# Tauroursodeoxycholate inhibits human cholangiocarcinoma growth via $Ca^{2+}$-, PKC-, and MAPK-dependent pathways

**Gianfranco Alpini, Noriatsu Kanno, Jo Lynne Phinizy, Shannon Glaser, Heather Francis, Silvia Taffetani and Gene LeSage**

*Am J Physiol Gastrointest Liver Physiol* 286:G973-G982, 2004. First published 30 December 2003; doi:10.1152/ajpgi.00270.2003



***AJP - Gastrointestinal and Liver Physiology*** publishes original articles pertaining to all aspects of research involving normal or abnormal function of the gastrointestinal tract, hepatobiliary system, and pancreas. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991.  ISSN: 0193-1857, ESSN: 1522-1547. Visit our website at http://www.the-aps.org/.

*Am J Physiol Gastrointest Liver Physiol* 286: G973–G982, 2004.
First published December 30, 2003; 10.1152/ajpgi.00270.2003.

# Tauroursodeoxycholate inhibits human cholangiocarcinoma growth via $Ca^{2+}$-, PKC-, and MAPK-dependent pathways

**Gianfranco Alpini,[1,2,4] Noriatsu Kanno,[1,2] Jo Lynne Phinizy,[3] Shannon Glaser,[3] Heather Francis,[3] Silvia Taffetani,[3] and Gene LeSage[1]**

[1]*Departments of Internal Medicine and* [2]*Medical Physiology and* [3]*Division of Research and Education, Scott & White Hospital and The Texas A&M University System Health Science Center, College of Medicine, and* [4]*Central Texas Veterans Health Care System, Temple, Texas 76504*

Submitted 24 June 2003; accepted in final form 26 December 2003

**Alpini, Gianfranco, Noriatsu Kanno, Jo Lynne Phinizy, Shannon Glaser, Heather Francis, Silvia Taffetani, and Gene LeSage.** Tauroursodeoxycholate inhibits human cholangiocarcinoma growth via $Ca^{2+}$-, PKC-, and MAPK-dependent pathways. *Am J Physiol Gastrointest Liver Physiol* 286: G973–G982, 2004. First published December 30, 2003; 10.1152/ajpgi.00270.2003.—Tauroursodeoxychate (TUDCA) is used for the treatment of cholangiopathies including primary sclerosing cholangitis, which is considered the primary risk factor for cholangiocarcinoma. The effect of TUDCA on cholangiocarcinoma growth is unknown. We evaluated the role of TUDCA in the regulation of growth of the cholangiocarcinoma cell line Mz-ChA-1. TUDCA inhibited the growth of Mz-ChA-1 cells in concentration- and time-dependent manners. TUDCA inhibition of cholangiocarcinoma growth was blocked by BAPTA-AM, an intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) chelator, and H7, a PKC-α inhibitor. TUDCA increased $[Ca^{2+}]_i$ and membrane translocation of the $Ca^{2+}$-dependent PKC-α in Mz-ChA-1 cells. TUDCA inhibited the activity of MAPK, and this inhibitory effect of TUDCA was abrogated by BAPTA-AM and H7. TUDCA did not alter the activity of Raf-1 and B-Raf and the phosphorylation of MAPK p38 and JNK/stress-activated protein kinase. TUDCA inhibits Mz-ChA-1 growth through a signal-transduction pathway involving MAPK p42/44 and PKC-α but independent from Raf proteins and MAPK p38 and JNK/stress-activated protein kinases. TUDCA may be important for the treatment of cholangiocarcinoma.

bile acids; bile ducts; cyclic adenosine monophosphate; cancer; mitosis

INTRAHEPATIC BILE DUCT epithelial cells (i.e., cholangiocytes) are the target cells in a number of chronic liver diseases including primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), graft-vs.-host disease, and cholangiocarcinoma (2). The growth of cholangiocarcinoma is modulated by a number of factors, including somatostatin (61), estrogens (57), adrenergic innervation (34), and gastrin (33). Due to our lack of understanding of the mechanisms of cholangiocarcinoma growth, there are no established measures to treat this neoplasm (2).

Bile acids regulate the growth of different epithelial cells (16, 18), including cholangiocytes (3). Bile acids have been implicated as tumor promoters (e.g., enhancing colonic epithelial cell proliferation) and are also associated with the development of tumors (16, 18, 42). On the other hand, other studies have shown that unconjugated hydrophobic bile acids do not enhance cell growth but rather have cytotoxic effects against various cell types (58), including cholangiocytes (10). The therapeutic bile acids ursodeoxycholate (UDCA) and its taurine conjugate tauroursodeoxycholate (TUDCA) have been effectively used for the treatment of cholestatic liver diseases including PBC (28, 52) and PSC (17, 55). The efficacy of UDCA or TUDCA is attributed to its cytoprotective effects (20, 63), preventing apoptosis (11) and choleretic effects on hepatocytes (48) by increasing bile flow and biliary acid secretion (9) and hepatocellular vesicular exocytosis (12). TUDCA has been shown to be more effective than UDCA in the enrichment of biliary UDCA and more effectively absorbed by the intestine (31).

PKC modulates the effects of bile acids on a number of epithelia, including cholangiocytes (1, 3, 13, 32, 44). For example, both primary and secondary bile acids have been shown to activate the expression of PKC isoenzymes, MAPK, and phosphointositol 3-kinase in normal colonial epithelial cells and colorectal cancer tissue (50). Furthermore, sodium taurolithocholate reduces hepatocyte canalicular secretion through activation of PKC-ϵ (13). In hepatocytes, glycochenodeoxycholate-induced apoptosis is associated with activation and membrane translocation of PKC-α, PKC-δ, and PKC-ϵ (32). In situ histological studies (51) have shown that feeding of UDCA to bile duct-ligated rats decreases the number of intrahepatic bile ducts. Both UDCA and TUDCA inhibit cholangiocyte proliferation of bile duct-ligated rats by activation of the $Ca^{2+}$-dependent PKC-α (1). Moreover, feeding of taurocholate and taurolithocholate to normal rats increases cholangiocyte proliferation and the number of ducts by activation and membrane translocation of PKC-α (3). However, no information exists regarding the role and mechanism of action of TUDCA in the regulation of cholangiocarcinoma growth.

Three main distinct signaling cascades exist in the MAPK family of serine/threonine kinases: p38 MAPK, JNK, and p44 and p42 (encoded by ERK1 and ERK2, respectively) (14). The MAPK ERK1 and ERK2 are proline-directed kinases that are activated through concomitant phosphorylation of tyrosine and threonine residues (4). p38 is a member of the MAPK family with features most closely resembling those of the *Saccharomyces cerevisiae* protein HOG1 (15). The JNK family, which includes JNK1, JNK2, and JNK3, is distantly related to the MAPK family, members of which are activated by dual phosphorylation at a Thr-Pro-Tyr motif, specifically at $Thr^{183}$ and $Tyr^{185}$ residues, in response to ultraviolet (UV) light (60). JNK

Address for reprint requests and other correspondence: G. LeSage, The Univ. of Texas Houston Medical School, 6431 Fannin St., MSB 4.234, Houston TX 77030 (E-mail: gene.lesage@uth.tmc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

is phosphorylated by JNK-activating kinases (JNKK1 and JNKK2), which are members of the MEK family (68).

The cAMP-dependent PKA-MEK-MAPK pathway is modulated via cross-talk with other intracellular signaling pathways (64), including PKC, which studies have shown to be associated with activation of MAPK (50). The activation of the PKC pathway is not always associated with activation of MAPK (43). For example, PKC-η inhibits UV-induced activation of caspase-3 in normal human keratinocytes by inhibition of p38 MAPK pathway (43). cAMP-dependent PKA is the major substrate of cAMP, and cAMP-dependent signaling is associated with a wide range of biological responses, including differentiation, survival, inhibition of growth, and apoptosis (19, 37, 45, 64). The activation of signal-transduction pathways by growth factors, hormones, and neurotransmitters is mediated through two closely related MAPK, p44 and p42, that are encoded by ERK1 and ERK2, respectively (14).

In this study, we evaluated the role and mechanisms of action of TUDCA in the regulation of growth of the cholangiocarcinoma cell line Mz-ChA-1. We posed the following questions: *1*) Does TUDCA inhibit the growth of the cholangiocarcinoma cell line, Mz-ChA-1? *2*) Are TUDCA-inhibitory effects on Mz-ChA-1 growth associated with increases in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and activation of the $Ca^{2+}$-dependent PKC-α, which plays an important role in the regulation of cholangiocyte functions (1, 3, 26, 27, 33, 41)? *3*) Does TUDCA stimulation of $Ca^{2+}$-dependent PKC lead to inhibition of MAPK activity? *4*) Is TUDCA inhibition of MAPK activity associated with changes in Raf-1 and B-Raf activities, upstream regulators of MAPK? *5*) Is TUDCA-inhibition of MAPK associated with changes in the phosphorylation of MAPK p42/44, p38, and JNK/stress-activated protein kinases (SAPKs)?

