Tauroursodeoxycholic Acid Stimulates Hepatocellular Exocytosis and Mobilizes Extracellular Ca^{++} Mechanisms Defective in Cholestasis

Ulrich Beuers, Michael H. Nathanson, Carlos M. Isales, and James L. Boyer
Liver Center and Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract

To assess the effects of tauroursodeoxycholic acid (TUDCA) on bile excretory function, we examined whether TUDCA modulates vesicular exocytosis in the isolated perfused liver of normal rats in the presence of high (1.9 mM) or low (0.19 mM) extracellular Ca^{++} and in cholestatic rats 24 h after bile duct ligation. In addition, the effects of TUDCA on Ca^{++} homeostasis were compared in normal and in cholestatic hepatocytes. In the isolated perfused rat liver, TUDCA (25 μM) stimulated a sustained increase in the biliary excretion of horseradish peroxidase, a marker of the vesicular pathway, in the presence of high, but not low extracellular Ca^{++} or in the cholestatic liver. In contrast, TUDCA stimulated bile flow to the same extent regardless of the concentration of extracellular Ca^{++} or the presence of cholestasis. In indo-1-loaded hepatocytes, basal cytosolic free Ca^{++} ($[Ca^{++}]_i$) levels were not different between normal and cholestatic cells. However, in cholestatic cells $[Ca^{++}]_i$ increases induced by TUDCA (10 μM) and its 7α-OH epimer taurochenodeoxycholic acid (50 μM) were reduced to 22% and 26%, respectively, compared to normal cells. The impairment of TUDCA-induced $[Ca^{++}]_i$ increase in cholestatic cells could be mimicked by exposing normal cells to low extracellular Ca^{++} (21%) or to the Ca^{++} channel blocker $NiCl_2$ (23%). These data indicate that (*a*) dihydroxy bile acid–induced Ca^{++} entry may be of functional importance in the regulation of hepatocellular vesicular exocytosis, and (*b*) this Ca^{++} entry mechanism across the plasma membrane is impaired in cholestatic hepatocytes. We speculate that the beneficial effect of ursodeoxycholic acid in cholestatic liver diseases may be related to the Ca^{++}-dependent stimulation of vesicular exocytosis by its conjugate. (*J. Clin. Invest.* 1993. 92:2984–2993.) Key words: calcium • cholestasis • exocytosis • hepatocytes • tauroursodeoxycholic acid

Introduction

The dihydroxy bile acid ursodeoxycholic acid (UDCA)[1] improves liver structure and function in chronic cholestatic liver diseases by yet unknown mechanisms (1–3). Increasing evidence suggests that the site of beneficial action of the taurine and glycine conjugates of UDCA (TUDCA and GUDCA) is the hepatocyte (4–9). We reported recently that TUDCA at physiologic concentrations induces a marked and sustained increase in cytosolic free Ca^{++} ($[Ca^{++}]_i$) in rat hepatocytes by depleting intracellular Ca^{++} stores and mobilizing extracellular Ca^{++} (10). Ca^{++} entry across the plasma membrane is the key signal for vesicular exocytosis in a number of secretory cell types (11–13). In the hepatocyte, however, a role for Ca^{++} in vesicular exocytosis has not been clearly defined. Because bile acids may be transported by a microtubule-dependent vesicular transcytotic pathway when they are present in increased amounts (14, 15) as observed normally for other substances such as high molecular weight proteins, lipids, and more lipophilic organic anions (16), we investigated whether TUDCA affects vesicular exocytosis and, in particular, whether its ability to mobilize Ca^{++} might activate exocytosis, the final step in the vesicular transcytotic pathway.

Vesicular exocytosis of the liver cell is impaired in experimental cholestasis as demonstrated by accumulation of 100–200-nm vesicles in the pericanalicular zone (17, 18). Because Ca^{++} entry plays a key role in the regulation of exocytosis in different types of secretory cells, we also evaluated whether disturbances in Ca^{++} homeostasis were present in cholestatic hepatocytes and whether this abnormality if present could contribute to the impairment in exocytosis in cholestatic liver injury. To assess Ca^{++} homeostasis we compared basal and bile acid–modulated Ca^{++} signals in normal and cholestatic hepatocytes.

To study the effect of Ca^{++} influx on vesicular exocytosis, we utilized the isolated perfused rat liver preloaded with horseradish peroxidase (HRP). HRP is a biochemically determined marker for the vesicular pathway in the intact liver and the isolated perfused rat liver (19–24). In that bile duct ligation is also a well-characterized experimental model of cholestasis, isolated hepatocytes from bile duct–ligated rats were used to examine Ca^{++} signals in the cholestatic cell, and livers from bile duct–ligated rats were used to examine the acute effect of TUDCA on vesicular exocytosis under cholestatic conditions.

This article is dedicated to Prof. Dr. G. Paumgartner in honor of his 60th birthday.

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Address correspondence to Dr. Ulrich Beuers, Department of Medicine II, Klinikum Grosshadern, University of Munich, Marchioninistrasse 15, 81377 Munich, Germany.

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1. *Abbreviations used in this paper:* HRP, horseradish peroxidase; IP_3, inositol triphosphate; tBuBHQ, 2,5-di(tert-butyl)-1,4-benzohydroquinone; TCDCA, taurochenodeoxycholic acid; UDCA, TUDCA and GUDCA, ursodeoxycholic acid and taurine and glycine conjugates of UDCA, respectively.

Methods

Materials. Collagenase (type I) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Leibovitz L-15 medium was obtained from Gibco (Grand Island, NY). Indo-1/acetoxymethyl ester (AM) was from Molecular Probes Inc. (Pitchford, OR). Trypsin inhibitor, L-phenylephrine, HRP, 4-aminoantipyrine, 4-methylumbelliferone, and 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide were from Sigma Chemical Co. (St. Louis, MO). Taurine-conjugated bile acids and ionomycin were from Calbiochem Corp. (La Jolla, CA). 2,5-Di(tert-butyl)-1,4-benzohydroquinone (tBuBHQ) was from Aldrich Chemical Co. (Milwaukee, WI). $^{45}Ca^{++}$ was from Amersham Corp. (Arlington Heights, IL). All other chemicals were of the highest purity commercially available.

