the supply of DG does not appear to cause the inhibition of PC biosynthesis.

Oleic Acid Prevents the Okadaic Acid-mediated Inhibition of PC Biosynthesis, Changes in DG Levels, Decrease in Membrane-associated CT Activity, and Phosphorylation of CT—We next determined if the okadaic acid effect on PC biosynthesis, DG levels, and CT activity on membranes could be prevented by the addition of oleate. Previous studies have shown that supplementation of hepatocytes with oleic acid increased DG levels (12, 17). Hepatocytes were pulse-labeled with radioactive choline for 30 min and subsequently chased for 60 min in the absence or presence of okadaic acid plus or minus various concentrations of oleic acid. The presence of okadaic acid in the medium caused a 31% decrease ($p < 0.05$) in PC biosynthesis compared with controls (Fig. 7A). Addition of various concentrations of oleate to cells incubated with okadaic acid caused a progressive increase in PC biosynthesis,
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Incubation Time (min)

FIG. 4. Densitometry scan of cytosolic CT. Hepatocytes were incubated as in Fig. 8, and the autoradiogram of 32P-labeled digitonin-released cytosolic CT was scanned at 460 nm on a densitometer. Closed symbols, control; open symbols, okadaic acid treated.

preventing the okadaic acid effect. The increase over okadaic acid-treated cells was greatest (2.74 \times 10^5 dpm, p < 0.05) with 2 mM oleate. Cells incubated with okadaic acid exhibited an increase (6.3 \times 10^4 dpm, p < 0.05) in radioactivity incorporated into phosphocholine (Fig. 7B). Addition of oleate to the cells resulted in a progressive decrease in phosphocholine levels. The decrease compared with controls was greatest (1.34 \times 10^5 dpm, p < 0.05) with 2 mM oleate.

Fatty acid and DG levels were determined in these cells. Fatty acid content was unaltered in okadaic acid or okadaic acid-oleate-supplemented cells compared with controls (Fig. 8A). Thus, these suspension cultures of hepatocytes had the capacity to utilize rapidly fatty acids in the presence of okadaic acid. There was 40% lower (p < 0.05) DG content in okadaic acid-treated cells compared with controls (Fig. 8B). Addition of various concentrations of oleate to okadaic acid-treated cells resulted in a parallel increase in DG content compared with cells incubated with okadaic acid alone. As shown in Fig. 8C the increase in DG concentration in these cells correlated positively with the increase in PC biosynthesis (r = 0.95).

In separate experiments, CT activity was assayed in hepatocytes treated as above. Okadaic acid caused a 37% decrease (p < 0.05) in cell membrane CT activity compared with untreated cells (Fig. 9A). Addition of oleate to okadaic acid-treated hepatocytes prevented the inhibition and increased membrane CT activity compared with cells incubated with okadaic acid alone. When DG content of hepatocytes was plotted against membrane CT activity, the correlation coefficient was 0.89 (Fig. 9B). Moreover, when CT activity was plotted as a function of PC biosynthesis (radioactivity incorporated into PC at 60 min of chase minus radioactivity incorporated into PC at the beginning of the chase), the correlation coefficient was 0.86 (Fig. 9C). Thus, a positive correlation exists among DG concentration, membrane CT activity, and PC biosynthesis in okadaic acid-oleate-treated hepatocytes.

In another set of experiments, the phosphorylation state of CT was investigated in okadaic acid ± oleate-treated cells. Hepatocytes were incubated with 32P for 1 h, and then the 32P was chased in the absence or presence of okadaic acid, oleate, or both, for 60 min. Subsequently, the cells were permeabilized with digitonin, CT in the resulting cell membranes and released cytosol was immunoprecipitated with antibody to CT, and the CT was analyzed by SDS-polyacryl-