## MATERIALS AND METHODS

### *Materials*

Reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA; clone PC10); rabbit polyclonal antibody against Raf-1 (clone C-12); mouse monoclonal antibody against B-Raf (clone F-7); horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG; HRP-conjugated goat anti-mouse IgG; mouse monoclonal (IgG1) antibody p-JNK (clone G-7), which detects JNK1, JNK2, and JNK3 phosphorylated at $Thr^{183}$ and $Tyr^{185}$ of human origin; rabbit polyclonal antibody (clone FL), which was produced by immunization with full-length (amino acids 1–384) human JNK1 produced in *Escherichia coli* and reacts with JNK1, JNK2 p54, and JNK3; mouse monoclonal (IgG) antibody pp38 (clone D-8), which detects $Tyr^{182}$-phosphorylated MAPK p38, Mxi2, and p38β; mouse monoclonal (IgG1) antibody p38 (clone A-12), which detects total MAPK p38 and p38β; rabbit monoclonal (IgG) antibody ERK1 (clone C-16), which detects the MAPK p44 and p42; rabbit monoclonal (IgG) antibody ERK2 (clone C-14), which detects the MAPK p44 and p42; and mouse monoclonal (IgG) antibody pERK (clone N-18), which detects phosphorylated MAPK p44 and p42; and rat $Ca^{2+}$-dependent PKC-α antibody (rabbit IgG). [Methyl-$^3$H]thymidine and [γ-$^{32}$P]ATP were purchased from New England Nuclear Life Science Products, (Boston, MA). Raf-1 immunoprecipitation-kinase cascade assay kit, MAPK immunoprecipitation cascade assay kit (which detects MAPK p44/p42), Ras activation assay kit, protein A agarose beads, and protein G agarose beads were purchased from Upstate Biotechnology (Lake Placid, NY). Nitrocellulose membrane (0.2 μm) and Bio-Rad protein assay were purchased from Bio-Rad Laboratories (Hercules, CA).

### *Cell Line Culture*

Mz-ChA-1 cells (human gallbladder in origin) (36) were a gift from Dr. Fitz (University of Colorado, Denver, CO). We have previously used this cell line to evaluate the effect of gastrin and the $\alpha_2$-adrenergic receptor agonist UK-14304 on cholangiocarcinoma growth (33). Cells were maintained at 37°C in a 5% $CO_2$ incubator with the conditioned culture medium (CCM) composed of CMRL Medium-1066 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin and streptomycin, and 2 mM l-glutamine.

### *Effect of TUDCA on the Growth of Mz-ChA-1 Cells*

*Measurement of [$^3$H]thymidine incorporation.* After trypsinization, Mz-ChA-1 cells were suspended in CCM at $5 \times 10^4$ cells/ml, and 200 μl of the cell suspension were seeded into flat-bottomed 96-well plates. After an initial incubation step of 4 h at 37°C in a 5% $CO_2$ incubator, the cells were incubated at 37°C with TUDCA (0.2–200 μM). After incubation for 24–96 h, [$^3$H]thymidine was put into each well at 5.0 μCi/ml and incubated for additional 4 h. [$^3$H]thymidine incorporation was measured by a scintillation counter. At the end of each incubation period, we evaluated cell viability by trypan blue exclusion.

*Measurement of PCNA protein expression.* Mz-ChA-1 cells ($1.5 \times 10^6$) were seeded into flat-bottomed six-well plates and incubated in CCM until 70% confluence. Subsequently, cells were incubated with *1*) 0.2% BSA (basal value) or *2*) TUDCA (200 μM) in the absence or presence of BAPTA-AM (a chelator of $[Ca^{2+}]_i$; 5 μM) (33, 34) or H7 (a PKC-α inhibitor; 2 μM) (33) for 48 h. After the selected treatment, cells were washed twice with ice-cold PBS and then lysis buffer (10 mM Tris, pH 7.4, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM $Na_3VO_4$) was added into each well. Culture plates were kept on ice for 30 min with gentle rocking, then cells were scraped, collected in a microcentrifuge tube, and centrifuged at 300 *g* for 10 min at 4°C. Following electrophoresis, protein samples (10 μg) were transferred to a nitrocellulose membrane. The membrane was immersed into a blocking solution consisting of 5% dry milk and 1× TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween-20) and incubated with gentle rocking for 2 h. The membrane was incubated with anti-PCNA antibody diluted to 1:200 as the primary antibody overnight at 4°C. After being washed, the membrane was incubated with HRP-conjugated anti-mouse IgG diluted to 1:3,000 for 1 h at room temperature. After washes, proteins were visualized by using chemiluminescence (ECL Plus kit; Amersham Life Science). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low-light imaging system (Alpha Innotech, San Leandro, CA).

### *Effect of TUDCA on $[Ca^{2+}]_i$, and Protein Expression and Membrane Translocation of the $Ca^{2+}$-dependent PKC-α*

After trypsinization, Mz-ChA-1 cells were transferred to a clean tube and incubated for 1 h at 37°C (33, 34) to regenerate membrane proteins damaged by trypsin digestion (35). Subsequently, cells were stimulated for 15 min at 22°C with 0.2% BSA (basal value) or TUDCA (200 μM) in the presence of 0.2% BSA. Mz-ChA-1 $[Ca^{2+}]_i$ levels were determined by a microfluorescent technique (1, 30) in Mz-ChA-1 cells previously loaded with the fluorescent $Ca^{2+}$ indicator fluo-3 (1 μM for 10 min). The fluo-3 fluorescence was converted to $[Ca^{2+}]_i$ levels by employing a calibration kit from Molecular Probes (Eugene, OR) (30).

Cells ($1.5 \times 10^6$) were seeded into flat-bottomed six-well plates and incubated in CCM until they were grown to 70% confluence.

Subsequently, cells were incubated with 0.2% BSA (basal) or TUDCA (200 μM) for 90 min as described by us and others (7, 25, 27, 33, 41). After two washes with ice-cold PBS, lysis buffer was added into each well (see *Measurement of PCNA protein expression*). Total protein expression for PKC-α in Mz-ChA-1 cells (treated with BSA or TUDCA for 90 min) was evaluated in whole cell lysate by immunoblots (25, 27, 33, 41).

PKC-α membrane translocation was evaluated by immunoblots (25, 27, 33, 41) in a cytosol or membrane fraction (54) isolated from Mz-ChA-1 cells treated with BSA or TUDCA for 90 min. The cytosol and membrane fractions were obtained from Mz-ChA-1 as described previously (54). Briefly, Mz-ChA-1 cells in 100-mm dishes were washed with PBS, extracted in 1 ml of *buffer A* (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml each aprotinin and leupeptin), and homogenized with 30 strokes of a Dounce homogenizer. The homogenate was transferred to a microcentrifuge tube and centrifuged in a microcentrifuge at 4°C at 10,000 *g* for 2 min to clarify. The clarified homogenate was centrifuged at 4°C in an ultracentrifuge at 45,000 *g* for 30 min. The supernatant was collected as the cytosol fraction. The pellet was washed twice with *buffer A* and resuspended in 500 μl of *buffer A* with 0.5% Triton X-100. The pellet solution was vortexed, incubated on ice for 30 min, and centrifuged in a microcentrifuge at 4°C at 10,000 *g* for 2 min. The supernatant was collected as the membrane fraction. Immunoblots for PKC-α were performed as described above for Western immunoblotting for PCNA expression except for the use of mouse anti-PKC-α (diluted to 1:1,000) as the primary antibody and HRP-conjugated anti-mouse IgG diluted to 1:3,000 as a secondary antibody. The intensity of the bands was determined by scanning video densitometry using the Chemi-Imager 4000. The effects of TUDCA on $[Ca^{2+}]_i$ and PKC-α protein expression were performed at different incubation times with TUDCA (15 min for $Ca^{2+}$ and 90 min for PKC), because we anticipated that the $Ca^{2+}$-dependent activation of PKC-α protein expression would occur at a later time than the increase in $[Ca^{2+}]_i$. This is due both to the fact that that the increased calcium signal is upstream to the increase in protein expression as well as the time lag required for synthesis of new proteins (90 min for activation of PKC) (7, 27, 41). The same time period (15 min for $Ca^{2+}$ and 90 min for PKC) has been used by us in studies aimed to evaluate the effect of bile acids (e.g., UDCA and TUDCA) (1), gastrointestinal hormones (e.g., insulin) (41), or nerve receptor agonists (e.g., the $D_2$ dopaminergic receptor agonist quinelorane) (25) on intracellular $Ca^{2+}$ levels and the expression and membrane translocation of $Ca^{2+}$-dependent PKC isoforms. Furthermore, other studies have shown that prolonged incubation time (i.e., 90 min) is necessary for the activation of PKC-γ and PKC-δ, which are involved in insulin-like growth factor-I migration of colonic epithelial cells (7).

