Taurolithocholic Acid Exerts Cholestatic Effects via Phosphatidylinositol 3-Kinase-dependent Mechanisms in Perfused Rat Livers and Rat Hepatocyte Couplets*

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 Taurolithocholic acid (TLCA) is a potent cholestatic agent. Our recent work suggested that TLCA impairs hepatobiliary exocytosis, insertion of transport proteins into apical hepatocyte membranes, and bile flow by protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) mechanisms. Prior studies showed that inhibitors for phosphatidylinositol 3-kinases (PI3K) stimulate PKCe. We studied the role of PI3K for TLCA-induced cholestasis in isolated perfused rat liver (IPRL) and isolated rat hepatocyte couplets (IRHC). In IPRL, TLCA (10 μmol/liter) impaired bile flow by 51%, biliary secretion of horseradish peroxidase, a marker of vesicular exocytosis, by 46%, and the Mrp2 substrate, 2,4-dinitrophenyl-S-glutathione, by 95% and stimulated PI3K-dependent protein kinase B, a marker of PI3K activity, by 154% and PKCe membrane binding by 23%. In IRHC, TLCA (2.5 μmol/liter) impaired canalicular secretion of the fluorescent bile acid, cholyglycylamido fluorescein, by 50%. The selective PI3K inhibitor, wortmannin (100 nmol/liter), and the anticholestatic bile acid tauroursodeoxycholic acid (TUDCA, 25 μmol/liter) independently and additively reversed the effects of TLCA on bile flow, exocytosis, organic anion secretion, PI3K-dependent protein kinase B activity, and PKCe membrane binding in IPRL. Wortmannin also reversed impaired bile acid secretion in IRHC. These data strongly suggest that TLCA exerts cholestatic effects by PI3K- and PKCe-dependent mechanisms that are reversed by tauroursodeoxycholic acid in a PI3K-independent way.

The hydrophobic bile acid taurolithocholic acid (TLCA) was identified as a potent cholestatic agent 35 years ago (1, 2).

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§ The abbreviations used are: TLCA, taurolithocholic acid; UDCA, ursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; IRHC, iso-

However, the mechanisms of this cholestatic effect are not yet clear (3, 4). TLCA induces cholestasis at low micromolar concentrations in vivo (1) as well as in the isolated perfused liver (5, 6) and in isolated hepatocyte couplets (7 in vitro. TLCA impairs hepatobiliary exocytosis, a key step for the insertion of apical carrier proteins into their target membrane, and lowers the density of the apical conjugate export pump, Mrp2, in canaliculared membranes of liver cells in association with reduced canalicular excretion of organic anions (6). In parallel, TLCA modulates a number of signaling events in liver cells that may contribute to membrane vesicle fusion and membrane protein insertion; TLCA may (i) impair Ca2+ influx across hepatocellular membranes (8–10), (ii) reduce hepatocellular membrane binding of the Ca2+-sensitive α-isofrom of protein kinase C (PKCα), a mediator of regulated exocytosis (6), and (iii) selectively translocate the Ca2+-independent ε-isofrom of PKC to canaliculared membranes and activate membrane-bound PKC (6, 11). The role of PKCe as a mediator of TLCA-induced cholestasis, however, remains elusive because specific PKCe inhibitors for in vivo use are not available.

Products of phosphatidylinositol-3 kinases (PI3Ks) are mediators of diverse cellular functions and may also modulate secretory activity of epithelial cells (12, 13). In hepatocytes, PI3K is involved in taurocholic acid (TCA)-induced biliary bile acid secretion (14, 15). Interestingly, products of PI3K, phosphatidylinositol-3,4,5-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate, are potent stimuli of the e-isofrom of PKC in transfected insect cells as well as in human hepatoma cells (16, 17), possibly via binding and recruitment to membranes of phosphoinositide-dependent kinase-1 (PDK-1) (18) and subsequent PDK-1-dependent phosphorylation and autophosphorylation of PKCε (19) in a way similar to activation of the best characterized PI3K effector, the proto-oncogene Akt/protein kinase B (PKB) (13). Therefore, we speculate that the TLCA cholestatic effects may be mediated by PI3K- and PKCe-dependent mechanisms. PI3K can be selectively blocked by specific P13K inhibitors, among which wortmannin is the best characterized in vivo and in vitro (20).

In contrast to TLCA, the hydrophilic bile acid ursodeoxycholic acid (UDCA) is a potent anticholestatic agent and is used in the treatment of a number of cholestatic disorders (21, 22).
The taurine conjugate of UDCA (TUDCA) reverses TCA-induced cholestasis by PKCα-dependent mechanisms (6). Recently, PI3K was proposed to contribute to TUDCA-induced stimulation of bile acid secretion in normal rat liver (26). In the present study we investigated the role of PI3K and PKCα in TCA-induced impairment of bile secretion in vivo in the model of the isolated perfused rat liver (IPRL) as well as in vitro in isolated rat hepatocyte coupled cultures (IRHCC) using the selective PI3K inhibitor wortmannin. We also investigated the role of PI3K in the ability of TUDCA to reverse PI3K activity and hepatobiliary exocytosis.

EXPERIMENTAL PROCEDURES

Materials—Anti-PI3K p85, p110α, and p110β antibodies and a PDK-1 immunoprecipitation kinase assay kit were from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-peptide antibodies anti-PKCα, fetal calf serum, Geneticin, and trypsin/EDTA were purchased from Invitrogen. Anti-phospho PKB (Ser-473) and anti-PKB antibodies were from Cell Signaling (Beverly, MA). Molecular weight markers and protein A/G plus-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA), and Hyperfilm ECL was from Amersham Biosciences. Immobilin-P membranes were from Millipore (Eschborn, Germany). A goat anti-rabbit IgG antibody was from Sigma. Anti-conjugated bile acids, dimethyl sulfoxide (Me2SO), horseradish peroxidase (HRP, type II), leupeptin, wortmannin, and albumin (fraction V) were from Sigma. 1-Chloro-2,4-dinitrobenzene (CDNB) was from ICN Biomedicals Inc. (Aurora, OH). Easitides (γ-P)ATP was from PerkinElmer Life Sciences. A renaissance Western blot chemiluminescence reagent was from PerkinElmer Life Sciences. All other chemicals were of the highest purity commercially available.