Isolated rat liver perfusion. Isolated rat liver perfusion studies were performed in the Perfusion Core Facility of the Yale Liver Center. The technical procedure used has been described in detail previously (25). In brief, rats were anesthetized with sodium pentobarbital (50 mg/kg body wt i.p.); after incision of the abdominal wall and ligation of the pancreatoduodenal branch of the portal vein, the bile duct was cannulated with a PE-10 tubing (35 cm; Clay Adams, Inc., Parsippany, NJ). The portal vein was cannulated with a 14-gauge Teflon intravenous catheter (Criticon, Tampa, FL) and the liver was perfused with a Krebs-Ringer bicarbonate solution (KRB: NaCl 118 mM, KCl 4.7 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.9 mM, D-glucose 5.5 mM, gassed with 95% O_2/5% CO_2) with 200 U heparin/100 ml, and the inferior vena cava was cannulated. The liver was then transferred into a chamber maintained at 37°C and continued to be perfused with KRB without heparin. The temperature was continuously monitored with a thermistor probe (Yellow Springs Instrument Co., Yellow Springs, OH) inserted between the lobes of the liver.

The perfusion protocol used for our study has been described previously (23, 24): livers were preloaded with HRP (0.05 mg/ml) for 25 min in a recirculating KRB perfusion (40 ml/min) containing 1 g/dl BSA; pH was continuously adjusted to 7.40 during this period. The perfusion was then switched to a nonrecirculating HRP- and BSA-free KRB perfusion (40 ml/min). For 5 min residual HRP in the vascular space was washed away. Then bile acids (or carrier) were infused for 50 min at a continuous rate (0.5 ml/min of a 2 mM stock solution) into the perfusion medium to reach a final physiologic concentration of 25 μM in the portal vein. For each series of experiments, control perfusions and TUDCA perfusions were randomly paired. Bile samples were collected in 2- and 5-min periods as were effluent samples. Bile volume was determined gravimetrically assuming the density of bile to be 1.0 g/ml. The viability of the perfused liver and adequacy of vascular perfusion were demonstrated in five ways: (*a*) Portal pressure was continuously monitored and was stable between 6 and 8 cm H_2O during perfusions of normal livers in the presence or absence of TUDCA. Portal pressure increased slightly from 7.4±0.5 cm H_2O to 8.9%±2.0 cm H_2O over an 80 min period during perfusions of cholestatic livers (n = 14). (*b*) The liver surface was examined to exclude mottling. (*c*) Release of lactate dehydrogenase (LDH) was measured at 40 and 80 min (26) in the perfusate and did not increase during the perfusions in the presence or absence of TUDCA. (*d*) O_2 consumption was measured at 10, 30, 50, and 70 min using a Lex-O_2-con instrument (Cavitron, Anaheim, CA) and was stable during the experiments. (*e*) Trypan blue was injected at the end of experiments to ascertain that the perfusion was homogenous.

Measurement of enzyme activities in bile. HRP activity was measured spectrophotometrically using 4-aminoantipyrine as substrate and recording the linear change in adsorption at 510 nm for 3 min (27). The quantity of HRP expressed as ng protein/min × g liver was calculated after establishing HRP standard curves. *N*-acetyl-β-D-glucosaminidase, a lysosomal marker enzyme, was determined in bile samples according to LaRusso and Fowler (28) with modifications. In brief, 10 μl of bile and 10 μl of assay buffer (100 mM Na citrate, 0.2% Triton X-100, 0.5 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide [10 mM stock in Me_2SO_4], pH 4.5) were incubated for 45 min at 37°C. Substrate and enzyme blanks were treated in the same way. 1.2 ml of NaOH-glycine buffer (50 mM NaOH, 50 ml of glycine, 5 mM EDTA, pH 10.4) was added to stop the reaction. Fluorescence of 4-methylumbelliferone was measured in a fluorescence spectrophotometer (Farrand Mark I, Farrand Optical Co., New York) with excitation at 365 nm and emission at 460 nm. Results were quantified by establishing standard curves for the same buffers containing known concentrations of 4-methylumbelliferone. TUDCA (3.3 mM) had no effect on the enzymatic reaction.

Calculation of biliary HRP and *N*-acetyl-β-D-glucosaminidase excretion was corrected for the volume of the biliary cannula (20 μl) and the "dead space" of the biliary tree taken as 2.3 μl/g liver according to Hacki and Paumgartner (29).

Hepatocyte preparation. Normal and cholestatic hepatocytes were isolated from livers of untreated male Sprague-Dawley rats or from rats 24 h after bile duct ligation (160–240 g; Camm Research Lab Animals, Wayne, NJ; maintained on Purina Rodent Chow under a constant 12-h light cycle) in the Hepatocyte Isolation Core of the Yale Liver Center as described previously (10, 30). In brief, rat livers were perfused with Hanks A and then Hanks B medium containing 0.05% collagenase and 0.8 U trypsin inhibitor/U tryptic activity and were then excised, minced, and passed through serial nylon mesh filters, and the resultant cells were washed. Mean viability by trypan blue exclusion was 84% for normal cells and 77% for cholestatic cells immediately after preparation. Cells were suspended at a concentration of 3.3×10^5 cells/ml in Leibovitz L-15 medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% FCS ("enriched L-15 medium") and plated onto 3 × 3-mm glass coverslip fragments for microspectrofluorometry. Cells were incubated at 37°C for 2 h before dye-loading and used from 2 to 6 h after plating. Cholestatic hepatocytes were isolated and studied 24 h after bile duct ligation since viability of cells isolated 2 or 7 d after bile duct ligation was considerably lower, and these cells were extensively blebbed 2 h after plating.

Bile duct ligation. Bile duct ligation was performed as described from this laboratory (31). In brief, rats were anesthetized with pentobarbital (30 mg/kg body wt). After a longitudinal midline incision of the abdominal wall, the common bile duct was dissected from the surrounding tissue, ligated with silk thread twice, and transsected between the ligatures. The incision of the abdominal wall was closed using separate sutures for the muscle layer and the skin. After 24 h, cholestasis was verified by examining the color of the urine, and by examining the ligated bile duct for proximal distention before cannulation. Intraabdominal bile leaks were not observed in any of the animals under study and ligatures were in place after 24 h in all animals.