*Expression of Raf-1 and B-Raf in Mz-ChA-1 Cells*

The protein expression of Raf-1 and B-Raf in Mz-ChA-1 cells was evaluated by immunoblotting (25, 27, 34) using anti-Raf-1 and anti-B-Raf primary antibodies and the corresponding secondary antibod-

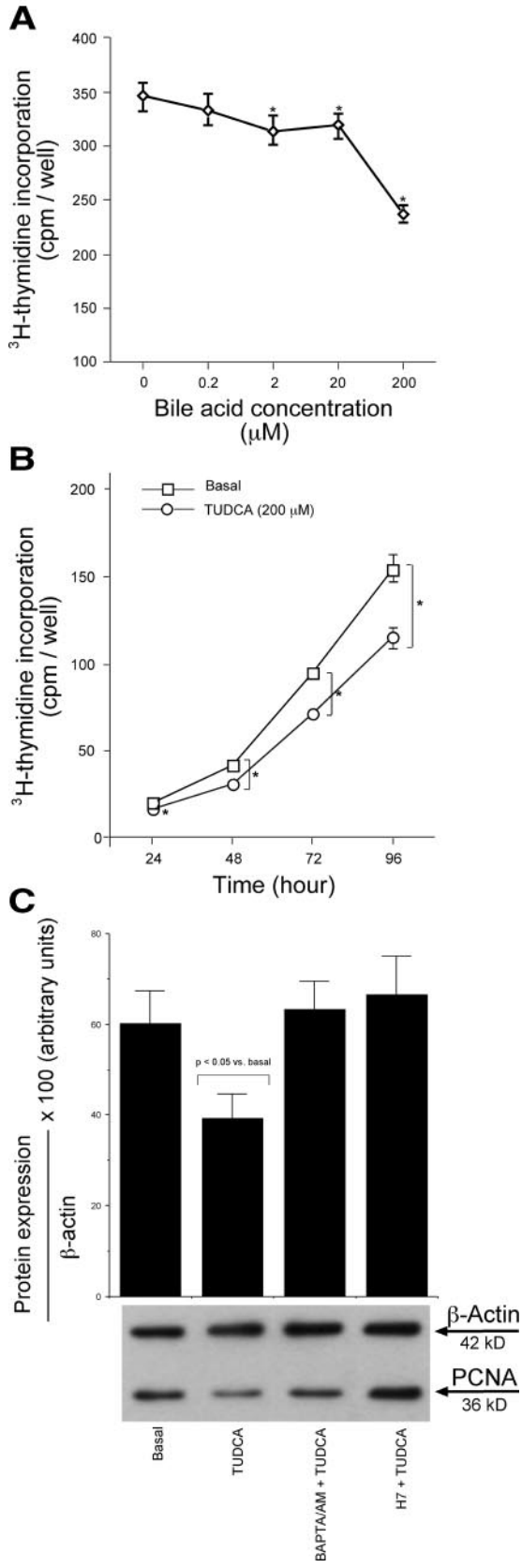
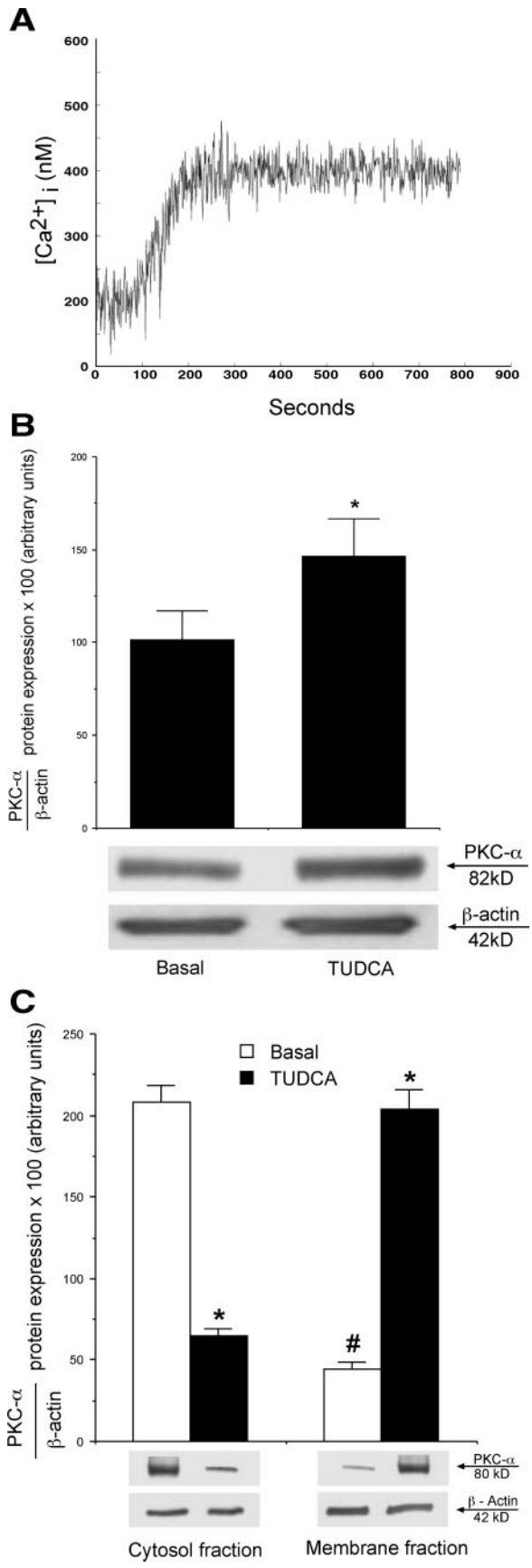



Fig. 1. *A*: Mz-ChA-1 cells were incubated with tauroursodeoxycholate (TUDCA; 0–200 μM) for 48 h. TUDCA inhibited [$^3$H]thymidine incorporation from 2 to 200 μM. Data are means ± SE of 6 experiments. *$P < 0.05$ vs. the corresponding basal values. *B*: at 200 μM, the effect of TUDCA on [$^3$H]thymidine incorporation of Mz-ChA-1 cells was determined from 24 to 96 h. TUDCA significantly inhibited [$^3$H]thymidine incorporation from 24 to 96 h of the incubation period. Data are means ± SE of 12 experiments. *$P < 0.05$ vs. the corresponding basal values. *C*: effects of TUDCA (200 μM for 48 h) on the proliferative capacity of Mz-ChA-1 cells were evaluated by immunoblots for proliferating cell nuclear antigen (PCNA) in the absence or presence of BAPTA-AM or H7. TUDCA inhibited PCNA protein expression in Mz-ChA-1. TUDCA inhibition of cholangiocarcinoma growth was blocked by BAPTA-AM and H7. Data are means ± SE of at least 3 experiments. *$P < 0.05$ vs. the corresponding basal values.




ies. After washes, bands were visualized by using chemiluminescence (ECL Plus). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000.

*Evaluation of the Transduction Pathways by Which TUDCA Regulates Cholangiocarcinoma Growth: Effect of TUDCA on Raf-1, B-Raf, and MAPK Activities and on Phosphorylation of MAPK p42/44, p38, and JNK/SAPKs*

The effect of TUDCA (200 μM) on the activity of Raf-1, B-Raf, and MAPK was performed as previously described by us (34). Cells in the culture medium were seeded into flat-bottomed six-well plates and incubated until they became 70% confluent. Subsequently, cells were placed in serum-free medium (CMRL Medium-1066 supplemented with 0.1% BSA, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) and cultured for an additional 24 h. After serum starvation, Mz-ChA-1 cells were incubated for 24 h with 100 nM EGF or TUDCA (200 μM containing 100 nM EGF) in the absence or presence of BAPTA-AM (5 μM) or H7 (2 μM). The effect of TUDCA on MAPK activity (using an antibody against MAPK1/2, ERK1/2) in Mz-ChA-1 cells was also evaluated in the presence of serum. Mz-ChA-1 cells ($1.5 \times 10^6$) were seeded into flat-bottomed six-well plates and incubated in CCM until 70% confluence. Subsequently, cells were incubated with 0.2% BSA (basal value) or TUDCA (200 μM) for 24 h. Subsequently, cells were washed twice with ice-cold PBS and incubated with lysis buffer (10 mM Tris, pH 7.4, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM $Na_3VO_4$) for 30 min on ice. Samples were collected, and total protein concentration was evaluated by using the Pierce protein assay system. The cell samples were treated according to the manufacturer's protocols (Upstate Biotechnology) of the Raf-1 immunoprecipitation kinase cascade assay kit and MAPK immunoprecipitation cascade assay kit, with the difference that in the B-raf assay goat anti-B-Raf antibodies (clone C-19) instead of anti-Raf-1 were used. Raf-1, B-Raf, and MAPK activities were evaluated with the phosphorylated MBP under the presence of [$\gamma$-$^{32}$P]ATP by using a scintillation counter. The kit for the detection of MAPK activity recognizes the MAPK p44/42 (i.e., ERK1/2).