Animals—Male Sprague-Dawley rats (229 ± 16 g) were obtained from Charles River (Sulzdorf, Germany). They were subjected to a 12-h day-night rhythm with free access to rodent food and water.

Isolated Rat Liver Perfusion—The technical procedure used has been described previously (6). Rats were euthanized with sodium pentobarbital (50 mg/kg of body weight, intraperitoneal). After cannulation of the bile duct, the portal vein, and the inferior vena cava, the latter was ligated above the right renal vein. The liver was perfused with Krebs-Ringer bicarbonate solution (6) at 37°C at a constant flow rate of 4.0–4.5 ml/min/g of liver. Temperature and perfusion pressure were continuously monitored and did not significantly change during any of the experimental conditions chosen in this study. Bile flow was measured gravimetrically in pretared tubes. Hepatopanefus oxygen of lactate dehydrogenase was measured as an indicator of liver cell damage by use of a standard enzymatic test (24). Two perfusion protocols were applied for determination of (i) hepatobiliary exocytosis and (ii) secretion of the model Mrp2 substrate, 2,4-dinitrophenyl-s-glutathione (GS-DNP).

Protocol I: Hepatobiliary Exocytosis (6, 25)—Livers were preloaded with 5 mg/dl HRP, 1 g/dl bovine serum albumin for 25 min in a recirculating Krebs-Ringer bicarbonate perfusion solution (40 ml/min). The perfusion was then switched to a non-recirculating HRP- and bovine serum albumin-free Krebs-Ringer bicarbonate perfusion solution (40 ml/min). At the end of the experiments, the left anterior liver lobe was clamped and excised. A sample of this lobe was immediately quick-frozen in liquid nitrogen and stored at −80 °C for determination of PI3K activity and PKB (Ser-473) phosphorylation (see below).

Biliary HRP activity was determined spectrophotometrically using 4-aminoantipyrine as substrate and recording the linear change in absorption at 510 nm for 3 min at a constant temperature of 25°C (6, 25). HRP (mg of protein/min/g of liver) was quantitated after establishing HRP standard curves. Biliary HRP secretion was expressed as % of secretion at min 45 (after HRP loading and washout period) to correct for differences in base-line total HRP activity.

Biliary secretion of GS-DNP was determined spectrophotometrically (6, 26). 5 μl of bile were added to 1000 μl of H2O in a cuvette. Absorption measurements of biliary GS-DNP levels were calculated using the formula C = EoVc−Vt/edVt where, Eo absorption at 335 nm; Vc cał; 1005 μl; ϵ, molar absorption coefficient 9.6 liters mmol−1 cm−2; d, 1 cm; Vt, μl, 5 μl). The low background absorption at 335 nm of the bile sample collected just before infusion of CDBN was set as 0.

PI3K activity in hepatic tissue was determined by use of a PI3K assay. In brief, 100 mg of shock-frozen tissue were homogenized in ice-cold lysis buffer (1 ml/50 mg of tissue; HEPES, 50 mMol/liter; NaCl, 137 mMol/liter; CaCl2, 1 mMol/liter; MgCl2, 1 mMol/liter; Na3PO4, 10 mMol/liter; Na3, 10 mMol/liter; EDTA, 2 mMol/liter; tritongol Noniden P40, 1% (v/v); glyceral, 10%, (v/v); antipain, 10 mg/mliter; aprotinin, 2 mg/mliter; benzamidine, 9.6 mMol/liter; leupeptin, 10 μMol/liter; Na2VO3, 2 mMol/liter; phenylmethylsulfonyl fluoride, 1 mMol/liter; pH 7.5). Samples were kept on ice for 30 min and then were centrifuged for 30 min (3000 × g, 4 °C). For immunoprecipitation, aliquots from the supernatant containing 500 μg of protein were incubated with 4 μg of anti-PI3K p85, p110α, or p110β antibodies at 4 °C overnight and precipitated with 90 μl of A/G-agarose for 2–4 h at 4 °C. After centrifugation (20,000 × g, 1 min, 4 °C), the pellet was washed thrice with lysis buffer, 3 times with buffer B (Tris, 100 mMol/liter; LiCl, 5 mMol/liter; Na2VO3, 0.1 mMol/liter; pH 7.4, 4 °C) and 2 times with buffer C (NaCl, 150 mMol/liter; Tris, 10 mMol/liter; EDTA, 5 mMol/liter; Na2VO3, 0.1 mMol/liter; pH 7.4, 4 °C). The PI3K assay was then started by adding 50 μl of a reaction buffer (Tris, 10 mMol/liter; MgSO4, 20 mMol/liter; EDTA, 5 mMol/liter; pH 7.5, 2 mMol/liter; supplemented with 20 μg of PtdIns, 10 μMol/liter γ-PATP, and 200 μMol/liter ATP, and samples were incubated at 37 °C for 10 min. The reaction was stopped by adding 150 μl of chloroform: methanol: HCl, 12 mlol/liter (33:66:0.6).

For lipid extraction, 120 μl of chloroform were added, and samples were centrifuged (20,000 × g, 10 min, 4 °C). The organic phase was washed by adding 150 µl of CH2OH:HCl, 1 mol/liter (1.1), 20 µl of HCl, 8 mol/liter, and 160 µl of CHCl3:CH2OH (1:1). After centrifugation (20,000 × g, 10 min, 4 °C), the organic phase was evaporated under N2 and spotted on TLC plates. Plates were developed in CHCl3:CH2OH:H2O 25% NH3 (60:47:11:3.2). Autoradiography and densitometry of spots representing the PI3K product, phosphatidylinositol 3-phosphate, and phosphatidylinositol 4-phosphate (PtdIns 3,4-P2) is performed.