Measurement of cytosolic free Ca^{++} by ratio microspectrofluorometry (10, 32). Cells attached to coverslip fragments were loaded with indo-1 by incubation for 45–60 min with 4 μM indo-1/AM at 37°C in enriched L-15 medium. Coverslip fragments were then transferred to the stage of an inverted Zeiss IM 35 microscope (Oberkochen, FRG) and were continuously superfused with a KRB solution (see above) at a flow rate of 3 ml/min and at 37°C. Fluorescence was measured from groups of cells (20±4 cells per group, n = 56) in 150-μm diam fields. Fields were selected to exclude damaged cells with blebs or cytosolic granulations. Cells were excited at 365 nm and emission signals were collected at 405 and 480 nm to allow ratio measurement of fluorescence (33).

Emission signals were calibrated at the end of each single experiment by superfusing cells with 10 μM ionomycin and then 5 mM EGTA. Calculation of $[Ca^{++}]_i$ from these measurements as well as validation of this approach have been described in detail recently (10).

$^{45}Ca^{++}$ influx in isolated hepatocytes. Calcium influx was measured using a modification of the method of Mauger et al. (34). In brief, after isolation hepatocytes were kept on ice for 60 min in enriched L-15 medium. Cells were then centrifuged at 50 *g*, the medium was replaced

by albumin-free L-15 medium ($CaCl_2$ 1.3 mM), and cells were suspended at a concentration of 2×10^6 cells/ml and kept on ice.

Experiments were performed 90–240 min after preparation of cells. 10 min before the start of the experiment, 1-ml aliquots of the cell suspension were transferred to a waterbath and incubated at 37°C. Aliquots of stock solutions of TUDCA, taurocholic acid, or vasopressin in albumin-free L-15 were added to 1 ml of suspended cells. After 60 s (vasopressin) or 180 s (TUDCA and taurocholic acid) 10 μCi of $^{45}Ca^{++}$ was added to the samples and cell aliquots of 100 μl were removed at 15, 45, 75, and 105 s, filtered (Whatman GF/C, Whatman Inc., Clifton, NJ) under constant vacuum, and washed three times with 3 ml of an ice-cold stop buffer (5 mM tris [hydroxymethyl] aminomethane, 5 mM $CaCl_2$, 144 mM NaCl; pH 7.40). The cell-associated radioactivity was counted in scintillation fluid using a liquid scintillation counter (35).

Statistical analysis of data. Data are expressed as mean±SD. Results were compared using the unpaired two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Effect of TUDCA on bile flow and biliary excretion of HRP in the isolated perfused rat liver. Livers were preloaded with HRP (0.05 mg/ml) for 25 min in a recirculating system and washed for 5 min by single pass perfusion with HRP-free KRB to remove extracellular HRP. Then the effect of TUDCA on bile flow and HRP excretion was studied over 50 min. TUDCA (25 μM) infusion resulted in a 68% increase in total bile flow ($P < 0.0001$) and a 68% increase in biliary HRP excretion ($P < 0.01$) relative to controls calculated as area under the curve of Fig. 1, *A* and *B* (see also Fig. 3). The excretion of HRP showed a biphasic pattern: TUDCA induced a net increase of HRP excretion during the first 15 min (Fig. 1 *A;* note also the decrease of HRP excretion in the controls). Excretion then slowly declined in TUDCA-treated livers parallel to, but higher than in control livers.

The effect of TUDCA on release into bile of *N*-acetyl-β-D-glucosaminidase, a specific marker enzyme of the lysosomal pathway (28) was studied, because HRP is processed in hepatocytes via both a direct vesicular pathway and a lysosomal pathway (19). TUDCA suppressed lysosomal enzyme release (Table I), indicating that stimulation of HRP excretion by TUDCA is not related to the lysosomal pathway of HRP.

Together, these data indicate that TUDCA, at a concentration that is equivalent to a low physiologic bile acid level in the portal vein of the rat, stimulates both bile flow and hepatocellular vesicular exocytosis.

Effect of low extracellular Ca^{++} on TUDCA-induced stimulation of bile flow and HRP excretion. To examine the importance of extracellular Ca^{++} in the TUDCA-induced stimulation of bile flow and vesicular exocytosis, livers were preloaded with HRP and then perfused with low Ca^{++} KRB (0.19 mM). This Ca^{++} concentration was chosen because it prevents the

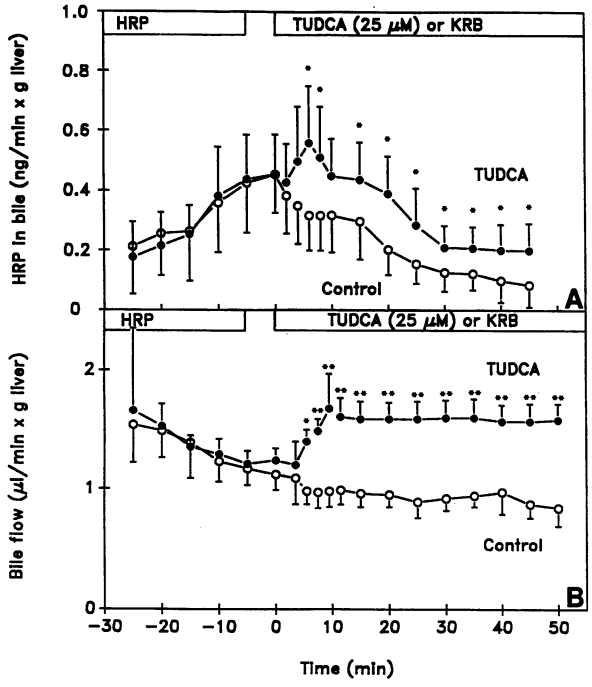

Figure 1. TUDCA (25 μM) stimulates (*A*) biliary excretion of HRP and (*B*) bile flow in the isolated perfused rat liver. Rat livers were perfused with KRB solution (gassed with 95% O_2/5% CO_2) containing 1.9 mM Ca^{++} at a constant flow (3.6–4.0 ml/min × g liver). In a first "loading period" livers were perfused in a recirculating way with KRB containing 0.05 mg/ml HRP. Perfusion was then switched to a nonrecirculating perfusion with HRP-free KRB. After a 5-min "wash period" (to wash away residual HRP from the vascular space) TUDCA stock (or the carrier KRB in controls) was infused into the perfusate to reach a final bile acid concentration of 25 μM in the portal vein. Results are given as mean±SD of eight experiments, each. $^*P < 0.05$, $^{**}P < 0.001$, unpaired *t* test.