Mz-ChA-1 cells ($1.5 \times 10^6$) were seeded into flat-bottomed six-well plates and incubated in CCM until 70% confluence. Subsequently, Mz-ChA-1 cells were incubated for 24 h with 0.2% BSA or TUDCA (200 μM) in the absence or presence of BAPTA-AM (5 μM) or H7 (2 μM). The protein expression of total and phosphorylated MAPK p42/44, p38, and JNK/SAPKs in Mz-ChA-1 cells was evaluated by immunoblotting (25, 27, 34) using the selected primary antibodies and the corresponding secondary antibodies. After washes,

Fig. 2. Effect of TUDCA on intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (*A*), total protein expression (*B*), and membrane translocation (*C*) for PKC-α in Mz-ChA-1 cells. *A*: $[Ca^{2+}]_i$ levels were determined by a microfluorescent technique in Mz-ChA-1 cells previously loaded with the fluorescent $Ca^{2+}$ indicator fluo-3 (1 μM for 10 min). The fluo-3 fluorescence was converted to $[Ca^{2+}]_i$ levels by employing a calibration kit from Molecular Probes. TUDCA (added at *time 0*) induced a 2-fold persistent increase in $[Ca^{2+}]_i$. Data are means ± SE of 3 experiments. *$P < 0.05$ vs. the corresponding basal values. *B*: TUDCA (200 μM for 90 min) increased total PKC-α protein expression in Mz-ChA-1 cells. Data are means ± SE of 23 experiments. *$P < 0.05$ vs. the corresponding basal values. *C*: immunoblots for PKC-α in a cytosol and membrane fraction isolated from Mz-ChA-1 cells treated with 0.2% BSA or TUDCA for 90 min. In Mz-ChA-1 cells treated with 0.2% BSA, the majority of PKC-α is found in the cytosol fraction. On the addition of TUDCA, PKC-α protein expression decreases significantly in the cytosol fraction. After TUDCA treatment, loss of PKC-α from the cytosol fraction was associated with an increase in PKC-α protein expression in the membrane fraction of Mz-ChA-1 cells. Data are means ± SE of 6 experiments. *$P < 0.05$ vs. the corresponding basal values. #$P < 0.05$ vs. the corresponding value of the cytosol fraction.

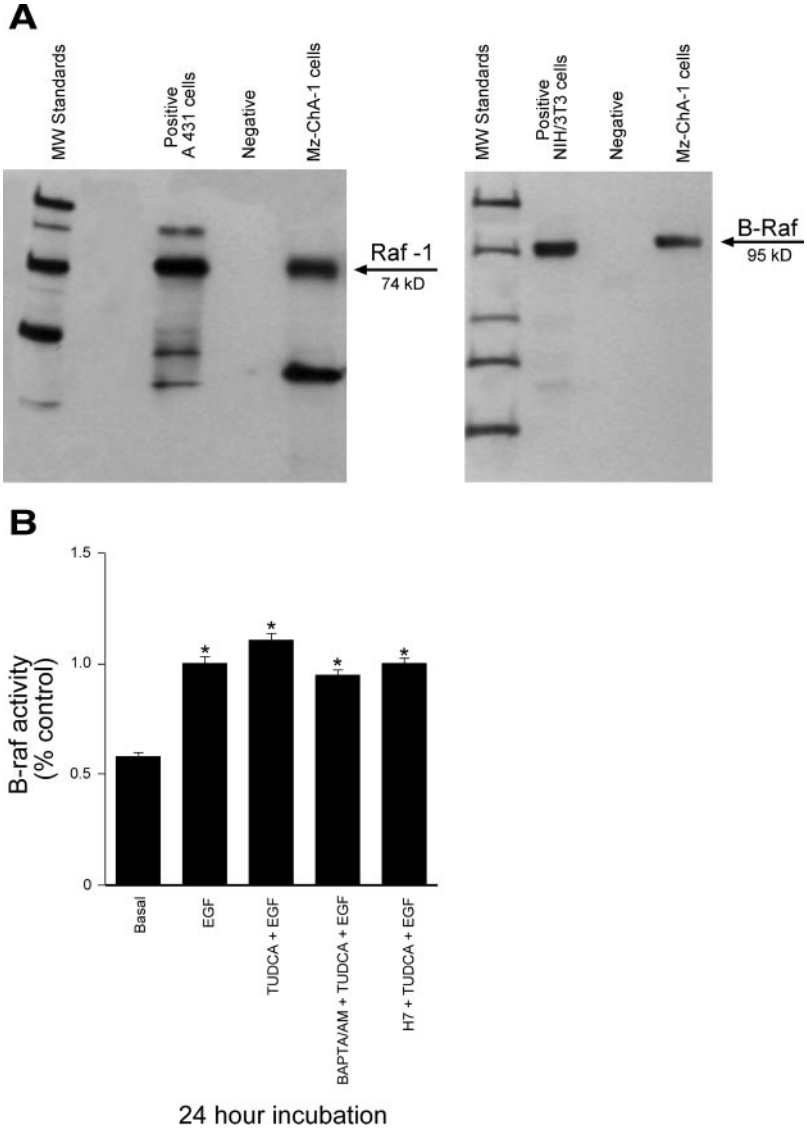



Fig. 3. *A*: Mz-ChA-1 cells express the protein for Raf-1 and B-Raf. MW, molecular weight. *B*: stimulation of Mz-ChA-1 cells with EGF (100 nM) for 24 h caused a significant increase in B-Raf activity. EGF stimulation of B-Raf activity was not prevented by TUDCA after 24 h of incubation. Data are means ± SE of 5 experiments. *$P < 0.05$ vs. the control value (stimulated with EGF).

bands were visualized by using chemiluminescence (ECL Plus). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000.

*Statistical Analysis*

All data are expressed as means ± SE. The differences between groups were analyzed by Student's *t*-test when two groups were analyzed or analysis of variance (ANOVA) if more than two groups were analyzed.

## RESULTS

*Time- and Concentration-Dependent Inhibition of the Growth of Mz-ChA-1 Cells by TUDCA*

After 48 h of incubation, TUDCA significantly inhibited [$^3$H]thymidine incorporation of Mz-ChA-1 cells at concentrations ranging from 2 to 200 μM (Fig. 1*A*). At the concentration of 200 μM, TUDCA significantly inhibited [$^3$H]thymidine incorporation in Mz-ChA-1 cells from 24 to 96 h of the incubation period (Fig. 1*B*). The data show that TUDCA inhibits the growth of Mz-ChA-1 cells in a time- and concentration-dependent fashion. TUDCA (48 h at 200 μM) inhibited PCNA protein expression (an index of cell replication) (40) of Mz-ChA-1 cells (Fig. 1*C*). Consistent with the concept that PKC-α regulates TUDCA modulation of cholangiocarcinoma growth, TUDCA inhibition of PCNA protein expression of Mz-ChA-1 was blocked by BAPTA-AM and H7 (Fig. 1*C*). Trypan blue exclusion analysis showed that TUDCA did not increase the percentage of dead cells compared with controls.

*TUDCA Increases $[Ca^{2+}]_i$ and PKC-α Protein Expression and Induces Membrane Translocation of PKC-α in Mz-ChA-1 cells*

TUDCA (200 μM) caused a marked and sustained increase in $[Ca^{2+}]_i$ levels in Mz-ChA-1 cells (Fig. 2*A*). Previous studies from our laboratory (3) in cholangiocytes isolated from bile duct-ligated rats demonstrated that TUDCA mobilizes $Ca^{2+}$ from intracellular stores rather than originating from extracellular stores. Further studies are needed to establish the source of mobilized calcium in cholangiocarcinoma cells.

Immunoblotting analysis shows that Mz-ChA-1 cells express the protein for the $Ca^{2+}$-dependent PKC-α and that TUDCA increased total PKC-α protein expression in Mz-ChA-1 cells (Fig. 2*B*). Figure 2*C* shows the subcellular distribution of PKC-α in Mz-ChA-1 cells treated with 0.2% BSA or TUDCA for 90 min. In Mz-ChA-1 cells treated with 0.2% BSA, the majority of PKC-α is found in the cytosol fraction (Fig. 2*C*); however, on addition of TUDCA, PKC-α protein expression significantly decreases in the cytosol fraction (Fig. 2*C*). After TUDCA treatment, loss of PKC-α from the cytosol fraction was associated with an increase in PKC-α protein expression in the membrane fraction of Mz-ChA-1 cells (Fig. 2*C*). The magnitude of increases of total PKC-α expression and membrane translocation of PKC-α by TUDCA were similar to that we previously described by gastrin (27). The activation of PKC is due to increases in membrane-bound PKC-α following TUDCA treatment. Figure 2*C* shows a significant increase in the membrane fraction for PKC-α following TUDCA treatment. The increase in total PKC-α is commonly seen for other activators of PKC (e.g., PMA) (24), which presumably provides more PKC-α available for translocation.