PKB/Akt activity in hepatic tissue was determined by an immunoblotting technique (27, 28). In brief, shock-frozen tissue (−80 °C) was homogenized in ice-cold lysis buffer (27) (1 ml/100 mg of tissue) and processed as described (27, 28). Aliquots were electrophoresed using sodium dodecyl sulfate, 10% polyacrylamide gel electrophoresis. Separated proteins were transferred to Immobilon-P membranes and probed with phospho-PKB (AktSer-473) antibodies at a dilution of 1:1000 overnight to detect the activated form of PKB. Then membranes were stripped and reprobed with a PKB antibody (1:1000) to detect total PKB in an identical procedure. After the use of a goat anti-rabbit IgG antibody (1:5000), a chemiluminescence reagent, and Hyperfilm ECL, the gel was exposed to X-ray film. The experiments, HRP activity, and densitometric analysis (NH Image Densitometric Analysis 1.54; Bethesda, MD, 1994).

Distribution of the protein kinase C isofrom ε in hepatic tissue was determined by an immunoblotting technique exactly as described previously (6, 11, 29) using affinity-purified isoenzyme-specific antibodies for PKCε. The PKC bands at 90 kDa (ε) were quantified by densitometry and expressed as % of total PKC activity. The PKC bands were measured by using densitometric analysis (1.54; Bethesda, MD). The bands were expressed as [optical density of the particular fraction/total optical density of cytosol and membrane fraction] × 100%.

PKD-I activity in hepatic tissue was determined by use of a PKD-1 immunoprecipitation kinase assay kit (Upstate, Lake Placid, NY). In brief, shock-frozen liver tissue (100 mg/ml) was homogenized on ice in 50 mMol/liter Tris, 50 mMol/liter NaCl, 100 µMol/liter EGTA, 50 mMol/liter; sodium β-glycerophosphate, 10 mMol/liter; Na3PO4, 5 mMol/liter; Na2VO3, 1 mMol/liter; 2-mercaptoethanol, 0.1%; H2O, 50 ml/liter; pH 7.5, 4 °C) and further processed exactly following the assay protocol provided by the manufacturer. After im-
munnprecipitation of PDK-1 with anti-PDK-1 sheep immunoffinity-purified IgG and protein G-agarose beads, PDK-1 activity was determined by phosphorylation and activation of recombinant serum and glucocorticoid-inducible kinase 1 (SGK1) and SGK1-dependent phosphorylation of an Akt/SGK substrate peptide using [γ-32P]ATP. The phosphorylated substrate peptide was separated from residual [γ-32P]ATP using P81 phosphocellulose paper and quantitated using a scintillation counter. PDK-1 activity was expressed in arbitrary units (AU). Mean PDK-1 activity in control livers was set as 1.00 AU.

Isolation and culture of rat hepatocyte couplets was performed as previously described (30). Cells were plated in 100-mm Petri dishes (27) (1 ml/100 mg) and processed as described above (27, 28). CGamF was synthesized according to Schteingart et al. (31) and was kindly provided by Dr. Alan Hofmann. Four hours after isolation, hepatocytes (on coverslips) were briefly transferred to HEPES buffer (30). Bile acid secretion by IRHC was assessed by measuring the hepatocellular uptake and secretion of 1 μmol/liter cholyglycylamido fluorescein (CGamF) into the canalicular space as previously described (30). CGamF was washed out when Me2SO was added (Fig. 1B) to allow adequate loading of the fluorescent bile acid and transferred back for 10 min to their previous dishes (i-vi). Hepatocyte secretion was stopped by placing coverslips in ice-cold HEPES buffer on ice, and cells were viewed immediately on a Zeiss LSM 510 microscope (Thornwood, NY). Laser settings were optimized for a dynamic range to avoid saturation of the fluorescence. The same settings were used for all conditions.

Cells were analyzed on the confocal laser scanning microscope by one investigator (C. J. Soroika) who was blinded to the experimental conditions. Couplets were selected based upon the presence of a well-defined canalicular space as determined under bright field optics. Images were then acquired with rapid scanning to avoid quenching of the fluorescence. Quantitation of uptake (uptake = [F° cell + F° can]/μm²) and secretion (% secretion = [F° can/F° cell + F° can] × 100) of CGamF was performed as previously published (30), except that NIH Image software was used.

PKB/Akt activity in isolated rat hepatocytes was determined by an immunoblotting technique (27, 28). In brief, 4 h after plating (see above) cells were incubated for 5, 15, 30, and 60 min with TCA (5 μmol/liter; at concentrations >5 μmol/liter, TCA caused visible damage in isolated hepatocytes in short term culture), TUDCA (10 μmol/liter), TCA (10 μmol/liter), or the carrier MeSO only (control, 0.1%, v/v). Culture dishes were then placed on ice, and cells were scraped and immediately shock-frozen (−80 °C). Shock-frozen cells were homogenized in ice-cold lysis buffer (27) (1 ml/100 mg) and processed as described above (27, 28).

Statistical Data are expressed as the mean ± S.D. Results were compared between groups using an unpaired two-tailed Student’s t test or ANOVA when indicated. p < 0.05 was considered statistically significant.

