Repression of cholesterol 7α-hydroxylase transcription by bile acids is mediated through protein kinase C in primary cultures of rat hepatocytes

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Abstract Inhibitors of protein kinases were screened for the ability to prevent the repression of cholesterol 7α-hydroxylase mRNA by taurocholate in primary cultures of adult rat hepatocytes. The addition of taurocholate (25 μM) for 6 h decreased cholesterol 7α-hydroxylase mRNA by 64 ± 3%. However, after a preincubation with the protein kinase C inhibitors calphostin C or chelerythrine, taurocholate had no significant effect on cholesterol 7α-hydroxylase mRNA, or decreased levels by only 23 ± 8%, respectively. Protein kinase C activation with phorbol 12-myristate, 13-acetate (100 nM) decreased cholesterol 7α-hydroxylase mRNA and transcriptional activity by 71 ± 5% and 60 ± 16%, respectively, within 3 h. mRNA levels recovered to control levels by 18–24 h, however, consistent with down-regulation of protein kinase C. Furthermore, after depletion of protein kinase C with a 24-h preincubation with phorbol diesters, taurocholate (25 μM) repressed cholesterol 7α-hydroxylase mRNA by only 14 ± 17%. The addition of taurocholate (50 μM) to the culture medium transiently increased membrane-associated protein kinase C activity by ~twofold, while causing a concomitant decrease in cytosolic activity. Other bile acids increased membrane-associated protein kinase C activity in approximate proportion to their relative hydrophobicity. Finally, immunoblotting experiments revealed translocation of the α isoform of protein kinase C to hepatocyte membranes in response to taurocholate. These data suggest that hydrophobic bile acids repress cholesterol 7α-hydroxylase transcription through a protein kinase C-dependent mechanism.—**Stravitz, R. T., Z. R. Vlahcevic, E. C. Gurley, and P. B. Hylemon.** Repression of cholesterol 7α-hydroxylase transcription by bile acids is mediated through protein kinase C in primary cultures of rat hepatocytes. *J. Lipid Res.* 1995. **36:** 1359–1369.

Supplementary key words cholesterol • bile acid • cholesterol 7α-hydroxylase • mRNA

Hepatic microsomal cholesterol 7α-hydroxylase (EC 1.14.13.17) catalyzes the first and rate-determining reaction in the bile acid biosynthetic pathway, a major route of cholesterol elimination from the body (1). Three classes of molecules regulate the activity of this enzyme: hormones (2, 3), which may include hormones directing its circadian periodicity (4, 5); cholesterol or metabolic oxidation product of cholesterol (6); and, hydrophobic bile acids (7). In the rat, cholesterol 7α-hydroxylase thus maintains the homeostasis of hepatic cholesterol by feed-forward stimulation in response to overabundant cholesterol, and feedback repression in response to a replete bile acid pool.

Numerous studies in rat models (7–9) and in humans (10, 11) indicate that the activity of cholesterol 7α-hydroxylase is subject to feedback repression by bile acids undergoing enterohepatic recirculation. Furthermore, the degree to which bile acids repress cholesterol 7α-hydroxylase activity parallels the relative hydrophobicity of bile acids in portal blood (12, 13); specifically, hydrophobic, but not hydrophilic, bile acids are effective down-regulators. In primary cultures of rat hepatocytes (14, 15) and intact rat models (16, 17), hydrophobic bile acids concommitantly inhibit cholesterol 7α-hydroxylase specific activity, steady-state mRNA levels, and transcriptional activity, suggesting an effect on the cholesterol 7α-hydroxylase 5'-flanking region. However, the intracellular mechanisms by which the hepatocyte senses the concentration and relative hydrophobicity of bile acids, and transduces this signal to the hepatocyte nucleus, remain obscure.

Two models might account for the transcriptional repression of cholesterol 7α-hydroxylase by bile acids. Bile acids may bind to a bile acid receptor, enter the nucleus, and interact with the cholesterol 7α-hydroxylase promoter (a "direct" or steroid hormone-type model). Alternatively, bile acids may generate one or more extranuclear signals, which ultimately result in the covalent modification of a *trans*-acting factor required for cholesterol 7α-hydroxylase transcription (an "indirect" or

Abbreviations: PKC, protein kinase C; TCA, taurocholic acid; PMA, phorbol 12-myristate, 13-acetate; PP-1, protein phosphatase 1; PP-2A, protein phosphatase-2A; PBS, phosphate-buffered saline; R_p-cAMPS, R_p-adenosine-3',5'-monophosphothioate; cPKCα, α isoform of PKC.

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second messenger-type model). To date, little scientific evidence strongly supports either model, although two observations argue against the former: bile acids may be excluded from hepatocyte nuclei (18, 19), and experiments using bile acid analogues have failed to show structural specificity of the response (15). While these findings do not rule out a direct effect of a putative bile acid receptor on the cholesterol 7α-hydroxylase promoter, they raise the possibility that the effects may be more indirect.

We screened several protein kinase inhibitors for the ability to block the repression of cholesterol 7α-hydroxylase mRNA by taurocholate (TCA) in primary cultures of adult rat hepatocytes. Highly selective inhibitors of protein kinase C (PKC) were found to prevent the feedback inhibition of cholesterol 7α-hydroxylase mRNA by TCA. In addition, phorbol di-esters in acute exposures reversibly inhibited cholesterol 7α-hydroxylase transcription. Finally, bile acids were shown to increase membrane-associated PKC activity in proportion to their relative hydrophobicity, and to cause translocation of the α isoform of PKC (cPKCα). These observations suggest that bile acids repress cholesterol 7α-hydroxylase through a PKC-dependent phosphorylation event.

MATERIALS AND METHODS

Materials

Bile acids, phorbol 12-myristate 13-acetate (PMA), and actinomycin D were obtained from Sigma. Chelerythrine chloride, calphostin C, okadaic acid (sodium salt), 1-*nor*-okadaone, calyculin A, and 4α-PMA were obtained from LC Laboratories (Woburn, MA). Affinity-purified rabbit antibody to cPKCα was obtained from GibcoBRL, and affinity-purified goat anti-rabbit IgG–horseradish peroxidase conjugate was from Bio-Rad. DEAE-Sephacel was obtained from Pharmacia, the PKC assay system and [^{32}P]ATP were from Amersham, and R_p-adenosine-3',5'-monophosphothioate (R_p-cAMPS) was from Calbiochem.