Table I. Effect of TUDCA on Biliary Excretion of the Lysosomal Marker Enzyme N-acetyl-β-D-Glucosaminidase in the Perfused Rat Liver

Time	Enzyme activity Control (n = 8)	TUDCA (n = 7)	P
min	$\mu U \times min^{-1} \times g\ liver^{-1}$		
1.9 mM extracellular Ca^{++}			
−25	1.37±1.02	1.33±0.56	NS
−15	2.33±1.55	1.29±0.58	NS
−5	3.48±2.02	2.04±0.75	NS
5	2.78±1.51	0.71±0.65	0.0053
15	2.39±1.70	0.40±0.42	0.0100
25	1.63±0.73	0.28±0.21	0.0006
35	1.61±0.89	0.38±0.40	0.0050
45	1.23±0.32	0.33±0.38	0.0005
0.19 mM extracellular Ca^{++}			
−25	1.87±2.87	1.09±0.43	NS
−15	1.61±1.44	0.90±0.17	NS
−5	2.27±1.39	1.43±0.50	NS
5	1.20±0.59	0.47±0.25	0.0104
15	1.18±0.58	0.48±0.34	0.0176
25	0.92±0.43	0.36±0.16	0.0067
35	0.76±0.46	0.31±0.18	0.0290
45	0.83±0.55	0.28±0.13	0.0239

Bile samples of experiments described in Figs. 1 and 2 were analyzed for enzymatic activity of *N*-acetyl-β-D-glucosaminidase as described in Methods. TUDCA was added to the perfusate at min 0. Data are corrected for the volume of the bile cannula and the "dead space" of the biliary tree and are expressed as mean±SD. Enzyme activity is expressed as units (1 U = hydrolysis of 1 μmol 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide per min).

sustained increase in cytosolic Ca^{++} induced by TUDCA in single hepatocytes (see Fig. 5), yet is adequate to prevent (*a*) depletion of intracellular Ca^{++} in single cells as we have previously observed in Ca^{++}-free medium (see Fig. 5 and reference 10), (*b*) Ca^{++} deprivation-induced cholestasis in the perfused rat liver (36, 37), and (*c*) Ca^{++} deprivation-induced reduction of biliary HRP excretion in the perfused rat liver (38). Under these conditions, the mean rate of basal bile flow during a 50-min period was identical to that in the presence of 1.9 mM Ca^{++} (48.1±7.2 vs. 46.6±4.8 μl/g liver). Similarly, the TUDCA-induced stimulation of bile flow in the presence of 0.19 mM Ca^{++} was identical to that observed in the presence of 1.9 mM Ca^{++} (Fig. 2 *B*): total bile flow was enhanced by 68% ($P < 0.002$; Fig. 3). In contrast, in low Ca^{++} medium TUDCA (25 μM) infusion did not result in stimulation of biliary HRP excretion (Fig. 2 *A* and Fig. 3).

Release of the lysosomal marker enzyme *N*-acetyl-β-D-glucosaminidase, however, was suppressed by TUDCA also in low Ca^{++} medium (Table I). These data suggest that bile production and vesicular exocytosis are independently regulated in the rat liver and that the stimulation of vesicular exocytosis by TUDCA depends on the TUDCA-induced Ca^{++} entry into the hepatocyte. Furthermore, TUDCA-induced exocytosis is not associated with stimulation of lysosomal enzyme secretion.

Effect of taurochenodeoxycholic acid (TCDCA) on bile flow, portal pressure, and biliary excretion of HRP in the isolated perfused rat liver. The effect of TCDCA, the hydrophobic 7α-OH epimer of TUDCA, which also may cause sustained increases in hepatocellular $[Ca^{++}]_i$ (10), was studied at a 25 μM concentration. TCDCA caused cholestasis in the presence of both 1.9 and 0.19 mM Ca^{++}: bile flow decreased from 1.10±0.11 and 1.21±0.22 to 0.73±0.16 and 0.77 μl/min × g liver after 20 min and to 0.28±0.16 and 0.55±0.33 μl/min × g liver after 40 min, respectively ($n = 4$, each). The portal pressure, which was not affected by equimolar TUDCA in the same experimental setting (see Methods) rose within 20 min of TCDCA treatment both in the presence of high or low extracellular Ca^{++} from 7.1±0.9 to 9.7±3.6 cm H_2O and from 6.6±0.4 to 10.4±3.1 cm H_2O, respectively. During the first 15 min, biliary HRP excretion tended to increase in livers perfused with 1.9 mM Ca^{++} in comparison to those perfused with 0.19 mM Ca^{++} (7.8±2.0 vs. 5.2±1.9 ng/g liver, $P < 0.1$). Thereafter, biliary HRP excretion decreased markedly parallel to the observed increase in perfusion pressure and decrease in bile flow and was not different in the presence of high or low extracellular Ca^{++} during the following 20 min (2.9±0.5 vs. 3.8±0.9 ng/g liver). These data indicate that TCDCA-induced disturbance of liver function at physiologic concentrations is initiated independently of extracellular Ca^{++}.