RESULTS

Hepatobiliary Exocytosis in Perfused Rat Livers (Protocol I)—Bile flow was 1.0 ± 0.1 μl/min/g of liver (n = 47) after loading of livers with HRP for 25 min and a wash-out period of 5 min and remained stable for the following 50 min in control experiments (52.2 ± 4.4 μl/50 min/g of liver, n = 5) in the presence of dimethyl sulfoxide (MeSO, 0.1%, v/v; Fig. 1A). Wortmannin (100 nmol/liter) did not affect bile flow under basal conditions (54.9 ± 5.1 μl/50 min/g of liver, n = 5; Fig. 1A). TUDCA (10 μmol/liter) caused a decrease of bile flow to 18% of controls (9.5 ± 1.6 μl/50 min/g of liver, n = 6, p < 0.01; Fig. 1A). The TUDCA-induced decrease of bile flow was partially reversed by wortmannin; bile flow was 50% (26.2 ± 3.0 μl/50 min/g of liver, n = 6, p < 0.01 versus TUDCA) and 47% (24.6 ± 3.5 μl/50 min/g of liver, n = 5, p < 0.01 versus TUDCA) of controls when wortmannin was administered at 100 nmol/liter and 500 nmol/liter, respectively (Fig. 1A). TUDCA (25 μmol/liter) increased bile flow to 139% of that controls (72.7 ± 16.1 μl/50 min/g of liver, n = 5, p < 0.05, Fig. 2A). Wortmannin did not affect TUDCA-induced bile flow (73.7 ± 14.4 μl/50 min/g of liver, n = 5; Fig. 2A). TUDCA reversed TUDCA-induced impairment of bile flow (57.4 ± 12.8 μl/50 min/g of liver, n = 5, p < 0.05 versus TLCA; Fig. 2A). Wortmannin (100 nmol/liter) and TUDCA additively counteracted TLCA-induced impairment of bile flow (83.9 ± 10.3 μl/50 min/g of liver, n = 5; p < 0.05 versus TUDCA+TLCA; Fig. 2A). Thus, the PI3K inhibitor, wortmannin, did not affect basal and TUDCA-induced bile flow but partly reversed TLCA-induced inhibition of bile flow. Wortmannin and TUDCA exerted additive anticholestatic effects.

Biliary HRP secretion was 0.88 ± 0.38 ng/min/g of liver (n = 47) after loading of livers with HRP for 25 min and a wash-out period of 5 min. HRP secretion slowly decreased during the following 50 min (Fig. 1B) leading to a total secretion of 19.5 ± 9.0 ng/50 min/g of liver (n = 5) in control livers treated with MeSO (0.1%, v/v) only (Fig. 1B). Wortmannin (100 nmol/liter) significantly increased biliary HRP secretion (47.5 ± 8.1 ng/50 min/g of liver, p < 0.05, Fig. 1B). TLCA (10 μmol/liter) markedly impaired biliary secretion of HRP (6.7 ± 2.3 ng/50 min/g of liver, p < 0.05; Fig. 1B). Wortmannin antagonized TLCA-induced impairment of HRP secretion both at low (100 nmol/liter) and high (500 nmol/liter) concentrations (22.1 ± 6.0 ng/50 min/g of liver n = 6 and 18.0 ± 5.5 ng/50 min/g of liver, n = 5, p < 0.01 versus TLCA; Fig. 1B).
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Co-administration of wortmannin (100 nmol/liter) and TUDCA (25 μmol/liter) led to a marked stimulation of biliary secretion of HRP in normal livers (30.3 ± 3.0 ng/50 min/g of liver, n = 5, p < 0.05, each, versus control and TUDCA only; Fig. 2B) as well as in livers treated with TLCA (10 μmol/liter) (46.6 ± 8.9 ng/50 min/g of liver, n = 5, p < 0.05 versus TLCA + TUDCA; Fig. 2B).

Thus, the PI3K inhibitor, wortmannin, stimulated biliary exocytosis both under basal conditions and in the presence of TUDCA and completely reversed TLCA-induced impairment of exocytosis. Again, wortmannin and TUDCA exerted additive anticholestatic effects.

**Organic Anion Secretion in Perfused Rat Livers (Protocol II)**—Bile flow was 1.3 ± 0.2 μl/min/g of liver (n = 40) after 20 min before bile acids or their carrier Me₃SO only (0.1%, v/v) were infused, indicating an adequate secretory capacity of livers under these experimental conditions. The addition of CDNB (30 μmol/liter for 10 min, min 41–50) led to a transient increase of bile flow under all experimental conditions due to the choleretic potential of CDNB-glutathione conjugate, GS-DNP, in rat liver. In controls, bile flow was 53.2 ± 7.7 μl/50 min/g of liver (n = 5) after CDNB infusion. Wortmannin (100 nmol/liter) did not significantly affect bile flow (92% of controls), whereas TLCA (10 μmol/liter) markedly reduced bile flow to 6% of controls (Table I). Wortmannin (100 nmol/liter) induced a marked increase of bile flow in TLCA-treated livers (33% of controls, p < 0.01 versus TLCA; Table I). TUDCA (25 μmol/liter) increased bile flow to 177% of controls, and this increase was not affected by concomitant treatment with wortmannin (170% of controls; Table I). TUDCA (25 μmol/liter) reversed the cholestatic effect of TLCA (10 μmol/liter) (128% of controls), and wortmannin (100 nmol/liter) and TUDCA (25 μmol/liter) additively counteracted the cholestatic effect of TLCA (10 μmol/liter) (176% of controls, 137% of TUDCA + TLCA, Table I).

Thus, as in the first perfusion protocol, wortmannin did not affect basal and TUDCA-induced bile flow but antagonized TLCA-induced impairment of bile flow. The anticholestatic effects of wortmannin and TUDCA on TLCA-induced impairment of bile flow were additive and independent.

Biliary secretion of GS-DNP, a model Mrp2 substrate, was 730 ± 120 nmol/50 min/g of liver (n = 5; Table 1) after administration of CDNB (30 μmol/liter) for 10 min as described previously (6). Wortmannin (100 nmol/liter) did not affect basal GS-DNP secretion (94.7% of controls) (Table I). In contrast, TLCA (10 μmol/liter) markedly reduced GS-DNP secretion to 5.3% of controls (Table I) as reported previously (6). Wortmannin (100 nmol/liter) increased GS-DNP secretion in TLCA-treated livers from 5.3 to 15.0% of controls (p < 0.05 versus TLCA). TUDCA (25 μmol/liter) stimulated GS-DNP secretion (127.0% of controls), and this increase was not affected by concomitant treatment with wortmannin (118.0% of controls). TUDCA (25 μmol/liter) largely antagonized the effect of TLCA on secretion of GS-DNP (72.6% of controls, p < 0.01 versus TLCA) as reported previously (6), and wortmannin (100 nmol/liter) and TUDCA (25 μmol/liter) tended to additively counteract the effect of TLCA (10 μmol/liter) on GS-DNP secretion (82.5% of controls) (Table I).