Primary cultures of rat hepatocytes

The isolation, plating, and maintenance of hepatocytes from male Sprague-Dawley rats were performed essentially as described in detail previously (20, 21). Hepatocytes (3.5×10^6) were plated in 3 ml serum-free Williams' E medium containing thyroxine (1.0 μM), dexamethasone (0.1 μM), insulin (0.25 U/ml), and penicillin (100 U/ml) on 60-mm Falcon culture dishes coated with rat tail collagen. Hepatocytes were harvested 18–30 h after plating, as indicated.

Isolation of total RNA from rat hepatocytes and quantification of cholesterol 7α-hydroxylase mRNA

Total RNA was isolated at the indicated times from five culture dishes/sample in 5 ml TriReagent (Molecular Research Center, Cincinnati), and was extracted by the chloroform–phenol–guanidinium thiocyanate method, as recommended by the manufacturer. Cholesterol 7α-hydroxylase and rat cyclophilin mRNAs were quantitated by Northern and dot blot hybridization exactly as described (21). Cholesterol 7α-hydroxylase mRNA was normalized for variation of loading to rat cyclophilin mRNA, which did not vary under any of the experimental conditions.

Isolation of hepatocyte nuclei and nuclear run-on determination

Hepatocyte nuclei were isolated (40 plates/sample) and nuclear run-on determinations were performed exactly as described previously (21). The transcriptional activity of cholesterol 7α-hydroxylase as determined by exposure of hybridized membranes to XAR film was normalized to that of cyclophilin.

Assays for toxic effects of protein kinase C inhibitors

Accumulation of lactate dehydrogenase in culture medium was assayed spectrophotometrically by decrease in NADH at 340 nm. For DNA fragmentation assays, hepatocytes from two individual plates were scraped into 300 μl lysis buffer (0.05% Triton X-100, 10 mM Tris HCl, 15 mM EGTA, 15 mM EDTA, pH 8.0), and centrifuged at 30,000 *g* for 40 min at 4°C. Nonsedimenting DNA fragments (≤3000 bp) were detected spectrofluorophotometrically in the presence of *bis*-benzimide with 365 nm excitation and 460 nm emission, as previously described (22).

Determination of PKC activity in hepatocyte homogenates

Hepatocytes (eight dishes/sample) were washed in phosphate-buffered saline (PBS) and scraped into 1.0 ml homogenization buffer on ice containing Tris-HCl (20 mM; pH 7.5), EDTA (2 mM), EGTA (5 mM), 2-mercaptoethanol (10 mM), leupeptin (50 μM), and PMSF (1 mM). Hepatocytes were homogenized on ice × 25 strokes and the lysate centrifuged for 1 h at 100,000 *g* (4°C). The supernatant (cytosol) was removed and membranes were resuspended in 1.0 ml homogenization buffer containing 0.5% Triton X-100. Membrane homogenates were then incubated on ice for 30 min, and centrifuged at 10,000 *g* for 2 min. Membrane supernatant and cytosol were then purified over 400 μl DEAE-Sephacel columns and eluted with 300 mM NaCl as described (23). PKC activity in cytosol and membrane fractions was determined using the PKC assay system from Amersham according to the manufacturer's recommendations. Briefly, dilutions of cytosol or membrane extract were incubated at 25°C for 15 min with a PKC-specific acceptor peptide, in the presence of calcium, phosphatidylserine, PMA, and [γ-^{32}P]ATP (0.25 μCi/reaction). An aliquot of the terminated

reaction mixture was then applied to filter paper, washed in 5% v/v acetic acid, and the extent of phosphorylation of the acceptor peptide was determined by scintillation counting. Counts from appropriate blank samples were subtracted from the total activity, and sample activity was corrected for protein concentration [determined by the method of Bradford (24)].

Western blotting of PKC isozymes

Hepatocyte, liver, and brain protein extracts were prepared as described for PKC activity assays, except that no DEAE purification was required. Proteins (75–200 μg) were separated on an 8% SDS-polyacrylamide gel, electrophoretically transferred for 16 h at 6 volts/cm^2 in Tris (25 mM, pH 8.3), glycine (192 mM), and methanol (20%) at 4°C to nitrocellulose. Membranes were air-dried and blocked for 1 h at room temperature in 5% Carnation non-fat dry milk in PBS containing 0.1% Tween-20. Membranes were then immunoblotted with isozyme-specific, affinity-purified rabbit anti-cPKCα (Gibco-BRL), 1:1000 dilution in PBS–5% milk–0.1% Tween 20 at room temperature for 1.5 h. After washing, affinity-purified goat anti-rabbit IgG–horseradish peroxidase (1:1000 dilution) was added in PBS–5% milk–0.1% Tween for 1 h, the membranes were washed, and detection was performed using the Renaissance ECL Reagent and Reflection film from DuPont NEN. Nitrocellulose membranes were stained with Ponceau S solution (0.2% w/v in 1% acetic acid; Sigma) to ensure equal loading and transfer of proteins. To demonstrate specificity of binding, primary antibody was also incubated with cPKCα-specific peptide (1:2 w/w × 10 min) before adding to some membranes.