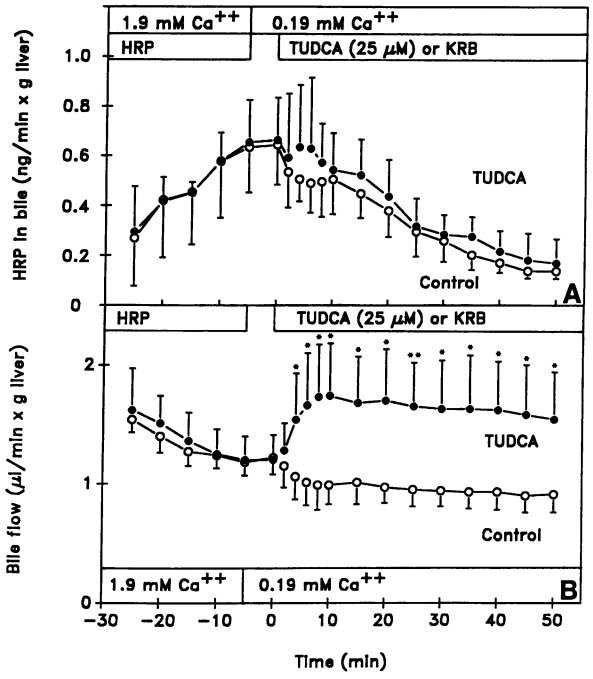

Figure 2. Effect of low (0.19 mM) extracellular Ca^{++} on TUDCA (25 μM)-induced stimulation of (*A*) biliary HRP excretion and (*B*) bile flow in the isolated perfused rat liver. Experimental conditions are as described in Fig. 1. Results are given as mean±SD of seven experiments, each. $*P < 0.05$, unpaired *t* test. Low extracellular Ca^{++} blocks TUDCA-induced stimulation of biliary HRP excretion but not bile flow.

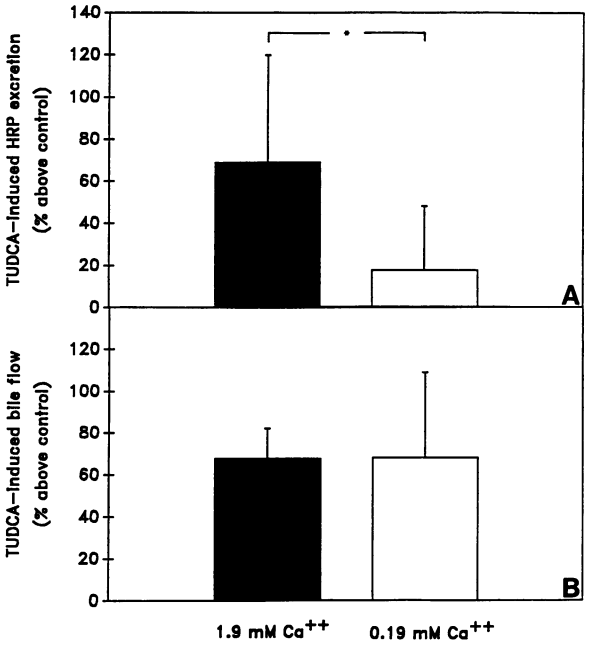

Figure 3. (*A*) Cumulative (total) HRP excretion and (*B*) total bile flow, during infusion of TUDCA (25 μM), expressed as percentage above control experiments in the isolated rat liver perfused with a standard KRB. (1.9 mM $CaCl_2$, *solid bars*) or a "low Ca^{++} KRB" (0.19 mM $CaCl_2$, *open bars*). Areas under the curve (AUC) of the experiments given in Figs. 1 and 2 were calculated in each single experiment. Each bar represents the mean percent deviation of eight or seven TUDCA experiments from the mean of eight or seven control experiments, respectively. Total bile flow was significantly stimulated by TUDCA in the presence of both standard 1.9 mM $CaCl_2$ KRB ($P < 0.001$) and "low Ca^{++} KRB" ($P < 0.002$), whereas cumulative (total) HRP excretion did only increase above control values in the presence of 1.9 mM extracellular Ca^{++} ($P < 0.01$), but not in the presence of low extracellular Ca^{++}. $*P < 0.05$; unpaired *t* test.

Effect of TUDCA on $^{45}Ca^{++}$ influx in isolated hepatocytes. The effect of TUDCA on $^{45}Ca^{++}$ influx in isolated hepatocytes was examined to assess more directly our previous observation using microspectrofluorometry (10) which suggested that TUDCA stimulates Ca^{++} entry across the plasma membrane.

As illustrated in Fig. 4, TUDCA (10 μM) increased Ca^{++} entry by 99% relative to unstimulated control cells ($P < 0.02$). Vasopressin (10 nM), which is known to stimulate Ca^{++} entry in hepatocytes (34), also increased Ca^{++} entry by 70% relative to unstimulated control cells ($P < 0.01$). In contrast, the trihydroxy bile acid taurocholic acid, which has minimal effects on hepatocellular Ca^{++} homeostasis at low physiologic concentrations (10 μM) (10; see also 39, 40) did not increase $^{45}Ca^{++}$ influx relative to control cells (Fig. 4). These data provide direct evidence that TUDCA, but not taurocholic acid at equimolar concentrations induces Ca^{++} entry into hepatocytes across the plasma membrane.

Basal $[Ca^{++}]_i$ levels in normal and cholestatic hepatocytes. Basal $[Ca^{++}]_i$ levels as measured by microspectrofluorometry were 198±98 nM ($n = 32$) in normal hepatocytes in agreement with values reported by many groups. Basal $[Ca^{++}]_i$ did not differ (178±67 nM; $n = 24$) in cholestatic cells prepared 24 h after bile duct ligation.