Thus, wortmannin did not affect basal or TUDCA-induced secretion of the model Mrp2 substrate, GS-DNP, but partly antagonized TLCA-induced impairment of GS-DNP secretion. The anticholestatic effects of wortmannin and TUDCA were independent.

Hepatovenous lactate dehydrogenase release after 85 min was not affected by administration of wortmannin (100 nmol/liter) or TUDCA (25 μmol/liter). TLCA (10 μmol/liter) alone or in combination with TUDCA (25 μmol/liter) markedly increased lactate dehydrogenase release (Table I). These effects were reversed by wortmannin (100 nmol/liter) (Table I). Thus, wortmannin did not induce liver cell damage under the experimental conditions chosen but reversed liver cell damage induced by TLCA alone or by TLCA and TUDCA.

**Kinase Activities in Tissue of Perfused Rat Livers—PI3K class IA activity, as determined by a PI3K assay after immunoprecipitation using an anti–PI3K p85 antibody, was reduced by wortmannin (100 nmol/liter) to 62% of controls (p < 0.01; Fig. 3). TLCA (10 μmol/liter) tended to increase and TUDCA (25 μmol/liter) tended to decrease total PI3K activity as determined by this methodological approach. Immunoprecipitation of PI3K class IA isoforms using PI3K p110 was not affected (33% of controls, data not shown).

Thus, wortmannin inhibited PI3K activity in liver tissue. The limited sensitivity of the methodological approach may have prevented unequivocal disclosure of the effects of bile acids at low micromolar concentrations on PI3K activity.

PI3K-dependent PKB (PKB/Akt) activity, a sensitive and convenient physiological read-out of the activation of the PI3K pathway (27, 32) as determined by the amount of phospho-PKB (Ser-473) in liver tissue, was reduced by wortmannin (100

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**Fig. 2.** The PI3K inhibitor, wortmannin (100 nmol/liter), and the anticholestatic bile acid, TUDCA, additively counteract TLCA-induced impairment of bile flow (A) and biliary secretion (B) of HRP in the isolated perfused rat liver (for experimental details, see “Experimental Procedures,” “Protocol I”). Livers were preloaded with HRP. After a wash-out phase of 5 min, TUDCA (25 μmol/liter; □), TUDCA (25 μmol/liter) + wortmannin (Wo; 100 nmol/liter; ■), TUDCA (25 μmol/liter) + TLCA (10 μmol/liter; ◀), and TUDCA (25 μmol/liter) + wortmannin (100 nmol/liter) + TLCA (10 μmol/liter; ◆) were administered for 50 min. Results are expressed as the mean ± S.D. of 5–6 experiments. For statistical evaluation, see “Results.”
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TABLE I

Effect of the PI3K inhibitor, wortmannin (100 nmol/liter), on bile flow, biliary secretion of the model Mrp2 substrate, GS-DNP, and lactate dehydrogenase (LDH) release into the hepatovenous effluent in isolated perfused rat liver (for experimental details, see “Experimental Procedures,” “Protocol II”)

Livers were treated in the presence or absence of the PI3K inhibitor, wortmannin (100 nmol/liter), for 75 min with Me 2 SO alone (0.1% (v/v) control), TLCA (10 μmol/liter), TUDCA (25 μmol/liter), or TLCA (10 μmol/liter) + TUDCA (25 μmol/liter). After 25 min, CDNB (30 μmol/liter), a precursor of GS-DNP, was administered for 10 min, and bile flow and biliary secretion of GS-DNP were measured for 50 min. Results are expressed as the mean ± S.D. of five experiments each.

|            | Me2SO (100 nmol/liter) | Wortmannin (100 nmol/liter) | TLCA (10 μmol/liter) | TLCA (10 μmol/liter) + Wortmannin (100 nmol/liter) | TUDCA (25 μmol/liter) | TUDCA (25 μmol/liter) + Wortmannin (100 nmol/liter) | TUDCA (25 μmol/liter) + TLCA (10 μmol/liter) | TUDCA (25 μmol/liter) + TLCA (10 μmol/liter) + Wortmannin (100 nmol/liter) |
|------------|------------------------|----------------------------|----------------------|-------------------------------------------------|-----------------------|--------------------------------------------------|---------------------------------------------|--------------------------------------------------------------------------------|
| Bile flow  | 53.2 ± 7.7             | 48.8 ± 8.7                 | 3.4 ± 1.6             | 17.3 ± 6.3                                       | 93.9 ± 13.1           | 90.2 ± 10.5                                      | 68.1 ± 2.7                                   | 93.3 ± 25.1                                                                                |
| GS-DNP secretion | 730 ± 120            | 692 ± 139                 | 39 ± 25               | 110 ± 61                                         | 924 ± 122             | 861 ± 177                                        | 530 ± 49                                    | 602 ± 185                                                                                 |
| LDH efflux after 85 min (milliunits/min/g liver) | 4.0 ± 0.9            | 5.5 ± 4.1                 | 3.0 ± 8.0             | 4.8 ± 1.1                                        | 6.4 ± 8.2             | 7.9 ± 6.8                                        | 55.9 ± 7.5                                  | 10.4 ± 5.8                                                                                 |

*p < 0.01 vs. control.

*p < 0.01 vs. TLCA alone.

*p = 0.05 vs. TLCA alone.

*p < 0.01 vs. TLCA + TUDCA, unpaired two-tailed Student’s t test.

Fig. 3. PI3K activity in liver tissue in the absence (white bars) or presence (black bars) of the PI3K inhibitor, wortmannin (Wo, 100 nmol/liter), under the experimental conditions described in Table I. PI3K activity was determined in shock-frozen liver tissue after immunoprecipitation using an anti-PI3K p85 antibody as described under “Experimental Procedures.” The product of PI3K, phosphatidylinositol 3-phosphate (PtdIns(3)P), was identified by TLC and autoradiography. The bar graph represents the amount of phosphatidylinositol 3-phosphate formed by immunoprecipitates of liver tissue from experiments shown in Table I as quantitated by phosphorimaging analysis. Immunoprecipitation with anti-PI3K p110α and p110β antibodies revealed similar results. Results are given as the mean ± S.D. of four experiments each. DMSO, Me2SO. *p < 0.01 versus control; #p < 0.05 versus TLCA.