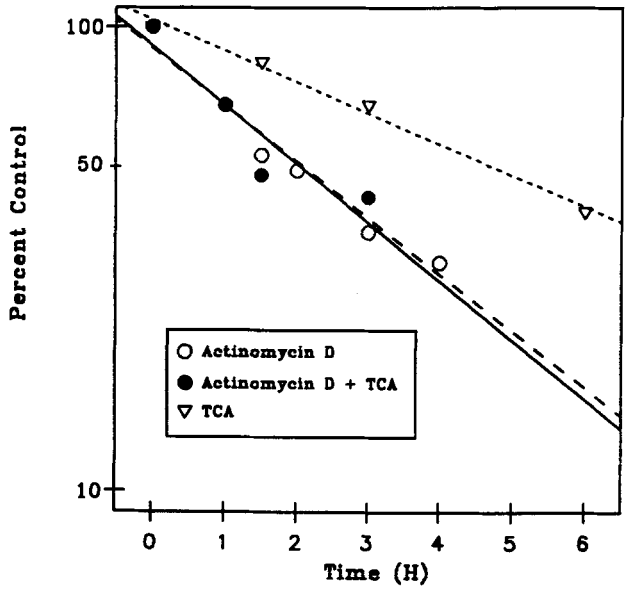

Fig. 1. Cholesterol 7α-hydroxylase mRNA turnover in primary cultures of rat hepatocytes. Semi-logarithmic plots of cholesterol 7α-hydroxylase mRNA decay after the addition of the general transcriptional inhibitor, actinomycin D (10 μg/ml), 18 h after plating. Cholesterol 7α-hydroxylase mRNA decay is compared in cultures treated or untreated with taurocholate (TCA; 50 μM). The time course of decay after the addition of TCA (25 μM) is also shown. Total RNA was isolated at the indicated times after the addition of actinomycin D or TCA. Cholesterol 7α-hydroxylase mRNA was quantified by Northern blot hybridization as described in Experimental Procedures and expressed as a percent of levels in cultures harvested at time 0. (Representative of duplicate experiments.)

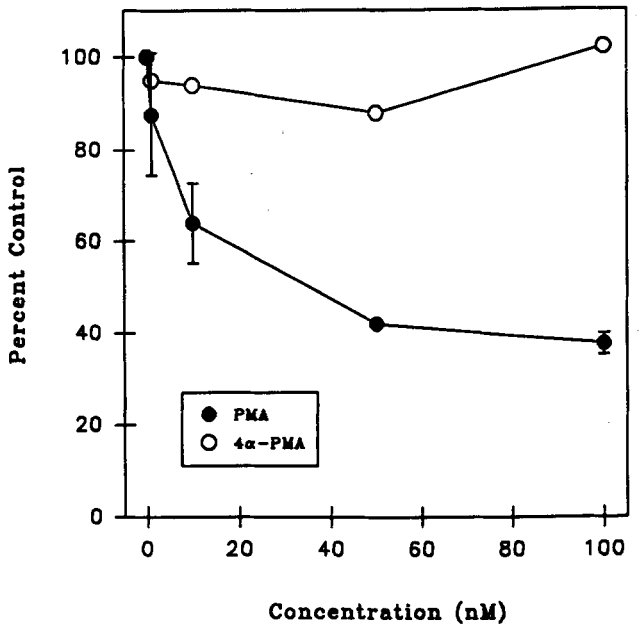

Fig. 2. Effect of PMA and 4α-PMA on cholesterol 7α-hydroxylase mRNA in primary cultures of rat hepatocytes. PMA or 4α-PMA in the indicated concentrations was added to the culture medium 18 h after plating, and total RNA was isolated 3 h after the addition. Cholesterol 7α-hydroxylase mRNA was quantified by dot-blot hybridization, loading-controlled to rat cyclophilin mRNA as described in Experimental Procedures, and expressed as percent of untreated controls harvested at the same time. Mean ± SE of three experiments for PMA; mean of two experiments for 4α-PMA.

Statistics

Results are reported as mean ± SE. Statistical significance was determined where appropriate by Student's t test for unpaired data.

RESULTS

Determination of cholesterol 7α-hydroxylase mRNA turnover

In order to optimize the timing of additions to cultured rat hepatocytes, preliminary experiments were performed to define the turnover rate of cholesterol 7α-hydroxylase mRNA under various culture conditions. In particular, these experiments sought to determine whether bile acids altered cholesterol 7α-hydroxylase mRNA turnover. After the inhibition of transcription with actinomycin D (10 μg/ml), cholesterol 7α-hydroxylase mRNA decayed with a half-life ($T_{½}$) of approximately 2 h (**Fig. 1**). Moreover, in

the presence of taurocholate (TCA; 50 μM), the addition of actinomycin D decreased cholesterol 7α-hydroxylase mRNA with a $T_{½}$ identical to that in actinomycin-treated cultures without TCA. The addition of TCA (25 μM) alone decreased steady-state cholesterol 7α-hydroxylase mRNA with a $T_{½}$ of approximately 4 h. Cholesterol 7α-hydroxylase mRNA in untreated hepatocytes over a similar time course did not change (data not shown). Thus, cholesterol 7α-hydroxylase mRNA is rapidly degraded under our culture conditions, but bile acids do not contribute to this rapid turnover.

Regulation of cholesterol 7α-hydroxylase mRNA levels and transcriptional activity by PKC

A possible role for PKC in the regulation of cholesterol 7α-hydroxylase was first explored using phorbol esters,