### *Mz-ChA-1 Cells Express Raf-1 and B-Raf: Effect of TUDCA on Raf-1, B-Raf, and MAPK Activities and on Phosphorylation of MAPK p42/44, p38, and JNK/SAPKs*

As shown in Fig. 3*A*, Mz-ChA-1 cells express the proteins for Raf-1 and B-Raf (74 and 95 kDa, respectively). Stimulation of Mz-ChA-1 cells with EGF (100 nM) for 24 h caused a significant ($P < 0.05$) increase in B-Raf (Fig. 3*B*) but not Raf-1 (results not shown) activity. EGF stimulation of B-Raf activity was not prevented by TUDCA after 24 h of incubation (Fig. 3*B*).

After serum starvation, stimulation of Mz-ChA-1 cells with EGF (100 nM) for 24 h significantly enhanced MAPK activity (i.e., ERK1/2) of these cells (Fig. 4*A*). EGF stimulation of MAPK activity of Mz-ChA-1 cells was inhibited by TUDCA (Fig. 4*A*). TUDCA inhibition of EGF-induced MAPK activity was blocked by pretreatment of Mz-ChA-1 cells with BAPTA-AM and H7 (Fig. 4*A*). Similar results (related to MAPK activity of ERK1/2) were obtained when Mz-ChA-1 cells were treated with TUDCA in the presence of serum (Fig. 4*B*). TUDCA inhibited basal MAPK activity of Mz-ChA-1 cells (Fig. 4*B*).

TUDCA (200 μM) inhibited the phosphorylation of MAPK p42/44 (expressed as ratio to total protein expression for p42/44), whose protein expression was similar to that of Mz-ChA-1 treated with BSA (Fig. 5*A*). Consistent with the concept that the $Ca^{2+}$-PKC pathway regulates cholangiocarcinoma growth by changes in MAPK activity, TUDCA inhibition of MAPK p42/44 phosphorylation was blocked by BAPTA-AM and H7 (Fig. 5*A*). TUDCA inhibition of cholan-

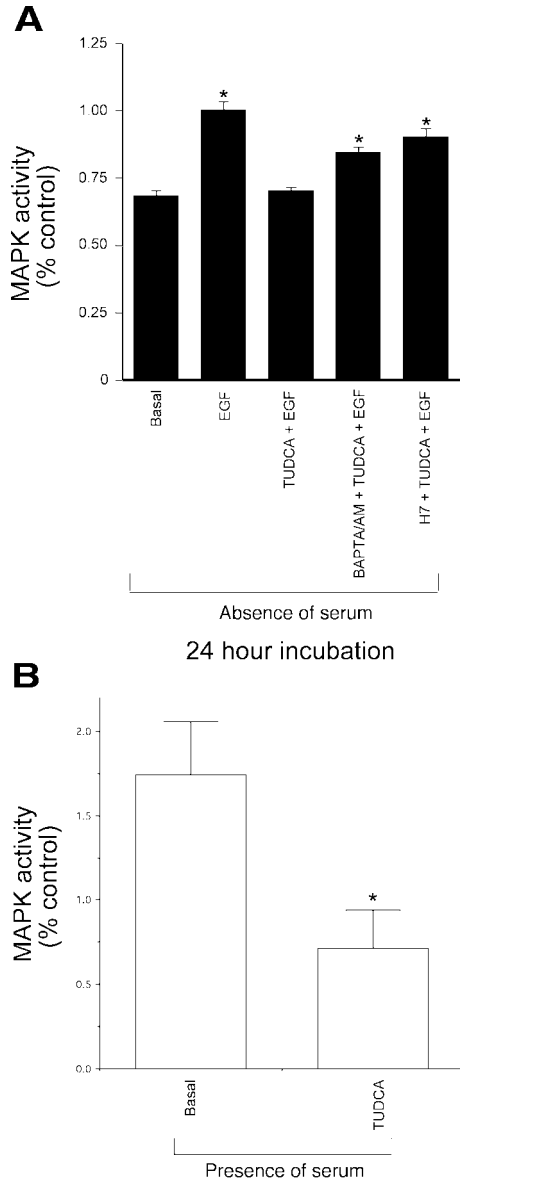
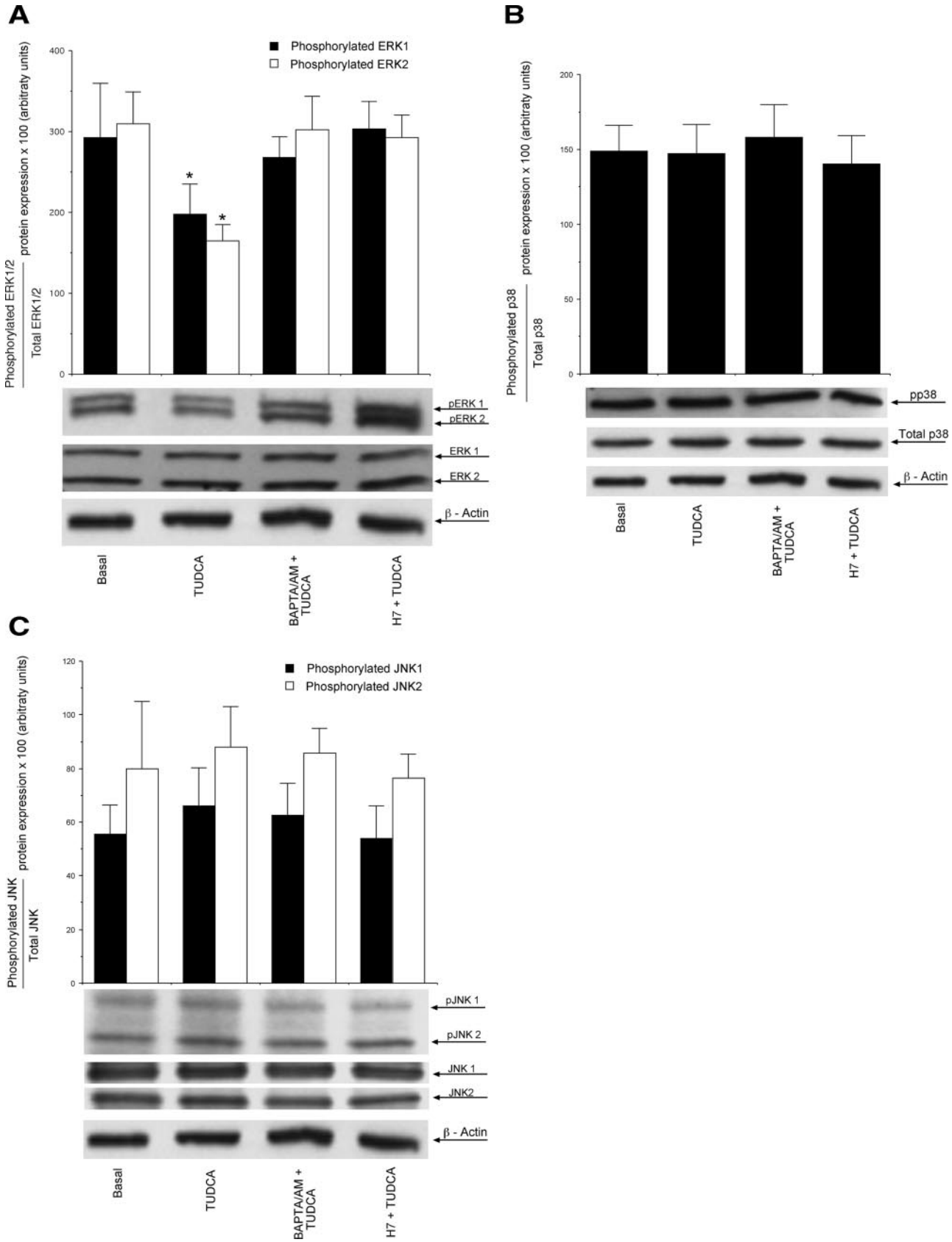



Fig. 4. *A*: effect of TUDCA (200 μM in the absence or presence of BAPTA-AM or H7) on EGF-induced MAPK activity in Mz-ChA-1 cells after 24 h of incubation time. Stimulation of Mz-ChA-1 cells with EGF for 24 h significantly enhanced MAPK activity of these cells. TUDCA inhibited EGF-induced increase in MAPK activity of Mz-ChA-1 cells. TUDCA inhibition of EGF-induced MAPK activity was blocked by BAPTA-AM and H7. Data are means ± SE of at least 3 experiments. *$P < 0.05$ vs. the control value (stimulated with EGF). *B*: effect of TUDCA (200 μM) on basal MAPK activity in Mz-ChA-1 cells after 24 h of incubation time. TUDCA inhibited basal MAPK activity (i.e., ERK1/2) of Mz-ChA-1 cells. Data are means ± SE of at least 3 experiments. *$P < 0.05$ vs. the corresponding basal value.