Fig. 4. TLCA-induced activation of PI3K-dependent PKB is reversed by the PI3K inhibitor, wortmannin (Wo, 100 nmol/liter), and the anticholestatic bile acid, TUDCA, in liver tissue under the experimental conditions described in Table I. PKB activity was determined in shock-frozen liver tissue as the amount of pPKB(Ser-473) using a specific pPKB(Ser-473) antibody and a Western blotting technique as described under “Experimental Procedures.” In parallel, total PKB mass was determined on each blot using a nonspecific PKB antibody to prove that the total amount of PKB was identical on each lane. Panel A shows representative immunoblots of which the upper bands represent pPKB(Ser-473), and the lower bands represent total PKB under different experimental conditions. The bar graph in B represents activated PKB as determined by the amount of pPKB(Ser-473) in liver tissue from experiments shown in Table I. Results are given as mean ± S.D. of five experiments each. *p < 0.01 versus control. DMSO, Me2SO.

TLCA (10 μmol/liter) markedly stimulated PKB activity to 250% (p < 0.001 versus control), and this effect was completely reversed by wortmannin (100 nmol/liter; Fig. 4). Interestingly, TUDCA (25 μmol/liter) reduced basal PKB activity to 54% of controls (p < 0.01, Fig. 4), and this effect was not further amplified by wortmannin (100 nmol/liter). In addition, TUDCA (25 μmol/liter) reduced TLCA-induced activation of PKB by 70% (p < 0.01 versus TLCA, Fig. 4). The addition of wortmannin (100 nmol/liter) led to an additional reduction of PKB activity below control levels (p < 0.02 versus TLCA + TUDCA, Fig. 4).

Thus, TLCA markedly enhanced PKB activity, a sensitive marker of PI3K activity, in liver tissue, whereas both wortmannin and TUDCA impaired basal and TLCA-induced PKB activity in IPRL. The effects of wortmannin and TUDCA on TLCA-induced PKB activity were additive and independent.
The e-isoenzyme of PKC was about equally distributed between cytosol (57.4 ± 5.8%, n=5) and membranes (42.6 ± 5.8%) in control livers treated with Me₂SO. Neither wortmannin (100 nmol/liter) nor TUDCA (25 μmol/liter) affected distribution of PKCe (Fig. 5). In contrast, TLCA (10 μmol/liter) significantly increased membrane binding of PKCe by 23.0% (p<0.05) as observed previously in isolated hepatocytes (11). Wortmannin (100 nmol/liter) as well as TUDCA (25 μmol/liter) reversed the effect of TLCA on PKCe membrane binding (Fig. 5).

Thus, wortmannin did not affect membrane binding of PKCe in liver tissue under basal conditions, but like TUDCA, reversed TLCA-induced membrane binding of PKCe. The effects of wortmannin and TUDCA on TLCA-induced membrane binding of PKCe were independent and additive.

In the present study, Dock-1 activity in liver tissue (controls: 1.00 ± 0.26 AU, n=5) was not significantly affected by wortmannin (100 nmol/liter; 0.78 ± 0.41 AU). Similarly, neither TLCA (10 μmol/liter) in the absence (0.98 ± 0.21 AU) or presence (0.94 ± 0.50 AU) of wortmannin nor TUDCA (25 μmol/liter) in the absence (1.04 ± 0.41 AU) or presence (1.05 ± 0.34 AU) of wortmannin or TUDCA + TUDCA (0.77 ± 0.61 AU) or presence (1.00 ± 0.56 AU) of wortmannin affected Dock-1 activity in liver tissue. Thus, Dock-1 activity was not altered by inhibition of PI3K or bile acid administration under the experimental conditions chosen.

**Secretion of Bile Acids by IRHC**—After incubation with the fluorescent bile acid, CGamF (1 μmol/liter, 5 min), IRHC took up 1044 ± 648 AU (n=7) CGamF. Wortmannin (100 nmol/liter) did not significantly affect uptake of CGamF (1493 ± 276 AU). Neither TLCA alone at 2.5 μmol/liter (971 ± 486 AU) or 5 μmol/liter (832 ± 208 AU) nor TLCA in the presence of wortmannin (100 nmol/liter) at 2.5 μmol/liter (1004 ± 348 AU) or 5 μmol/liter (1248 ± 606 AU) affected uptake of CGamF in IRHC. After 15 min, IRHC secreted 12.2 ± 3.9% (n=7) of CGamF into their canalicular space. Wortmannin (100 nmol/liter) did not affect biliary secretion of CGamF (97% of controls, Fig. 6). TLCA (2.5 μmol/liter) markedly reduced canalicular secretion of CGamF by 54% (p<0.05; Fig. 6). Wortmannin completely reversed the effect of TLCA (106% of controls, Fig. 6). Higher concentrations of TLCA (5 μmol/liter) also impaired CGamF secretion (60% of controls), and wortmannin again reversed the inhibiting effect of TLCA on canalicular secretion of CGamF (93% of controls). Thus, wortmannin did not affect basal bile acid secretion in IRHC but completely reversed the cholestatic effect of TLCA on canalicular bile acid secretion.

**PI3K-dependent PKB (PKB/Akt) Activity in Isolated Rat Hepatocytes**—The amount of phospho-PKB(Ser-473), a sensitive read-out of the activation of the PI3K pathway (27, 32), was markedly enhanced by TLCA (5 μmol/liter) in hepatocytes in short term culture (Fig. 7) and reached levels up to 194 ± 46% of controls after 60 min (p<0.005 versus control; p<0.05 versus TUDCA; p<0.01 versus TCA). In contrast, TUDCA (10 μmol/liter) only transiently increased PKB activity, whereas TCA (10 μmol/liter) had no effect under the experimental conditions chosen (Fig. 7). Thus, TLCA markedly affected PI3K activity in isolated hepatocytes in vitro, whereas TUDCA exerted only minor transient effects on the PI3K pathway when administered at low micromolar concentrations.