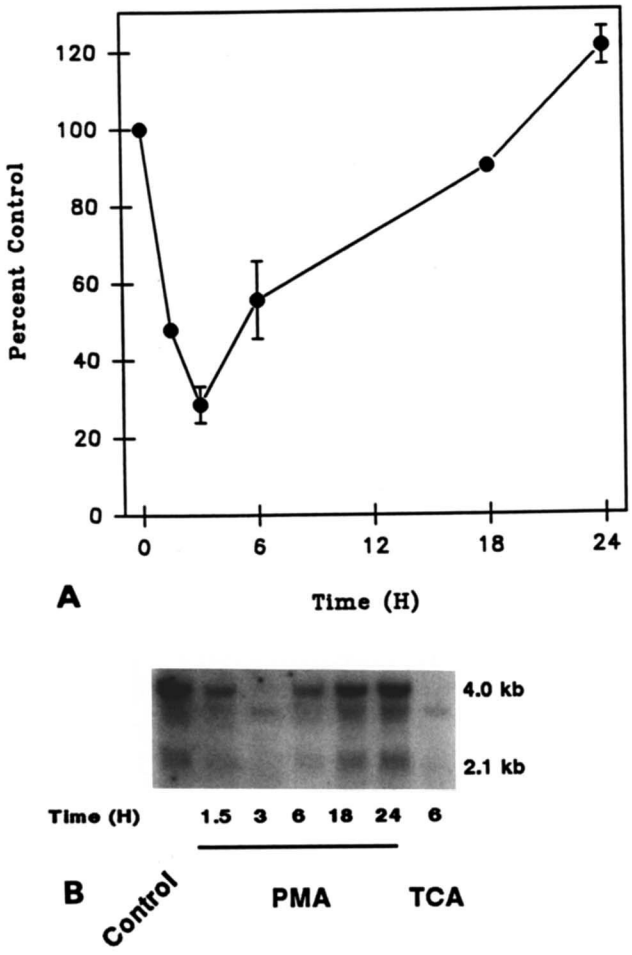

Fig. 3. Time course of the effects of PMA on cholesterol 7α-hydroxylase mRNA in primary cultures of rat hepatocytes. PMA (100 nM) was added to hepatocyte culture medium at the indicated times before harvest, beginning 4 h after plating. All cultures were harvested for total RNA 28 h after plating, and cholesterol 7α-hydroxylase mRNA was quantified by dot (A) or Northern blot (B) hybridization and loading-controlled to rat cyclophilin mRNA as described in Experimental Procedures. Cholesterol 7α-hydroxylase mRNA levels after a 6-h incubation with TCA (25 μM) is also depicted in (B). Mean ± SE of three experiments.

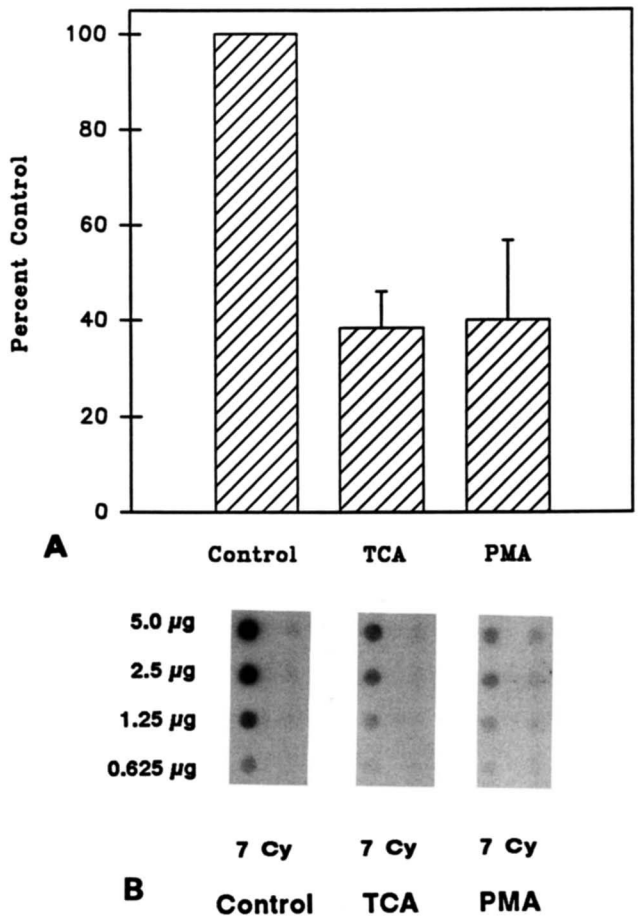

Fig. 4. Cholesterol 7α-hydroxylase transcriptional activity in primary cultures of rat hepatocytes. Taurocholate (TCA; 25 μM) or PMA (100 nM) was added to hepatocyte culture medium 18 h after plating, and hepatocyte nuclei were harvested 1.5 h later. Nascent cholesterol 7α-hydroxylase and cyclophilin mRNAs were elongated in vitro in the presence of [^{32}P]GTP ("nuclear run-on assays"), as described in Experimental Procedures. (A) Mean ± SE of three experiments, expressed as percent of untreated controls. (Both are significantly different at $P < 0.05$.) (B) Representative hybridization of radiolabeled cholesterol 7α-hydroxylase (7) and cyclophilin (Cy) mRNAs to their respective linearized cDNAs, under the same conditions.

which potently activate most PKC isozymes (25). The addition of PMA to primary cultures of rat hepatocytes for 3 h resulted in a dose-dependent decline in cholesterol 7α-hydroxylase mRNA between 1–50 nM (**Fig. 2**). The PMA-induced decline was maximal at 50 nM (decrease of 60% compared to no-addition controls); higher concentrations had no further effect. In contrast, the addition of the inactive isomer 4α-PMA (1–100 nM) had no significant effect on cholesterol 7α-hydroxylase mRNA.

The effects of PMA (100 nM) on cholesterol 7α-hydroxylase mRNA were biphasic (**Fig. 3**). Initially, PMA caused a rapid decline in cholesterol 7α-hydroxylase mRNA to 29 ± 5% of control cultures 3 h after the addition. This decline was followed by a recovery phase to control levels approximately 18–20 h after the addition, and an overshoot to 121 ± 5% of control at 24 h.

Cholesterol 7α-hydroxylase transcriptional activity declined in parallel with mRNA levels after short exposure to PMA (**Fig. 4**). One and one-half hour after the addition of PMA (100 nM), cholesterol 7α-hydroxylase transcription decreased by 60 ± 16% ($P < 0.05$ compared to untreated controls). In the same hepatocyte cultures, the addition of TCA (25 μM) for 1.5 h repressed cholesterol 7α-hydroxylase transcriptional activity by 62 ± 8%. In contrast, the transcriptional activity of rat cyclophilin was not affected by TCA or PMA (Fig. 4).

Effects of protein phosphatase inhibitors on cholesterol 7α-hydroxylase mRNA

Further evidence of cholesterol 7α-hydroxylase mRNA regulation by phosphorylation events was obtained using ser-/thr-protein phosphatase inhibitors. Okadaic acid and calyculin A inhibit protein phosphatase 2A (PP2A) with equal potency, but calyculin A inhibits protein phosphatase 1 (PP-1) more potently than okadaic acid (10- to 100-fold; 26). As the two compounds have similar IC_{50} values for PP-2A but markedly different IC_{50} values for PP-1, a comparison of the dose–response curves for the two inhibitors can be used to identify the phosphatase responsible for the observed effect. As shown in **Fig. 5**, cholesterol 7α-hydroxylase mRNA decreased with an IC_{50} of 10–20 nM after a 6-h incubation with either okadaic acid or calyculin A. These data suggest that the inhibition of PP-2A, rather than PP-1, represses cholesterol 7α-hydroxylase mRNA. 1-*Nor*-okadaone (50 nM), an inactive okadaic acid analogue, had no significant effect on cholesterol 7α-hydroxylase mRNA (Fig. 5).