Effect of the dihydroxy bile acids TUDCA and TCDCA on $[Ca^{++}]_i$ in normal and cholestatic isolated rat hepatocytes. In normal hepatocytes, maximally effective concentrations of TUDCA (10 μM) and TCDCA (50 μM) led to a sustained increase in $[Ca^{++}]_i$ by 423±131 nM ($n = 4$, maximum after 7.3±2.8 min) and 322±115 nM ($n = 5$, maximum after 8.8±2.2 min), respectively (Figs. 5 *A* and 6). In cholestatic cells, TUDCA (10 μM) led to a $[Ca^{++}]_i$ increase of only 22% of the increase seen in normal hepatocytes (95±39 nM, $P < 0.005$), and this increase was transient rather than sustained (maximum attained at 1.6±1.0 min, $P < 0.005$; Figs. 5 *B* and 6). A 10-fold increase in concentration of TUDCA (100 μM) had no additional effect, increasing $[Ca^{++}]_i$ by only 114±66 nM or 27% ($n = 5$; $P < 0.005$ vs. 10 μM TUDCA in normal cells), although this increase was more sustained (maximum after 5.8±4.1 min, not significantly different from 10 μM TUDCA in normal cells). Similarly, TCDCA (50 μM) increased $[Ca^{++}]_i$ in cholestatic hepatocytes by only 26% of the rise observed in normal cells ($P < 0.01$; Fig. 6). These data show that in cholestatic hepatocytes both TUDCA- and TCDCA-induced increases in $[Ca^{++}]_i$ are markedly reduced and more transient relative to the increases seen in normal hepatocytes.

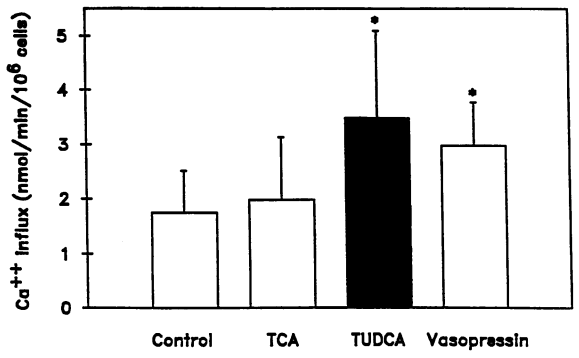

Figure 4. Effect of taurocholic acid (10 μM), TUDCA (10 μM), and vasopressin (10 nM) on $^{45}Ca^{++}$ influx in isolated rat hepatocytes. Hepatocytes were suspended at 2×10^6 cells/ml in a Hepes-buffered Leibovitz-15 medium (pH 7.40, Ca^{++} 1.3 mM). After preincubation for 10 min at 37°C, cells were stimulated with a bile acid for 180 s or with vasopressin for 60 s. Then 10 μCi $^{45}Ca^{++}$ was added and cell aliquots were removed, filtered, and analyzed for $^{45}Ca^{++}$ content (for further details see Methods). Results are given as mean±SD of six to nine experiments from different cell preparations. $^*P < 0.02$; unpaired *t* test.

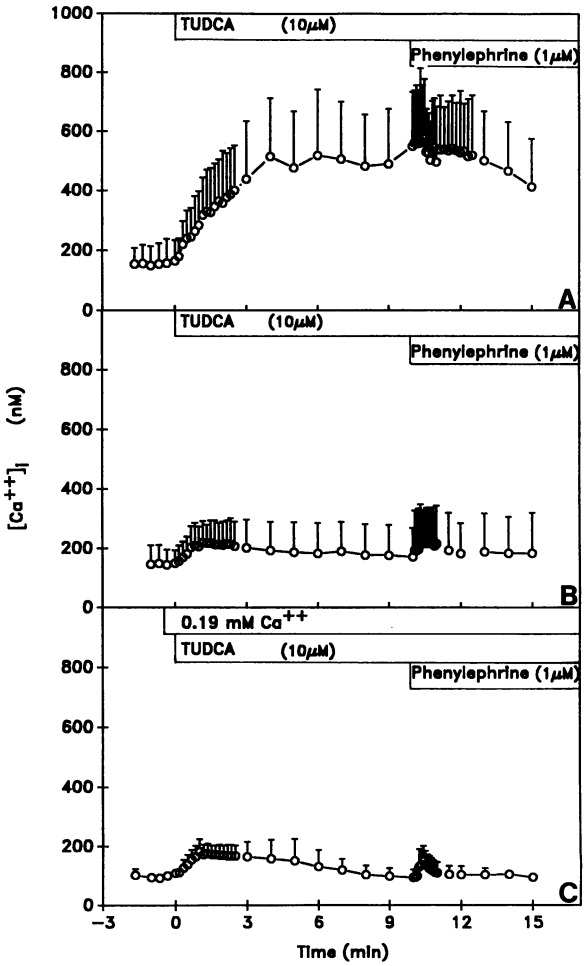

Figure 5. Effect of TUDCA (10 μM) and phenylephrine (1 μM) on $[Ca^{++}]_i$ in isolated rat hepatocytes. (*A*) Normal hepatocytes, Ca^{++} 1.9 mM in the medium. (*B*) Cholestatic hepatocytes of 24-h bile duct–ligated rats, Ca^{++} 1.9 mM in the medium. (*C*) Normal hepatocytes, Ca^{++} 0.19 mM in the medium (switch from 1.9 to 0.19 mM Ca^{++} 30 s before addition of TUDCA). $[Ca^{++}]_i$ was measured microspectrofluorometrically in small groups of cells loaded with indo-1 and superfused with KRB medium. Results are given as mean±SD of four, five, and three experiments from different cell preparations. The effect of TUDCA on stimulation of $[Ca^{++}]_i$ is impaired in cholestatic hepatocytes as observed when extracellular Ca^{++} is low.

Effect of inhibition of Ca^{++} influx on TUDCA-induced $[Ca^{++}]_i$ increase in normal rat hepatocytes. Pretreatment of normal hepatocytes with the Ca^{++} channel blocker $NiCl_2$ (5 mM; 41) for 5 min markedly reduced the TUDCA (10 μM)-induced $[Ca^{++}]_i$ increase to only 23% of that observed in the absence of $NiCl_2$ (121±37 nM, $P < 0.005$). In addition, the $[Ca^{++}]_i$ increase in the presence of Ni^{++} was only transient (maximum after 1.3±0.3 min; $P < 0.02$). ($NiCl_2$ itself caused a small and transient unexplained increase in $[Ca^{++}]_i$ which returned to basal levels after 2–3 min.)