**DISCUSSION**

The present study indicates that the monohydroxy bile acid, TLCA, impairs bile flow, hepatobiliary exocytosis, and secretion of bile acids and other cholephiles by PI3K- and putatively PKCe-dependent mechanisms. The major finding of this study is that TLCA-induced cholestasis can be reversed by specific PI3K inhibitors. This is demonstrated by the reversal of TLCA-induced impairment of bile flow and HRP secretion in IPRL (Figs. 1 and 2) as well as the reversal of TLCA-induced impairment of CGamF secretion in IRHC (Fig. 6) after administration of wortmannin. Thus, this study confirms that an individual bile acid can modulate liver cell function including bile secretion by interacting with specific signal transduction pathways in hepatocytes.

TLCA was the first human bile acid identified to cause cholestasis and jaundice (1), yet the molecular mechanisms by which TLCA induces cholestasis have remained obscure. TLCA induces selective damage of canalicular membranes leading to an increase in membrane rigidity and loss of microvilli (33, 34). TLCA impairs transcellular movement of vesicles (35) as well as vesicle fusion at the apical pole (6) and inhibits secretion of organic anions and bile acids across the canalicular membrane (6, 7, 36). The recent finding that TLCA markedly reduces the density of the conjugate export pump, Mrp2, in the canalicular membrane (6) strongly supports the concept that the mecha-
nism of TLCA-induced cholestasis involves inhibition of vesicle-mediated carrier insertion in the apical liver cell membrane. This view is further supported by the present study demonstrating that the PI3K inhibitor, wortmannin, completely reverses TLCA-induced inhibition of hepatobiliary exocytosis in IPRL in vitro as well as canicular bile acid secretion in IRHC in vitro.

Effects of the PI3K inhibitor, wortmannin, and of bile acids on total activity of class Ia PI3K were determined in IPRL in the present study. Class Ia PI3K are assumed to represent a predominant form of PI3K in secretory cells. Wortmannin inhibited PI3K activity in IPRL (Fig. 3), confirming that the effects of wortmannin on TLCA-induced cholestasis were mediated by PI3K in the present study. Bile acids at low micromolar concentrations did not induce significant changes of total class Ia PI3K activity as determined by a PI3K assay in IPRL (Fig. 3), although the PI3K inhibitor, wortmannin, markedly affected TLCA-induced changes of secretion (Figs. 1 and 2). Thus, we speculate that low micromolar concentrations of bile acids may modulate PI3K activity in hepatocytes in vivo at a subcellular level that is not technically detectable when using a PI3K assay in liver homogenates.

The serine/threonine protein kinase Akt/PKB is a well characterized target and effector of PI3K (13) and is used as a sensitive read-out of PI3K activity, in isolated rat hepatocytes in short term culture, whereas TUDCA (10 μmol/liter) induces transient activation, and TCA (10 μmol/liter) is ineffective under the experimental conditions chosen. Results are given as the mean ± S.D. of seven-eight experiments from eight different preparations. *, p < 0.05 versus control; **, p < 0.005 versus control; $, p < 0.05 versus TLCA.

TLCA Induces Cholestasis via PI3 Kinase-dependent Mechanisms

TLCA has already previously been shown to affect hepatocellular signaling cascades, which control vesicular exocytosis and membrane protein targeting in secretory cells. (i) TLCA specifically induces translocation of the ε-isofrom of PKC to the canicular membrane, increases intracellular levels of the physiological PKC activator, sn-1,2-diacylglycerol, and activates membrane-bound PKC (11, 29). (ii) TLCA modulates [Ca2+]cytosolic free calcium in isolated hepatocytes (8–10, 37, 38) and may inhibit Ca2⁺ influx in vitro at concentrations ≥10 μmol/liter (8–10). Both, activation of PKCs and impairment of Ca2⁺ influx have been related to impairment of exocytosis and membrane targeting of proteins (39, 40).

The ε-isofrom of PKC is specifically activated in vitro by products of PI3K, PtdIns-3,4-bisphosphate and PtdIns-3,4,5-trisphosphate (16, 17), possibly via phosphoinositide-dependent kinase I (PDK-1)-induced phosphorylation of Thr-566 in the activation loop and subsequent autophosphorylation of Ser-729 in the C-terminal hydrophobic motif (19). PDK-1 activity was not affected by wortmannin and bile acids in the present study (see “Results”). In human HepG2 hepatoma cells, activation of PI3K via stimulation of a mutant platelet-derived growth factor receptor led to specific translocation of PKCe from cytosol to membranes, a key step for activation of PKCe. This process was reversed by the addition of the PI3K inhibitor, wortmannin (17). The in vivo findings in the present study are consistent with these observations. TLCA-induced translocation of PKCe to membranes was reversed by wortmannin and, as recently shown, by the anticholestatic bile acid TUDCA (6). PKCe membrane binding was even more strongly inhibited when wortmannin was co-administered with TUDCA (Fig. 5). Thus, TLCA-induced membrane translocation of PKCe seems to be mediated by PI3K-dependent mechanisms.

Interestingly, wortmannin and TUDCA exerted additive and independent anticholestatic effects on bile flow and organic anion secretion as well as hepatobiliary exocytosis in TLCA-treated IPRL in the present study. Submaximal dosing of wortmannin was virtually excluded as a potential explanation for these additive effects of wortmannin and TUDCA because administration of the PI3K inhibitor at doses of 100 and 500 nmol/liter resulted in comparable effects on TLCA-induced impairment of bile flow in IPRL (Fig. 1a). As shown previously, the anticholestatic effect of TUDCA on TLCA-induced impairment of organic anion secretion (and bile flow)² was mediated by PKCa- and putatively Ca²⁺-dependent mechanisms (6) as documented by reversal of the anticholestatic effect of TUDCA by use of the PKC inhibitor, bisindolylmaleimide I. Bisindolylmaleimide I predominantly blocks the Ca²⁺-sensitive ε-isofrom of PKC. PKCa is selectively translocated by TUDCA to hepatocellular membranes (29, 41). TLCA impaired membrane binding of the Ca²⁺-sensitive PKCa (6), whereas TUDCA reversed TLCA-induced impairment of PKCa membrane binding (6). Thus, we speculate that TLCA may impair targeting of apical carrier proteins and, thereby, hepatobiliary secretion by a dual mechanism that includes activation of PI3K and, subsequently, PKCe at the apical pole of the hepatocyte on one hand and impairment of Ca²⁺ influx and PKCa membrane binding on the other hand. Further work is needed to corroborate this assumption.