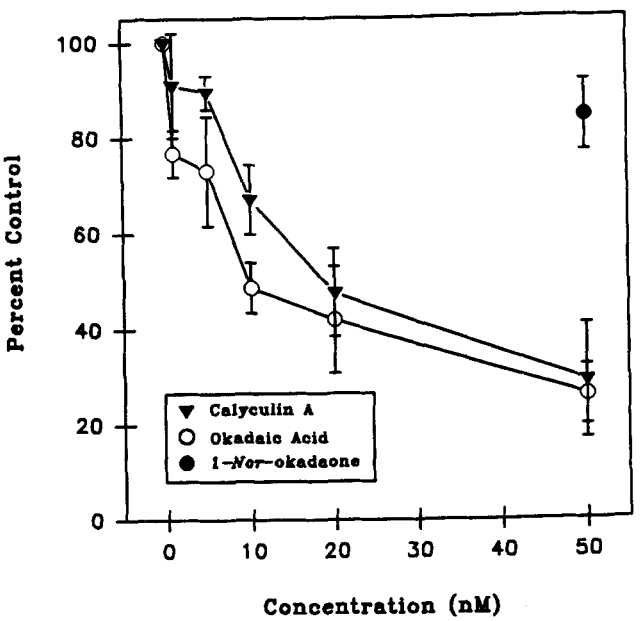

Fig. 5. Effects of ser-/thr- protein phosphatase inhibitors on cholesterol 7α-hydroxylase mRNA levels in primary cultures of rat hepatocytes. Okadaic acid (sodium salt) or calyculin A were added to the culture medium in the indicated concentrations 18 h after plating, and hepatocytes were harvested for total RNA 6 h later. Cholesterol 7α-hydroxylase mRNA was quantified by dot-blot hybridization and loading-controlled to rat cyclophilin mRNA as described in Experimental Procedures. Mean ± SE of three experiments.

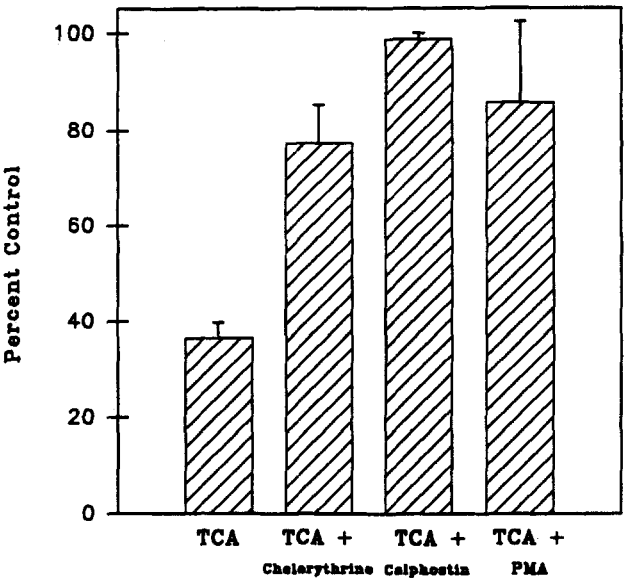

Fig. 6. Effects of PKC inhibitors on taurocholate-induced repression of cholesterol 7α-hydroxylase mRNA in primary cultures of rat hepatocytes. Hepatocytes (18 h after plating) were incubated with TCA (25 μM) for 6 h, either without PKC inhibitor, or after a 1-h preincubation with calphostin C (100 nM, in an illuminated incubator) or chelerythrine chloride (7 μM). In separate experiments, PMA (100 nM) was added 2 h after plating and TCA was added 24 h later. Cholesterol 7α-hydroxylase mRNA was determined by dot-blot hybridization, and loading-controlled to rat cyclophilin mRNA, as described in Experimental Procedures. Data are expressed as percent of untreated controls for cultures not treated with PKC inhibitor, or as percent of cultures treated with the respective inhibitor alone. Cholesterol 7α-hydroxylase mRNA levels in hepatocyte cultures treated only with inhibitor were within 20% of no-addition control levels. Mean ± SE of three to four experiments.

PKC inhibitors block the repression of cholesterol 7α-hydroxylase mRNA by bile acids

Two structurally unrelated and highly selective PKC inhibitors, calphostin C and chelerythrine, were next used to attempt to block the repression of cholesterol 7α-hydroxylase mRNA by bile acids. The addition of TCA (25 μM) for 6 h decreased cholesterol 7α-hydroxylase mRNA levels by 64 ± 3% compared to untreated controls (**Fig. 6**). However, after a 1-h preincubation with calphostin C (100 nM) or chelerythrine (7 μM), the addition of TCA (25 μM for 6 h) had no significant effect on cholesterol 7α-hydroxylase mRNA, or decreased it by only 23 ± 8%, respectively, compared to cultures incubated with the inhibitors alone. Individually, both PKC inhibitors decreased cholesterol 7α-hydroxylase mRNA by 10–20% compared to untreated cultures. As these compounds have been reported to cause DNA fragmentation and initiate apoptosis in the promyelocytic leukemia cell line, HL-60 (22), we attempted to rule out toxic effects by determining lactate dehydrogenase (LDH) levels in the

culture medium and apoptotic DNA fragmentation by spectrofluorophotometry (22). In triplicate experiments, DNA fragments (<3000 base pairs) and culture medium LDH activities were not increased after a 7-h exposure to either inhibitor (data not shown). In contrast to the PKC inhibitors, a similar preincubation with the protein kinase A inhibitor, R_p-cAMPS (100 μM), was unable to block the repression of cholesterol 7α-hydroxylase mRNA by TCA (decrease of 57% compared to cultures treated with R_p-cAMPS alone; data not shown).

As described above, prolonged stimulation of PKC by phorbol esters results in its proteolytic down-regulation (25). We therefore preincubated cultured rat hepatocytes with PMA (100 nM) for 24 h to deplete hepatocytes of PKC, and then examined the ability of TCA to repress cholesterol 7α-hydroxylase mRNA (Fig. 6). The addition of TCA (25 μM) to PMA-treated cultures resulted in a 14 ± 17% decrease in cholesterol 7α-hydroxylase mRNA levels, compared to cultures treated with 24 h of PMA alone (114% of untreated cultures in this set of experiments).