Fig. 5. Effect of TUDCA on the phosphorylation of MAPK p44/p42 (*A*), p38 (*B*), and JNK/stress-activated protein kinases (SAPKs) (*C*) in Mz-ChA-1 cells after 24 h of incubation time. *A*: TUDCA (200 μM) inhibited the phosphorylation of MAPK p42/44 (expressed as ratio to total protein expression for p42/44), whose protein expression was similar to that Mz-ChA-1 treated with BSA. Consistent with the concept that the $Ca^{2+}$-PKC pathway regulates cholangiocarcinoma growth by changes in MAPK activity, TUDCA inhibition of MAPK p42/44 phosphorylation was blocked by BAPTA-AM and H7. *B* and *C*: TUDCA inhibition of cholangiocarcinoma growth was not associated with changes in the phosphorylation of MAPK p38 and JNK/SAPKs (expressed as ratio to total protein expression for MAPK p38 and JNK/SAPKs, respectively). Data are means ± SE of 7 experiments. *$P < 0.05$ vs. the corresponding basal value.



giocarcinoma growth was not associated with changes in the phosphorylation of MAPK p38 and JNK/SAPKs (expressed as ratio to total protein expression for MAPK p38 and JNK/SAPKs, respectively) (Fig. 5, *B* and *C*). The data suggest that TUDCA inhibits Mz-ChA-1 growth through a signal transduction pathway involving MAPK p42/44 and PKC-α but independent from Raf proteins and MAPK p38 and JNK/SAPKs.

## DISCUSSION

The study shows that TUDCA inhibits the growth of the human cholangiocarcinoma cell line Mz-ChA-1. TUDCA inhibition of growth occurs in a dose- (2–200 μM) and time- (24–96 h) dependent fashion. We also show that TUDCA increased $[Ca^{2+}]_i$ and PKC-α protein expression and PKC-α membrane translocation and that TUDCA inhibition of cholangiocarcinoma growth was partially blocked by the $[Ca^{2+}]_i$ chelator BAPTA-AM and the PKC-α inhibitor H7. Furthermore, TUDCA inhibited MAPK activity, and the inhibitory effects of TUDCA on EGF-stimulated MAPK activity were partially blocked by BAPTA-AM and H7. TUDCA inhibited phosphorylation of MAPK p42/44 but not p38 and JNK/SAPKs. Consistent with the concept that the $Ca^{2+}$-PKC pathway regulates cholangiocarcinoma growth by changes in MAPK activity, TUDCA inhibition of phosphorylation of MAPK p42/44 was blocked by BAPTA-AM and H7. TUDCA did not inhibit Raf-1 or B-Raf activities. The data suggest that TUDCA inhibition of MAPK and Mz-ChA-1 growth occurs by activation of $Ca^{2+}$-dependent PKC-α upstream to MAPK but that the inhibition occurs independent of the Raf proteins.

PKC-α regulates a variety of cellular responses, including proliferation and apoptosis, through the activation (by phosphorylation) of Ras-Raf-MEK-MAPK (39, 66). Whereas in most cells PKC, activated by hormones and/or growth factors, mediates the stimulation of cell growth, in other cell types it exerts a negative control (5). The differential pattern of PKC in the modulation of cell growth may be due to the varying expressions of different isoforms of the PKC superfamily (47). For example, in the crypt-villus axis, PKC-α mediates inhibition of growth of intestinal epithelial cells (46). The activation of PKC-α decreases cell growth and tumorigenicity of intestinal cell lines (8). In the liver, PKC-α has been shown to play an important role in the regulation of cholangiocyte proliferation (1, 3, 26, 27, 33). For example, with activation of PKC-α (by gastrin) we found inhibition of cAMP levels and cell replication in cholangiocytes from bile duct-ligated rats (26, 27) and cholangiocarcinoma cell lines (33). Whereas activation of PKC-α (by taurocholate and taurolithocholate) leads to activation of cholangiocyte proliferation of normal cholangiocytes (3), PKC-α membrane translocation (by UDCA and TUDCA) induces inhibition of cholangiocyte proliferation from bile duct-ligated rats (1). Phorbol 12,13-dibutyrate (a PKC activator) (6) also inhibits the proliferation of Mz-ChA-1 cells (33). The different cross-talk between $Ca^{2+}$-dependent PKC and cAMP (which leads to stimulatory or inhibitory effects on MAPK and cell proliferation) (22, 65) is due to the type of receptor [gastrin (26, 27)] or transporter ($Na^+$-dependent bile acid transporter) up- or downregulated (1, 3), which differentially activates different PKC isoforms (1, 3, 26, 27), thus leading to activation or inhibition of cholangiocyte proliferation. These interactions may result in a different cross-talk between intracellular $Ca^{2+}$-PKC and specific adenylate cyclase isoforms, leading to inhibition or stimulation of adenylate cyclase and therefore of cAMP, MAPK, and cell proliferation.

In addition, PKC has been shown to mediate the effects of bile acids on a number of epithelia, including cholangiocytes (1, 3, 13, 32, 44). In our study, TUDCA inhibition of MAPK activities and cholangiocyte PCNA protein expression was abrogated by the presence of the $[Ca^{2+}]_i$ chelator (BAPTA-AM) (27) and a $Ca^{2+}$-dependent PKC-α inhibitor (H7) (27), compounds that were previously used to ascertain the role of the $Ca^{2+}$-dependent PKC-α in the regulation of cholangiocyte functions (27). These findings support the idea that TUDCA inhibition of MAPK and cholangiocarcinoma growth is PKC-α dependent. Although phosphorylation of MAPK by PKC-α may induce the activation of MAPK (66), in this study and our previous study involving gastrin inhibition of Mz-ChA-1 cholangiocarcinoma growth (33) PKC-α inhibits MAPK. In support of our findings, recent studies have shown that the activation of PKC pathway is not always associated with activation of MAPK (43). For example, PKC-η inhibits UV-induced activation of caspase-3 in normal human keratinocytes by inhibition of p38 MAPK pathway (43). Cross-talk between the PKC-α pathway and other not yet identified pathways may also be involved in TUDCA inhibition of cholangiocarcinoma growth. TUDCA may also inhibit cholangiocarcinoma growth by reducing cyclooxygenase-2 (COX-2) since COX-2 overexpression in cholangiocarcinoma (59) may promote growth, and endogenous bile acids have been shown to alter gene expression of COX-2 by a PKC-dependent mechanism (67).

Bile acid modulation of MAPK has been shown to alter bile secretion (38), cell proliferation (49), and apoptosis (29). Although bile acids have been shown to modify cell growth and MAPK through phosphorylation of the EGF receptor (53), the subsequent bile acid-induced changes in MAPK (in contrast to this study) were dependent on the activity of Ras and Raf. Thus it is unlikely that TUDCA inhibits growth by altering EGF receptor in Mz-ChA-1 cells. In other studies (38), TUDCA enhances MAPK-dependent bile secretion in hepatocytes by increasing phosphoinositol 3-kinase, which leads to Ras-dependent activation of ERK1/2. Because the TUDCA stimulation of MAPK in hepatocytes was independent of PKC (38), TUDCA inhibition of cholangiocarcinoma MAPK may be due to the ability of TUDCA to increase PKC-α in this cell line.

Cholangiocarcinoma is a liver neoplasm arising from intrahepatic bile duct and the extrahepatic bile ducts (62). Cholangiocarcinoma exhibits a poor prognosis, and surgical resection is virtually the only measure for the curative treatment, although other attempts, including radiotherapy (23) and photodynamic therapy (56), to relieve biliary obstruction due to unresectable tumors have been demonstrated successfully as an adjuvant therapy following surgery or as palliative therapy. However, to date, some gastrointestinal hormones and neuropeptides have been reported to be effective in the modulation of the growth of cholangiocarcinoma (21, 27, 61). Somatostatin, for example, prevents the growth of human cholangiocarcinoma cells implanted in athymic mice through somatostatin receptors (61). Also, as we have previously shown, gastrin inhibits the growth of human cholangiocarcinoma cell lines through inostiol 1,4,5-trisphosphate- and PKC-α-dependent

pathways (33). $\alpha_2$-Adrenergic receptor stimulation also inhibits the growth via cAMP-PKA-Raf-MAPK-dependent pathways (34). Because in humans, cholestatic liver diseases are important risk factors for cholangiocarcinoma, the finding that TUDCA is effective in improving clinical and histological features of cholestatic liver diseases, including PBC and PSC (2, 52), has important pathophysiological relevance. Our findings may suggest that the use of TUDCA in these clinical settings may not only improve clinical outcomes of cholestatic liver diseases by slowing the development of these diseases but has the potential to prevent the initiation or the development of cholangiocarcinoma.

## GRANTS

This work was supported by a grant award to G. LeSage and G. Alpini from Scott & White Hospital and The Texas A&M University System, by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant DK-54208 (to G. LeSage), and by a Veterans Affairs Merit Award and NIDDK Grant DK-58411 (to G. Alpini).