amide gel electrophoresis. As seen in Fig. 10, okadaic acid caused an increase in the phosphorylation of cytosolic CT compared with control. This increase in phosphorylation was prevented in the presence of oleate correlating with an increased CT activity associated with the membranes (Fig. 9A). Furthermore, the presence of oleate alone caused a reduction in the phosphorylation of cytosolic CT compared with control (Fig. 10) in which membrane CT activity is increased and cytosolic CT activity decreased (23). Thus, when the CT is in the cytosol it is in a highly phosphorylated state,
FIG. 6. DG concentration in hepatocytes and microsomes prepared from hepatocytes incubated in the absence or presence of okadaic acid and cholinephosphotransferase activity in hepatocytes incubated in the absence or presence of okadaic acid or okadaic acid plus 2 mM oleate. Hepatocytes were incubated in the absence or presence of 1 μM okadaic acid for up to 120 min. Subsequently, the DG concentration was determined in cells (panel A) and microsomes (panel B). Closed symbols, control; open squares, okadaic acid treated; open circle, okadaic acid addition at 60 min. Panel A, each point represents the mean ± S.D. of three experiments; panel B, each point represents the mean of two experiments; panel C, hepatocytes were incubated for 60 min in the absence (C) or presence of okadaic acid (OA) or okadaic acid with 2 mM oleate (OA+2) and permeabilized with digitonin. Subsequently, cholinephosphotransferase (CPT) activity in the cell membranes was determined. Results represent the mean ± S.D. of three experiments.

whereas when the CT is on the membrane it is significantly dephosphorylated.

DISCUSSION

The Mechanism by Which Okadaic Acid Inhibits PC Biosynthesis—Our objective in these studies was to determine the mechanism by which okadaic acid inhibits the biosynthesis of PC in suspension cultures of rat hepatocytes as we first reported (6, 7). Reaching a conclusion from the results was initially complicated because the okadaic acid had two major effects which could be responsible for the inhibition of PC biosynthesis. 1) We observed a rapid increase in the phosphorylation of CT and its release from membranes into the cytosol. Since CT activity on membranes can limit the rate of PC biosynthesis (1–3), the release of membrane CT might explain the reduced rate of PC biosynthesis. 2) We observed an increase of DG levels in control hepatocytes which did not occur in cells treated with okadaic acid. The concentration of DG, as a substrate for cholinephosphotransferase reaction, can also limit the rate of PC biosynthesis (18). A decrease in the supply of DG appears to be the mechanism by which PC biosynthesis is inhibited in cultured hepatocytes incubated with cAMP analogues (12). Other potential mechanisms for the inhibition of PC biosynthesis such as a decrease in fatty acid or in PC levels (1–3) did not seem to be involved. Thus, either the lower concentration of DG or an increase in CT phosphorylation in the okadaic acid-treated hepatocytes might potentially be responsible for the decrease in PC biosynthesis. In order to distinguish between these two possibilities, we evaluated the effects of okadaic acid as a function of time on PC biosynthesis, DG levels, CT activity on membranes, and the state of phosphorylation of CT.

The time course studies were very revealing. First, there was an increase in the DG levels of control hepatocytes (Fig. 6A) without an effect on PC biosynthesis (Fig. 1). If DG were limiting PC biosynthesis, an increase in DG should have been reflected by an increased rate of [methyl-3H]choline incorporation into PC which did not occur. This strongly suggests that some other factor is limiting PC biosynthesis (most likely the activity of CT on the membranes). In the okadaic acid-treated cells the DG levels did not change, yet there was a decrease in PC biosynthesis after 15 min of incubation. At the same time there was a loss of CT activity from cellular membranes with a concomitant increase in the cytosolic fraction. The loss of CT activity from the membranes was accompanied by at least a 2-fold increase in the phosphorylation of CT (Fig. 4). Peptide map studies indicated that the phosphorylation state of several phosphopeptides of cytosolic CT was increased in okadaic acid-treated cells compared with controls. Based on the above data we conclude that okadaic
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Fig. 8. Effect of okadaic acid or okadaic acid and oleate on PC biosynthesis, DG levels, and levels of fatty acids. Hepatocytes were incubated as described in Fig. 4. Subsequently, the concentration of fatty acid (FA, panel A) and DG (panel B) was determined. Panel C, the DG concentration from Fig. 8B was plotted against the radioactivity incorporated into PC from Fig. 4A and the correlation coefficient determined. Abbreviations used are the same as listed in the legend to Fig. 7.