Hydrophobic bile acids cause translocation of PKC activity to cultured rat hepatocyte membranes

The ability of bile acids to activate PKC was next explored. Most isozymes of PKC reside in hepatocyte cytosol, and translocate to membranes as a prerequisite for protein phosphorylation (23, 27, 28). Three min after the addition of TCA (50 μM) to cultured rat hepatocytes, PKC activity in partially purified membrane fractions increased to 139 ± 10% of levels in untreated hepatocytes (**Fig. 7**). Membrane-associated PKC activity peaked 5 min after the addition of TCA at 240 ± 48% of control levels, and persisted for 15 min. Concomitant with the increase in membrane activity, PKC activity decreased between 25–30% in the cytosolic fraction at 5 and 15 min after the addition of TCA. The percent decrease in cytosolic activity was less than the increase in membrane activity because cytosolic PKC activity in unstimulated hepatocytes was higher. Specifically, PKC activity in crude membrane extracts (not fractionated on DEAE-cellulose) was barely detectable, and 3- to 5-fold lower than in crude cytosolic fractions (data not shown).

Membrane-associated PKC activity was also determined 5 min after the addition of bile acids of various hydrophobicity (**Fig. 8**). Tauroursodeoxycholate (50 μM), a hydrophilic bile acid, slightly increased membrane activity to 124 ± 7% of no-addition controls. In this set of experiments, TCA (50 μM), a bile acid of intermediate hydrophobicity, increased membrane-associated activity to 173 ± 9% of untreated hepatocytes. In hepatocytes from the same rats, however, two hydrophobic bile acids, taurochenodeoxycholate and taurodeoxycholate, increased membrane protein kinase C activity to 220 ± 40% and 201 ± 31% of control hepatocytes, respectively. Thus, bile

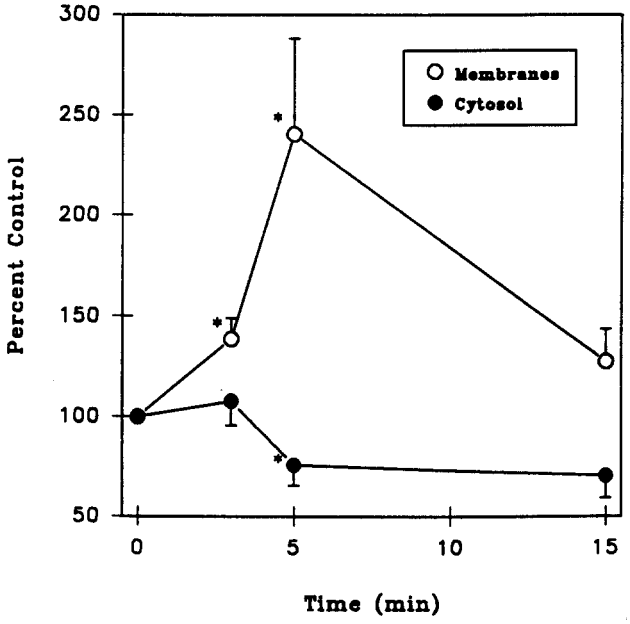

Fig. 7. Effects of taurocholate on PKC activity in primary cultures of rat hepatocytes. TCA (50 μM) was added to hepatocyte culture medium 18 h after plating and cells were scraped into iced homogenization buffer at the indicated times after the addition. Hepatocytes were homogenized, centrifuged at 100,000 *g*, and extracts were purified over DEAE-cellulose columns. PKC activity was determined in 25-μl aliquots using the PKC Assay Kit from Amersham, as described in Experimental Procedures; activity was controlled for protein concentration and expressed as percent of activity in the respective fractions of untreated cultures. Mean ± SE of four experiments; *P < 0.05 compared to no-addition control cultures.

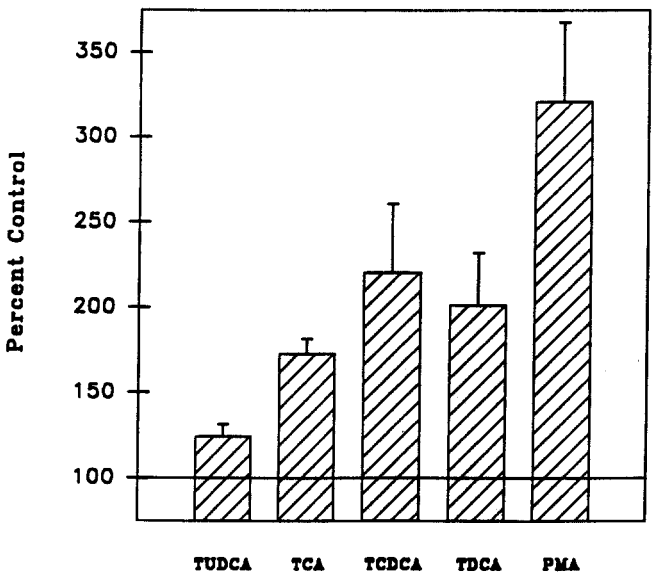

Fig. 8. Effects of PMA and bile acids of different hydrophobicity on PKC activity in cultured rat hepatocyte membranes. Bile acids (50 μM) or PMA (100 nM) were added to hepatocyte culture medium 18 h after plating, and hepatocytes were harvested 5 min later. Hepatocyte membranes were isolated, purified, and assayed for PKC activity as described in Experimental Procedures. Mean ± SE of four experiments; P < 0.05 for all conditions compared to untreated controls; TUDCA, tauroursodeoxycholate; TCDCA, taurochenodeoxycholate; TDCA, taurodeoxycholate.

acids increased membrane-associated PKC activity ($P < 0.05$ compared to controls) in rough proportion to their relative hydrophobicity. Hydrophobic bile acids, however, were not as effective as PMA (100 nM for 5 min), which increased levels to 321 ± 47% of control.

cPKCα isozyme translocation to hepatocyte membranes in response to phorbol di-esters and bile acids

Immunopurified antiserum against cPKCα was next used to determine whether bile acids induced the membrane translocation of this calcium-dependent isoform. Immunodetection with anti-cPKCα (**Fig. 9**) revealed a single ~80-kDa band in whole rat brain extracts; three bands were detected in cultured hepatocyte membrane extracts (~80, ~65, and ~45 kDa), and in cytosol from cultured hepatocytes and whole liver (80, 65, and ~50 kDa). In unstimulated hepatocytes, cPKCα was detected predominantly in the cytosolic fraction. The 80- and 45–50-kDa bands were undetectable after the primary antibody was preincubated with cPKCα-specific peptide, implying that they represent specific mature (catalytically active) and degradation fragments, respectively (29). The 65-kDa band persisted, implying that it is an artifact.