## REFERENCES

1. **Alpini G, Baiocchi L, Glaser S, Ueno Y, Marzioni M, Francis H, Phinizy JL, Angelico M, and LeSage G.** Ursodeoxycholate and tauroursodeoxycholate inhibit cholangiocyte growth and secretion of BDL rats through activation of PKC alpha. *Hepatology* 35: 1041–1052, 2002.
2. **Alpini G, Prall RT, and LaRusso NF.** The pathobiology of biliary epithelia. In: *The Liver: Biology and Pathobiology* (4th ed), edited by Arias IM, Boyer JL, Chisari FV, Fausto N, Jakoby W, Schachter D, and Shafritz DA. Philadelphia, PA: Lippincott Williams & Wilkins, 2001, p. 421–435.
3. **Alpini G, Ueno Y, Glaser SS, Marzioni M, Phinizy JL, Francis H, and LeSage G.** Bile acid feeding increased proliferative activity and apical bile acid transporter expression in both small and large rat cholangiocytes. *Hepatology* 34: 868–876, 2001.
4. **Alvarez E, Northwood IC, Gonzalez FA, Latour DA, Seth A, Abate C, Curran T, and Davis RJ.** Pro-Leu-Ser/Trh-Pro is a consensus primary sequence for substrate protein phosphorylation: characterization of the phosphorylation of c-Myc and c-Jun proteins by an epidermal growth factor receptor threonine 669 protein kinase. *J Biol Chem* 266: 15277–15825, 1991.
5. **Alvaro D, Della Guardia P, Bini A, Gigliozzi A, Furfaro S, La Rosa T, Piat C, and Capocaccia L.** Effect of glucagon on intracellular pH regulation in isolated rat hepatocyte couplets. *J Clin Invest* 96: 665–674, 1995.
6. **Alvaro D, Mennone A, and Boyer JL.** Role of kinases and phosphatases in the regulation of fluid secretion and $Cl^-/HCO_3^-$ exchange in cholangiocytes. *Am J Physiol Gastrointest Liver Physiol* 273: G303–G313, 1997.
7. **André F, Rigot V, Remacle-Bonnet M, Luis J, Pommier G, and Marvaldi J.** Protein kinase C-γ and -δ are involved in insulin-like growth factor-I migration of colonic epithelial cells. *Gastroenterology* 116: 64–77, 1999.
8. **Batlle E, Verdù J, Dominguez D, del Mont Liosas M, Diaz V, Loukili N, Paciucci R, Alameda F, and Garcia de Herreros A.** Protein kinase C-alpha activity inversely modulates invasion and growth of intestinal cells. *J Biol Chem* 273: 15091–15098, 1998.
9. **Baumgartner U, Scholmerich J, Sellinger M, Reinhardt M, Ruf G, and Farthmann EH.** Different protective effects of tauroursodeoxycholate, ursodeoxycholate, and 23-methyl-ursodeoxycholate against taurolithocholate-induced cholestasis. *Dig Dis Sci* 41: 250–255, 1996.
10. **Benedetti A, Alvaro D, Bassotti C, Gigliozzi A, Ferretti G, La Rosa T, Di Sario A, Baiocchi L, and Jezequel AM.** Cytotoxicity of bile salts against biliary epithelium: a study in isolated bile ductule fragments and isolated perfused rat liver. *Hepatology* 26: 9–21, 1997.
11. **Benz C, Angermuller S, Otto G, Sauer P, Stremmel W, and Stiehl A.** Effect of tauroursodeoxycholic acid on bile acid-induced apoptosis in primary human hepatocytes. *Eur J Clin Invest* 30: 203–209, 2000.
12. **Beuers U, Nathanson MH, Isales CM, and Boyer JL.** Tauroursodeoxycholic acid stimulates hepatocellular exocytosis and mobilizes extracellular $Ca^{++}$ mechanisms defective in cholestasis. *J Clin Invest* 92: 2984–2993, 1993.
13. **Beuers U, Probst I, Soroka C, Boyer JL, Kullak-Ublick GA, and Paumgartner G.** Modulation of protein kinase C by taurolithocholic acid in isolated rat hepatocytes. *Hepatology* 29: 477–482, 1999.
14. **Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, and Yancopoulos GD.** ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65: 663–675, 1991.
15. **Brewster JL, de Valoir T, Dwyer ND, Winter E, and Gustin MC.** An osmosensing signal transduction pathway in yeast. *Science* 259: 1760–1763, 1993.
16. **Craven PA, Pfanstiel J, Saito R, and DeRubertis FR.** Relationship between loss of rat colonic surface epithelium induced by deoxycholate and initiation of the subsequent proliferative response. *Cancer Res* 46: 5754–5759, 1986.
17. **Degott C, Zafrani ES, Callard P, Balkau B, Poupon RE, and Poupon R.** Histopathological study of primary biliary cirrhosis and the effect of ursodeoxycholic acid treatment on histology progression. *Hepatology* 29: 1007–1012, 1999.
18. **DeRubertis FR, Craven PA, and Saito R.** Bile salt stimulation of colonic epithelial proliferation. Evidence for involvement of lipoxygenase products. *J Clin Invest* 74: 1614–1624, 1984.
19. **Dugan LL, Kim JS, Zhang Y, Bart RD, Sun Y, Holtzman DM, and Gutmann DH.** Differential effects of cAMP in neurons and astrocytes. Role of B-raf. *J Biol Chem* 274: 25842–25848, 1999.
20. **Ejiri S, Eguchi Y, Kishida A, Ishigami F, Kurumi Y, Tani T, and Kodama M.** Cellular distribution of thrombomodulin as an early marker for warm ischemic liver injury in porcine liver transplantation: protective effect of prostaglandin I2 analogue and tauroursodeoxycholic acid. *Transplantation* 71: 721–726, 2001.
21. **Evers BM, Gomez G, Townsend CM Jr, Rajaraman S, and Thompson JC.** Endogenous cholecystokinin regulates growth of human cholangiocarcinoma. *Ann Surg* 210: 317–323, 1989.
22. **Fernandez M, Sanchez-Franco F, Palacios N, Sanchez I, Villuendas G, and Cacicedo L.** Involvement of vasoactive intestinal peptide on insulin-like growth factor I-induced proliferation of rat pituitary lactotropes in primary culture: evidence for an autocrine and/or paracrine regulatory system. *Neuroendocrinology* 77: 341–352, 2003.
23. **Fletcher MS, Brinkley D, Dawson JL, Nunnerley H, and Williams R.** Treatment of hilar carcinoma by bile drainage combined with internal radiotherapy using 192iridium wire. *Br J Surg* 70: 733–735, 1983.
24. **Gauthier ML, Torretto C, Ly J, Francescutti V, and O'Day DH.** Protein kinase Cα negatively regulates cell spreading and motility in MDA-MB-231 human breast cancer cells downstream of epidermal growth factor receptor. *Biochem Biophys Res Commun* 307: 839–846, 2003.
25. **Glaser S, Alvaro D, Roskams T, Phinizy JL, Stoica G, Francis H, Ueno Y, Barbaro B, Marzioni M, Mauldin J, Rashid S, Mancino MG, LeSage G, and Alpini G.** Dopaminergic inhibition of secretin-stimulated choleresis by increased PKC-γ expression and decrease of PKA activity. *Am J Physiol Gastrointest Liver Physiol* 284: G683–G694, 2003.
26. **Glaser S, Alvaro D, Ueno Y, Francis H, Marzioni M, Phinizy JL, Baumann B, Venter J, Marzioni M, LeSage G, and Alpini G.** Gastrin reverses established cholangiocyte proliferation and enhanced secretin-stimulated ductal secretion of BDL rats by activation of apoptosis through increased expression of $Ca^{2+}$-dependent PKC isoforms. *Liver Int* 23:78–88, 2003.
27. **Glaser S, Benedetti A, Marucci L, Alvaro D, Baiocchi L, Kanno N, Caligiuri A, Phinizy JL, Chowdury U, Papa E, LeSage G, and Alpini G.** Gastrin inhibits cholangiocyte growth in bile duct-ligated rats by interaction with cholecystokinin-B/gastrin receptors via d-myo-inositol 1,4,5-triphosphate-, Ca(2+)-, and protein kinase C alpha-dependent mechanisms. *Hepatology* 32: 17–25, 2000.
28. **Heathcote EJ.** Management of primary biliary cirrhosis. The American Association for the Study of Liver Diseases practice guidelines. *Hepatology* 31: 1005–1013, 2000.
29. **Higuchi H, Yoon JH, Grambihler A, Werneburg N, Bronk SF, and Gores GJ.** Bile acids stimulate cFLIP phosphorylation enhancing TRAIL-mediated apoptosis. *J Biol Chem* 278: 454–461, 2003.
30. **Hovis JG, Meyer T, Teasdale RM, Albrecht BN, Yorek MA, and Lowe WLJ.** Intracellular calcium regulates insulin-like growth factor-I messenger ribonucleic acid levels. *Endocrinology* 132: 1931–1938, 1993.
31. **Invernizzi P, Setchell KD, Crosignani A, Battezzati PM, Larghi A, O'Connell NC, and Podda M.** Differences in the metabolism and