Acid inhibits PC biosynthesis by causing an increase in the state of phosphorylation of CT. This results in a release of CT into its inactive cytosolic form from cellular membranes. Because CT activity on membranes is apparently limiting under the conditions of this experiment, there is a parallel decrease in PC biosynthesis.

In this report we have demonstrated for the first time that the state of CT phosphorylation could be altered in intact hepatocytes. The rapid doubling of phosphorylation of CT in 15 min suggests that under steady state conditions CT is constantly being phosphorylated and dephosphorylated. Since okadaic acid is specific for phosphatases 1 and 2A (4), the inhibition of dephosphorylation of CT observed in the present studies implies the involvement of either or both of these phosphatases. The identity of the protein kinase(s) involved is not clear. Current evidence suggests that CT is neither a substrate for cAMP-dependent protein kinase in liver cells (12) nor a substrate for protein kinase C (19, 20).

On the Mechanism by Which Oleic Acid Prevents the Okadaic Acid-mediated Inhibition of PC Biosynthesis in Hepatocytes—Initially, we thought that an increase in the fatty acid levels in the cells might mediate the translocation of CT to membranes as clearly demonstrated in HeLa cells (21) and in vitro (22, 23). However, the free fatty acid levels were not elevated in the hepatocytes even when incubated with 2 mM oleate, probably because of the large capacity of hepatocytes to activate and esterify fatty acids in these cells. However, the concentration of DG was increased as a function of the fatty acid concentration in the medium (Fig. 8B), and this correlated very well with CT translocation to membranes (Fig. 9B) and a reduction in the phosphorylation state of cytosolic CT (Fig. 10). We, therefore, postulate that the increased DG in the membranes of these cells causes the translocation of CT to membranes. This conclusion is supported by considerable in vitro evidence that shows that DG can mediate the binding of CT to membranes and lipid vesicles (21, 24–27). Moreover, it appears that the activation of PC biosynthesis in HeLa cells by phorbol esters is due to movement of CT from cytosol to membranes caused by an increased level of DG in membranes (19). It, therefore, seems likely that the stimulation of PC biosynthesis in the okadaic acid-oleate-treated hepatocytes was due to an increased production of CDP-choline mediated by increased association of CT activity with the cell membrane caused by the increase in DG.

The DG concentration did not increase in okadaic acid-treated compared with control hepatocytes. This was probably because of decreased availability of newly synthesized fatty acids by okadaic acid-mediated inhibition of acetyl-CoA carboxylase activity (28). An equally plausible explanation for the decrease in DG concentration in okadaic acid-treated hepatocytes might be the observed inhibition of phosphatidate phosphohydrolase activity (29).
Okadaic acid did not affect the activity of pure CT when added directly to the assay mixture (6) nor was the effect of okadaic acid on reactivation of cytosolic CT by PC:oleate vesicles altered. Thus, phosphorylation of CT in the presence of okadaic acid does not appear to affect directly its activation by lipids. It is likely that phosphorylation of cytosolic CT prevents its binding or interaction with membranes.

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While this article was under review a manuscript appeared that showed that okadaic acid could block the translocation of CT from cytosol to membranes in Chinese hamster ovary cells treated with phospholipase C (30). The authors did not elucidate the mechanism in these studies, but the evidence reported was consistent with an increase in the phosphorylation of CT as a result of the okadaic acid treatment. Their results are, therefore, in agreement with the mechanism proposed in our studies on hepatocytes.

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