In the present study wortmannin did not affect basal bile flow but stimulated biliary exocytosis in IPRL preloaded with HRP. These findings are in contrast to a study by Folli et al. (42) who show that wortmannin reduces bile flow and biliary HRP secretion in IPRL. However, the experimental protocols of

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2 U. Beuers, G. U. Denk, and R. Wimmer, unpublished observation.
the two studies differed. Folli et al. (42) investigated the effect of wortmannin on hepatic uptake (endocytosis), transcellular trafficking, and biliary excretion of HRP in IPRL (42), whereas the present study mainly focused on the role of PI3K in exocytosis. In the previous study, inhibition of PI3K impaired endoand transcytosis of fluid phase markers in IRHC and may, therefore, have impaired HRP uptake and transport across the hepatocyte in IPRL. In the present study, HRP was endocytosed before administration of wortmannin. Thus, in livers preloaded with HRP, stimulation of exocytosis by wortmannin may have antagonized a weak inhibiting effect of wortmannin on total bile flow, although the vesicular pathway may contribute less than 10% to total bile flow in IPRL (43). Altogether, the findings of these two studies suggest that basolateral endocytosis is stimulated, and apical exocytosis is suppressed by intrinsic PI3K activity in IPRL.

PI3K has also recently been demonstrated to be involved in regulation of canalicular bile acid secretion. Misra et al. (14, 15) observed that secretion of TCA by IPRL is mediated in part via PI3K-dependent mechanisms. Transport of TCA across the canalicular membrane was markedly reduced by wortmannin in IPRL and canalicular membrane vesicles. In contrast, the present study indicates that TLCA-induced impairment of bile acid secretion (Fig. 6) as well as bile flow, exocytosis, and organic anion secretion (Fig. 1, Table I) is reversed by wortmannin. Can these differences be explained? Different classes and subclasses of PI3K have been described that are all inhibited by the PI3K inhibitor, wortmannin (44). Class I PI3K are heterodimers made up of a 110-kDa catalytic subunit (p110) and an adaptor/regulatory subunit. Three p110 isoforms (α, β, δ) and at least seven adaptor proteins (p85, p55) may form class I PI3K family members. In contrast, class Iγ PI3K (p110/γ p101) are only abundant in mammalian white blood cells. PtdIns 4,5-bisphosphate appears to be the preferred substrate of class I PI3K in vivo, although these PI3K can also utilize PtdIns and PtdIns 4-phosphate as substrates in vitro (44). Three class II isoforms (PI3K-C2α, -β, -γ) have been detected in mammalian tissue. Their molecular mass is above 170 kDa, and their preferred substrate is PtdIns 4-phosphate. The γ-isoform is mainly detected in liver (44). Class III PI3K are homologues of yeast vesicular-sorting protein Vsp34p and use only PtdIns as substrate (44). As cellular levels of PtdIns 3-phosphate are quite constant under physiological conditions, their role in short-term regulation of cellular metabolism is regarded as limited. Thus, it appears possible that different bile acids such as TCA or TLCA affect different subclasses of PI3K that are involved in regulation of biliary secretion. Future development of specific inhibitors may permit differentiation of the actions of these different PI3K isoforms.

TUDCA has been shown to stimulate TCA secretion in normal IPRL in part by a PI3K-dependent mechanism and to stimulate PI3K activity at least transiently in isolated hepatocytes when administered at 500 μmol/liter (23). The present study confirmed transient stimulation of PI3K by TUDCA at 10 μmol/liter in isolated hepatocytes as determined by phosphorylation of PI3K-dependent PKB (Fig. 7). However, the present study did not reveal a role of PI3K in mediating choleretic and anticholestatic effects of TUDCA in vivo; bile flow, exocytosis, and organic anion secretion in IPRLs treated with TUDCA were not affected by wortmannin (Fig. 2, Table I). In addition, the anticholestatic effects of TUDCA in TLCA-treated livers were even enhanced when wortmannin was co-administered (Fig. 2). Thus, a mediator function of PI3K in TUDCA-induced bile secretion may be restricted to secretion of bile acids in normal liver.

In the present study, co-administration of a PI3K inhibitor not only reversed TLCA-induced impairment of bile secretion but also cellular damage as determined by lactate dehydrogenase release (Table I). The improvement in bile flow alone could not account for this effect since TUDCA also improved secretion in TLCA-treated livers but failed to abolish the cell damage induced by TLCA in IPRL. Future studies will be necessary to elucidate the role of PI3K in TUDCA-induced acute liver cell damage.

The present data suggest that PI3K represents a potential target of future anticholestatic treatment strategies. It should be mentioned, however, that PI3K may activate a survival pathway in rat hepatocytes treated with the hydrophobic bile acid, taurochenodeoxycholic acid (TCDCA) which protects liver cells from TCDCA-induced damage in vitro (45) as well as in vivo (Rust C, unpublished observation). Interestingly, the taurochenodeoxycholic acid-induced survival pathway did not involve PKB activation in vitro (45). Thus, different bile acids may exert differential effects on PI3K- and PKB-mediated processes in liver cells. It remains to be clarified whether involvement of different PI3K isoforms or action in different subcellular compartments may contribute to these diverse effects of bile acids on PI3K and PKB.

In summary, the present study demonstrates that TLCA-induced impairment of bile flow, hepatobiliary exocytosis, secretion of bile acids, and other organic anions as well as liver cell damage is mediated by PI3K- and putatively PKC-dependent mechanisms. TUDCA reversed the inhibitory effects of TLCA on bile secretion by a PI3K-independent mechanism.

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