As an alternative method to examine the role of Ca^{++} influx, Ca^{++} was reduced in the extracellular medium by 90% to

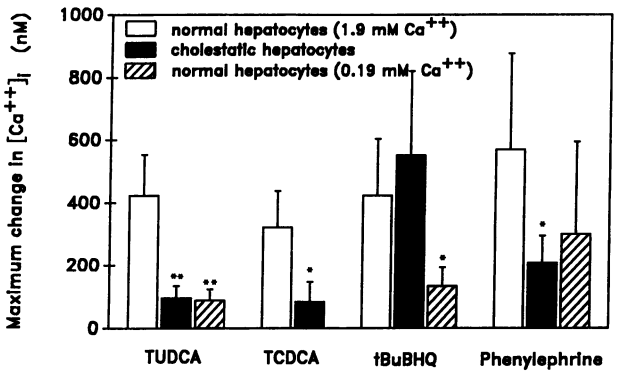

Figure 6. Maximum change in $[Ca^{++}]_i$ in isolated rat hepatocytes induced by TUDCA (10 μM), TCDCA (50 μM), tBuBHQ (25 μM), or phenylephrine (1 μM), under different conditions: (*a*) normal cells, Ca^{++} 1.9 mM (*open bars*); (*b*) cholestatic cells prepared 24 h after bile duct ligation, Ca^{++} 1.9 mM (*solid bars*); (*c*) normal cells, Ca^{++} 0.19 mM (*hatched bars*). Results are given as mean±SD of three to six experiments from different cell preparations and include experiments shown in Figs. 5 and 7. $^{**}P < 0.005$; $^{*}P < 0.05$; unpaired *t* test. The rise in $[Ca^{++}]_i$ after TUDCA, TCDCA, and phenylephrine, but not tBuBHQ is impaired in cholestatic hepatocytes.

0.19 mM (see above). Under these conditions, subsequent stimulation of cells with TUDCA (10 μM) led only to a transient 21% increase in $[Ca^{++}]_i$ (maximum after 1.7±0.6 min; $P < 0.03$) compared to normal cells in the presence of 1.9 mM extracellular Ca^{++} ($P < 0.005$; Figs. 5 *C* and 6). These data demonstrate that inhibition of Ca^{++} entry in normal hepatocytes markedly reduces bile acid–induced $[Ca^{++}]_i$ increases and also prevents the sustained $[Ca^{++}]_i$ increase which is thought to be dependent on influx of extracellular Ca^{++} (e.g., 42–45).

Effect of tBuBHQ and phenylephrine on $[Ca^{++}]_i$ levels in normal and cholestatic rat hepatocytes. At a maximally effective concentration (25 μM; 46), the microsomal Ca^{++}-ATPase inhibitor tBuBHQ caused a marked and sustained increase in $[Ca^{++}]_i$ in both normal hepatocytes (Figs. 6 and 7 *A*) and cholestatic cells (Figs. 6 and 7 *B*). When preincubated for 30 s in low Ca^{++} medium, normal cells displayed only a transient $[Ca^{++}]_i$ increase after addition of 25 μM tBuBHQ. This represented only 31% of the increase observed in the presence of 1.9 mM Ca^{++} ($P < 0.05$; Figs. 6 and 7 *C*).

The α-adrenergic agonist phenylephrine (1 μM) caused a 2.2-fold higher Ca^{++} transient in normal hepatocytes than in cholestatic cells ($P < 0.05$). In low Ca^{++} medium the phenylephrine-induced $[Ca^{++}]_i$ increase also tended to be reduced when compared to higher extracellular Ca^{++} (Fig. 6). The response to phenylephrine in bile acid- or tBuBHQ-pretreated cells was blunted under all experimental conditions studied (see Figs. 5 and 7) consistent with depletion of intracellular inositol triphosphate (IP_3)–sensitive Ca^{++} stores by these agents.

Together, these findings suggest that (*a*) IP_3-sensitive microsomal Ca^{++} stores are not reduced in cholestatic cells from 24-h bile duct–ligated animals, and (*b*) phenylephrine-induced Ca^{++} signals, but not tBuBHQ-induced Ca^{++} signals are impaired in cholestasis.

Effect of TUDCA on bile flow and HRP excretion in the isolated perfused liver of cholestatic rats. Livers isolated 24 h