disposition of ursodeoxycholic acid and of its taurine-conjugated species in patients with primary biliary cirrhosis. *Hepatology* 29: 320–327, 1999.
32. **Jones BA, Rao YP, Stravitz RT, and Gores GJ.** Bile salt-induced apoptosis of hepatocytes involves activation of protein kinase C. *Am J Physiol Gastrointest Liver Physiol* 272: G1109–G1115, 1997.
33. **Kanno N, Glaser S, Chowdhury U, Phinizy JL, Baiocchi L, Francis H, LeSage G, and Alpini G.** Gastrin inhibits cholangiocarcinoma growth through increased apoptosis by activation of $Ca^{2+}$-dependent protein kinase C-alpha. *J Hepatol* 34: 284–291, 2001.
34. **Kanno N, LeSage G, Phinizy JL, Glaser S, Francis H, and Alpini G.** Stimulation of alpha2-adrenergic receptor inhibits cholangiocarcinoma growth through modulation of Raf-1 and B-Raf activities. *Hepatology* 35: 1329–1340, 2002.
35. **Kato A, Gores GJ, and LaRusso NF.** Secretin stimulates exocytosis in isolated bile duct epithelial cells by a cyclic AMP-mediated mechanism. *J Biol Chem* 267: 15523–15529, 1992.
36. **Knuth A, Gabbert H, Dippold W, Klein O, Sachsse W, Bitter-Suermann D, Prellwitz W, and Meyer zum Buschenfelde KH.** Biliary adenocarcinoma. Characterisation of three new human tumor cell lines. *J Hepatol* 1: 579–596, 1985.
37. **Kurino M, Fukunaga K, Ushio Y, and Miyamoto E.** Cyclic AMP inhibits activation of mitogen-activated protein kinase and cell proliferation in response to growth factors in cultured rat cortical astrocytes. *J Neurochem* 67: 2246–2255, 1996.
38. **Kurz AK, Block C, Graf D, Dahl SV, Schliess F, and Haussinger D.** Phosphoinositide 3-kinase-dependent Ras activation by tauroursodeoxycholate in rat liver. *Biochem J* 350: 207–213, 2000.
39. **Leirdal M and Sioud M.** Protein kinase Cα isoform regulates the activation of the MAP kinase ERK1/2 in human glioma cells: involvement in cell survival and gene expression. *Mol Cell Biol Res Commun* 4: 106–110, 2000.
40. **LeSage G, Glaser S, Ueno Y, Alvaro D, Baiocchi L, Kanno N, Phinizy JL, Francis H, and Alpini G.** Regression of cholangiocyte proliferation after cessation of ANIT feeding is coupled with increased apoptosis. *Am J Physiol Gastrointest Liver Physiol* 281: G182–G190, 2001.
41. **LeSage GD, Marucci L, Alvaro D, Glaser SS, Benedetti A, Marzioni M, Patel T, Francis H, Phinizy JL, and Alpini G.** Insulin inhibits secretin-induced ductal secretion by activation of PKC alpha and inhibition of PKA activity. *Hepatology* 36: 641–651, 2002.
42. **Marchesa P, Lashner BA, Lavery IC, Milsom J, Hull TL, Strong SA, Church JM, Navarro G, and Fazio VW.** The risk of cancer and dysplasia among ulcerative colitis patients with primary sclerosing cholangitis. *Am J Gastroenterol* 92: 1285–1288, 1997.
43. **Matsumura M, Tanaka N, Kuroki T, Ichihashi M, and Ohba M.** The eta isoform of protein kinase C inhibits UV-induced activation of caspase-3 in normal human keratinocytes. *Biochem Biophys Res Commun* 303: 350–356, 2003.
44. **Matsuzaki Y, Bouscarel B, Le M, Ceryak S, Gettys TW, Shoda J, and Fromm H.** Effect of cholestasis on regulation of cAMP synthesis by glucagon and bile acids in isolated hepatocytes. *Am J Physiol Gastrointest Liver Physiol* 273: G164–G174, 1997.
45. **McConkey DJ, Orrenius S, and Jondal M.** Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. *J Immunol* 145: 1227–1230, 1990.
46. **McGarrity TJ, Pfeiffer LP, Neely EB, Palavarapu RG, Koltun WA, Parker P, and Howett MK.** Localization of protein kinase C alpha isoform expression in the human gastrointestinal tract. *Cell Growth Differ* 7: 953–959, 1996.
47. **Mellor H and Parker PJ.** The extended protein kinase C superfamily. *Biochem J* 332: 281–292, 1998.
48. **Milkiewicz P, Roma MG, Cardenas R, Mills CO, Elias E, and Coleman R.** Effect of tauroursodeoxycholate and *S*-adenosyl-l-methionine on 17β-estradiol glucuronide-induced cholestasis. *J Hepatol* 34: 184–191, 2001.
49. **Milovic V, Teller IC, Faust D, Caspary WF, and Stein J.** Effects of deoxycholate on human colon cancer cells: apoptosis or proliferation. *Eur J Clin Invest* 32: 29–34, 2002.
50. **Pongracz J, Clark P, Neoptolemos JP, and Lord JM.** Expression of protein kinase C isoenzymes in colorectal cancer tissue and their differential activation by different bile acids. *Int J Cancer* 61: 35–39, 1995.
51. **Poo JL, Feldmann G, Erlinger S, Braillon A, Gaudin C, Dumont M, and Lebrec D.** Ursodeoxycholic acid limits liver histologic alterations and portal hypertension induced by bile duct ligation in the rat. *Gastroenterology* 102: 1752–1759, 1992.
52. **Poupon RE, Poupon R, and Balkau B.** Ursodiol for the long-term treatment of primary biliary cirrhosis. *N Engl J Med* 330: 1342–1347, 1994.
53. **Rao YP, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P, and Hylemon PB.** Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. *Hepatology* 35: 307–314, 2002.
54. **Reyland ME, Barzen KA, Anderson SM, Quissell DO, and Matassa AA.** Activation of PKC is sufficient to induce an apoptotic program in salivary acinar cells. *Cell Death Differ* 7: 1200–1209, 2002.
55. **Rudolph G, Endele R, Senn M, and Stiehl A.** Effect of ursodeoxycholic acid on the kinetics of cholic acid and chenodeoxycholic acid in patients with primary sclerosing cholangitis. *Hepatology* 17: 1028–1032, 1993.
56. **Rumalla A, Baron TH, Wang KK, Gores GJ, Stadheim LM, and de Groen PC.** Endoscopic application of photodynamic therapy for cholangiocarcinoma. *Gastrointest Endosc* 53: 500–504, 2001.
57. **Sampson LK, Vickers SM, Ying W, and Phillips JO.** Tamoxifen-mediated growth inhibition of human cholangiocarcinoma. *Cancer Res* 57: 1743–1749, 1997.
58. **Shekels LL, Beste JE, and Ho SB.** Tauroursodeoxycholic acid protects in vitro models of human colonic cancer cells from cytotoxic effects of hydrophobic bile acids. *J Lab Clin Med* 127: 57–66, 1996.
59. **Sirica AE, Lai GH, Endo K, Zhang Z, and Yoon BI.** Cyclooxygenase-2 and ERBB-2 in cholangiocarcinoma: potential therapeutic targets. *Semin Liver Dis* 22: 303–313, 2002.
60. **Smeal T, Binetruy B, Mercola D, Grover BA, Heldecker G, Rapp UR, and Karin M.** Oncoprotein-mediated signaling cascade stimulates c-Jun activity by phosphorylation of serines 63 and 73. *Mol Cell Biol* 12: 3507–3513, 1992.
61. **Tan CK, Podila PV, Taylor JE, Nagorney DM, Wiseman GA, Gores GJ, and LaRusso NF.** Human cholangiocarcinomas express somatostatin receptors and respond to somatostatin with growth inhibition. *Gastroenterology* 108: 1908–1916, 1995.
62. **Thuluvath PJ, Rai R, Venbrux AC, and Yeo CJ.** Cholangiocarcinoma: a review. *Gastroenterologist* 5: 306–315, 1997.
63. **Tsukahara K, Kanai S, Ohta M, and Kitani K.** Taurine conjugate of ursodeoxycholate plays a major role in the hepatoprotective effect against cholestasis induced by taurochenodeoxycholate in rats. *Liver* 13: 262–269, 1993.
64. **Vossler MR, Yao H, York RD, Pan MG, Rim CS, and Stork PJ.** cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* 89: 73–82, 1997.
65. **Yamaguchi T, Nagao S, Wallace DP, Belibi FA, Cowley BD, Pelling JC, and Grantham JJ.** Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys. *Kidney Int* 63: 1983–1994, 2003.
66. **Yoon YM, Kim SJ, Oh CD, Ju JW, Song WK, Yoo YJ, Huh TL, and Chun JS.** Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J Biol Chem* 277: 8412–8420, 2002.
67. **Zhang F, Subbaramaiah K, Altorki N, and Dannenberg AJ.** Dihydroxy bile acids activate the transcription of cyclooxygenase-2. *J Biol Chem* 23: 2424–2428, 1998.
68. **Zheng C, Xiang J, Hunter T, and Lin A.** The JNKK2-JNK1 fusion protein acts as a constitutively active c-Jun kinase that stimulates c-Jun transcription activity. *J Biol Chem* 274: 28966–28971, 1990.