In triplicate experiments from different preparations, the 80-kDa band of cPKCα increased in hepatocyte membranes after incubation with TCA (50 μM) or PMA (100 nM; Fig. 9). Quantification of membrane-associated 80 kDa cPKCα by laser densitometry of chemiluminescent exposures revealed a mean 2.5-fold increase within 15 min of the addition of TCA (50 μM), compared to untreated controls. Conversely, the 80-kDa form of cytosolic cPKCα decreased by 10–15% 15 min after the addition of TCA. PMA (100 nM) caused a 4-fold increase in the 80-kDa band of hepatocyte membrane extracts after 5 min, and a more substantial (50%) decrease in cytosolic immunoreactivity.

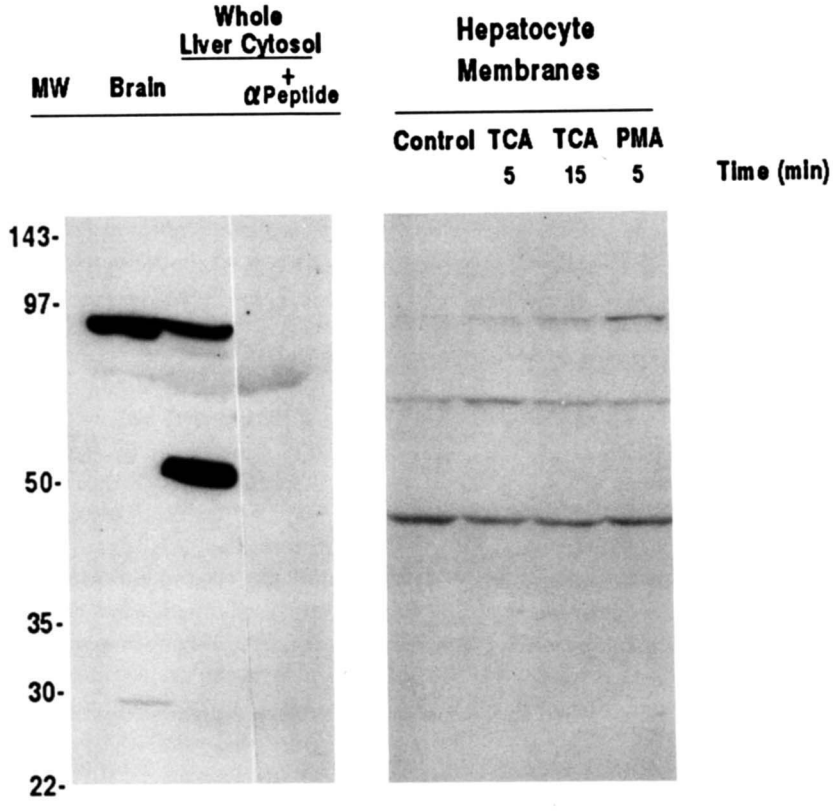

Fig. 9. Western immunoblots of cPKCα in rat brain, liver, and hepatocytes. Proteins from whole rat brain, rat liver cytosol, and hepatocyte membrane extracts (200 μg/lane) were separated in an 8% polyacrylamide gel, transferred to nitrocellulose, and blocked in 5% non-fat dry milk as described in Experimental Procedures. Rabbit anti-cPKCα (1:1000) and goat anti-rabbit IgG–horseradish peroxidase conjugate (1:1000) were sequentially added to blocking solution, and detection was accomplished by chemiluminescence. The blots depicted were exposed to DuPont Reflections film for 1.5 min. Lanes (left to right): molecular weight markers (MW), rat brain extract, rat liver cytosol, rat liver cytosol incubated with anti-cPKCα previously exposed to cPKCα-specific peptide (1:2 w/w), and cultured rat hepatocyte membrane extracts after the indicated experimental manipulations. Staining of nitrocellulose membranes with Ponceau S solution demonstrated approximately equal transfer of hepatocyte membrane proteins. Representative of three individual experiments.

DISCUSSION

The evidence presented strongly implicates PKC in mediating the repression of the cholesterol 7α-hydroxylase gene by hydrophobic bile acids. First, similar to the effects of hydrophobic bile acids, PKC activation by PMA inhibits cholesterol 7α-hydroxylase transcription and lowers mRNA levels. Equimolar concentrations of 4α-PMA, which does not activate PKC, had no effect. Second, bile acids repress cholesterol 7α-hydroxylase mRNA and transcriptional activity, and increase membrane-associated PKC activity, in approximate proportion to their relative hydrophobicity. This observation suggests that the dependence of cholesterol 7α-hydroxylase repression on bile acid hydrophobicity may be due to the more potent activation of PKC by hydrophobic bile acids. Third, PKC inhibition or depletion limits the ability of bile acids to repress cholesterol 7α-hydroxylase mRNA. Finally, TCA and PMA induce cPKCα translocation to hepatocyte membranes.

Indirect evidence suggests that the earliest event in the repression of cholesterol 7α-hydroxylase transcription by bile acids may be bile acid uptake into the hepatocyte. We have previously reported that the addition of TCA to cultured rat hepatocytes 48 h after plating decreased cholesterol 7α-hydroxylase mRNA with a $T_{½}$ of 22 h (15). However, after an identical addition at earlier culture times (18 h), Twisk, Lehmann, and Princen (14) observed a much more rapid decrease ($T_{½}$ = 3–4 h), similar to the present studies (Fig. 1). Recently, Liang et al. (30), have described a substantial decline in sodium-dependent bile acid transporter expression in rat hepatocytes cultured for longer than 24 h. One interpretation of these apparently discrepant time courses, therefore, is that bile acids must be internalized by an adequately expressed transport protein in order to rapidly regulate the cholesterol 7α-hydroxylase gene.

The biphasic response of cholesterol 7α-hydroxylase mRNA to PMA (Fig. 3) is consistent with the current understanding of the stimulation and termination of PKC activity. Short-term exposure to phorbol di-esters induces translocation of cytosolic PKC to membranes, where phosphorylation of target proteins occurs (25). Prolonged stimulation of PKC initiates its proteolytic degradation by calcium-dependent neutral proteases (calpains; ref. 31), ultimately depleting the cell of PKC (PKC "down-regulation"). The early decline in cholesterol 7α-hydroxylase mRNA after the addition of PMA may thus represent the phosphorylation of a positive *trans*-acting factor, perhaps limiting its ability to traverse the nuclear envelope or activate the cholesterol 7α-hydroxylase gene. Indeed, transcription factor phosphorylation events are commonly involved in regulating gene transcription (32). Subsequent down-regulation of PKC, dephosphorylation of the *trans*-acting factor, and restoration of cholesterol 7α-hydroxylase transcription may account for the rebound phase. Potentially, PP-2A, the activity of which may be regulated by sphingolipid-derived second messengers (33), may mediate this dephosphorylation (Fig. 5). Additional studies will be required to determine whether the PKC and PP-2A substrates implicated by these studies are the same proteins.

The inability of TCA to repress cholesterol 7α-hydroxylase mRNA after long-term incubation with PMA supports our interpretation of the recovery phase as the result of PKC depletion. Although the addition of TCA to hepatocyte cultures pretreated with PMA for 24 h yielded cholesterol 7α-hydroxylase mRNA levels 14% lower than cultures treated with PMA alone, cultures treated with PMA alone displayed levels 14% above untreated controls. Thus, PKC depletion had the net effect of completely preventing the bile acid-induced decline. Two highly specific PKC inhibitors were also used to attempt to block the bile acid effect. These inhibitors alone caused modest but possibly significant (10–20%) decreases in cholesterol 7α-hydroxylase mRNA compared to untreated cultures. Neither compound, however, appeared to initiate apoptotic DNA degradation as recently shown in a malignant cell line (22), or to liberate LDH from hepatocytes. These controls indicate that the decrease was not the result of cell death, but may not rule out nonspecific toxic effects. Nevertheless, the inability of TCA to repress cholesterol 7α-hydroxylase mRNA in the presence of these inhibitors and after long-term PMA treatment are consistent.

Three laboratories have recently implicated the proximal 5'-flanking region of the rat cholesterol 7α-hydroxylase gene in conferring bile acid responsiveness (34–36). Hoekman et al. (34) have proposed the presence of a bile acid-responsive element between −49 and −79 bp of the start site of transcription. In transgenic mice and SV-40-transformed mouse hepatocytes, Ramirez et al. (35) have reported bile acid repression of a reporter gene transcribed by −342 bp of the cholesterol 7α-hydroxylase 5'-flanking region. Optimal regulation of these chimeric genes by bile acids, however, required the inclusion of a liver-specific enhancer region located 6–7 kb upstream of the start site of transcription. Most recently, Chiang and Stroupe (36) have identified a DNase I footprint between −81 and −35 bp, containing several consensus sequences for the binding of putative transcription factors. Electrophoretic mobility shift assays revealed two retarded bands after incubation with nuclear extracts from rats fed a normal diet, but not after incubation with extracts from rats fed deoxycholate. These authors have proposed a model in which bile acids bind to a bile acid receptor and displace a positive *trans*-acting factor from the proximal cholesterol 7α-hydroxylase promoter.

Our data may be compatible with these promoter studies. PKC, or PKC-dependent transcription factors,

may antagonize the activity of positive *trans*-acting factors, such as the CCAAT-enhancer binding protein (C/EBP; 37), or the glucocorticoid receptor (38), respectively. Consensus sequences for both of these factors reside in the proximal cholesterol 7α-hydroxylase 5'-flanking region of the rat (39), although not within the nucleotides identified by Chiang and Stroupe (36). In addition, a putative AP-1 recognition motif, which may bind the phorbol di-ester-inducible transcription factors, c-Fos and c-Jun, also exists far upstream (approximately −1200 bp; 39). The functional significance of these sequences and those identified by Chiang and Stroupe (36) in reference to bile acid- and phorboid-responsiveness awaits further investigation.

Immunoblots of hepatocyte protein fractions indicate that a calcium-dependent isoform (cPKCα) translocates to membranes in response to a naturally occurring bile acid in physiological concentrations. The magnitude and time course of this translocation closely parallels the increase in membrane-associated PKC activity after the addition of TCA. In rat liver, calcium-dependent (α, β_{II}) and independent (δ, ϵ, ζ) PKC isoforms have been variably detected in previous studies (27, 40, 41). The functional significance of these isoforms remains unsettled, although evidence for different roles in cell signalling is accumulating (25, 42). Both calcium-dependent (α, β, 40, 43) and -independent (δ; 40) PKC isoforms have also been detected in rat hepatocyte nuclei. Although the functional significance of nuclear PKC in terms of gene regulation has not been firmly established, nuclear levels of specific isoforms have been suggested to be differentially regulated (40, 44). Interestingly, bile acid-induced PKC activation has also been documented in cells of non-hepatic origin (45–49).

Several important questions remain to be addressed. First, the mechanisms by which bile acids activate PKC await definition. Bile acids liberate intracellular calcium stores in isolated hepatocytes (50, 51), and may thereby contribute to the translocation and activation of PKC through a calcium signal. Second, the functional significance and intracellular destination of bile acid-activated PKC isozymes requires further study. In addition to cholesterol 7α-hydroxylase, PKC also appears to regulate the activities of the hepatocyte canalicular bile acid exporter (52) and sinusoidal sodium-taurocholate cotransporter (53). Bile acid-induced PKC isozyme activation may thus serve to coordinate bile acid homeostasis on a cellular level. Finally, the *trans*-acting factors and *cis*-acting nucleotides in the cholesterol 7α-hydroxylase 5'-flanking region mediating its transcriptional repression by bile acids, and presumably phorbol di-esters, must be precisely identified.

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