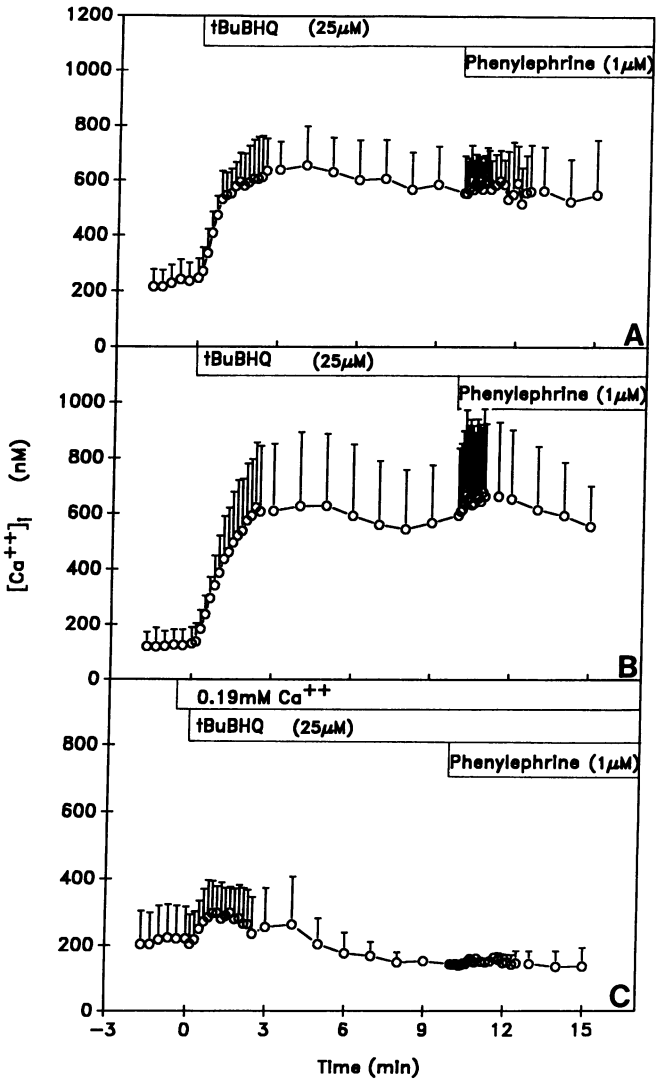

Figure 7. Effect of the microsomal Ca^{++}-ATPase inhibitor tBuBHQ (25 μM) and phenylephrine (1 μM) on $[Ca^{++}]_i$ in isolated rat hepatocytes. (*A*) Normal hepatocytes, Ca^{++} 1.9 mM in the medium. (*B*) Cholestatic hepatocytes of bile duct-ligated rats, Ca^{++} 1.9 mM in the medium. (*C*) Normal hepatocytes, Ca^{++} 0.19 mM in the medium. Experimental methods are as described in the legend of Fig. 5. Results are given as mean±SD of four, four, and three experiments from different cell preparations. The tBuBHQ-induced Ca^{++} signal is normal in cholestatic hepatocytes, but is blunted in normal hepatocytes in the presence of low extracellular Ca^{++}.

after bile duct ligation were preloaded with HRP (0.05 mg/ml) in a recirculating system and washed for 5 min by single-pass perfusion with HRP-free KRB as described above. HRP concentration in bile during HRP infusion into the portal vein exceeded that in normal livers five- to nine-fold (Fig. 8 *A*) probably due to paracellular HRP movement via disrupted hepatocellular tight junctions during acute bile duct obstruction (31). After the wash period HRP concentration in bile dropped sharply. TUDCA infusion did not significantly stimulate HRP secretion at any given time point between 0 and 30 min (Fig. 8 *A*). However, bile flow was significantly higher in livers treated with TUDCA than in control livers (Fig. 8 *B*)

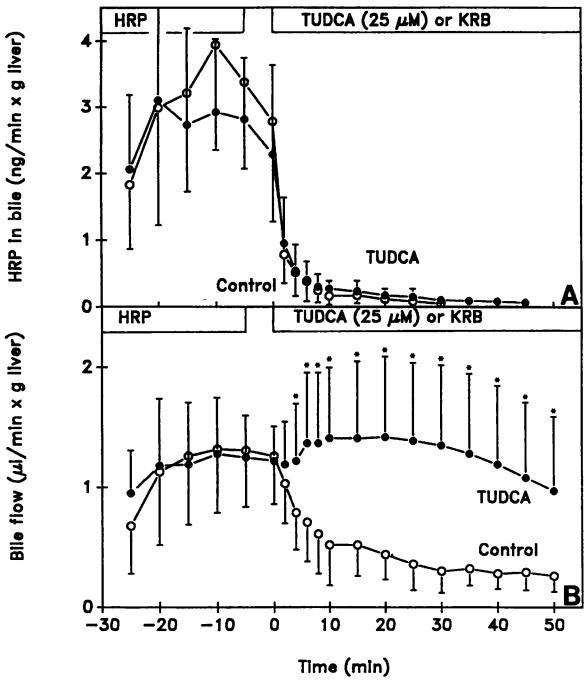

Figure 8. Effect of TUDCA (25 μM) on (*A*) biliary HRP excretion and (*B*) bile flow in the isolated perfused liver of 24-h bile duct–ligated rats. Experimental conditions are as described in Fig. 1. Note the five- to nine-fold increase in the concentrations of HRP in bile in the "early" peak as compared to normal liver (see Figs. 1 and 2; different scale of the *y*-axis), which may be attributed to disruption of tight junctions between hepatocytes in the bile duct–ligated liver. Results are given as mean±SD of seven experiments, each. TUDCA stimulates bile flow, but not HRP excretion in the 24-h bile duct–ligated perfused rat liver.

where bile flow decreased progressively after switching to a single pass perfusion. The difference in bile flow between TUDCA-induced livers and control livers during a 50-min period (calculated as the difference between the means of the areas under the curve of TUDCA and control experiments) was similar between cholestatic livers (42.5 μl/g liver) and normal livers in the presence of high (31.5 μl/g liver) or low (32.8 μl/g liver) extracellular Ca^{++}. These data show that TUDCA is able to stimulate bile flow in cholestatic livers of 24 h bile duct–ligated rats to values similarly to livers of normal rats, but that TUDCA (25 μM) does not stimulate biliary HRP excretion acutely in cholestatic livers similarly to the finding in normal livers in the presence of low extracellular Ca^{++}.

Discussion

Previous observations indicated that TUDCA at physiologic concentrations induces a sustained elevation of $[Ca^{++}]_i$ in hepatocytes by mobilizing both intra- and extracellular sources of Ca^{++} comparable to physiologic agonists (10). The present study indicates that TUDCA-induced Ca^{++} entry across the hepatocellular plasma membrane is associated with stimulation of vesicular exocytosis in the perfused rat liver. In contrast, this dihydroxy bile acid-induced, sustained $[Ca^{++}]_i$ increase is impaired in cholestatic hepatocytes isolated from bile duct–ligated rats, a phenomenon reproduced in normal cells by lowering extracellular Ca^{++} or blocking Ca^{++} entry across the plasma membrane with $NiCl_2$. The findings suggest that this defect in mobilizing extracellular Ca^{++} in cholestatic hepatocytes may be linked to the impaired effect of TUDCA on vesicular exocytosis in the cholestatic livers of bile duct-ligated rats.

A sustained $[Ca^{++}]_i$ increase due to Ca^{++} entry across the plasma membrane is required for sustained stimulation of vesicular exocytosis, the final step in the transcytotic vesicle pathway in a variety of secretory cells such as adrenal chromaffine cells (11), pancreatic acinar cells (12), or platelets (13). It has been speculated that localized $[Ca^{++}]_i$ increases beneath the plasma membrane rather than average $[Ca^{++}]_i$ increases throughout the cell might determine the degree of activation of exocytosis (11). However, measurements of the subplasmalemmal $[Ca^{++}]_i$ increases required to stimulate exocytosis have not yet been reported, due to limited spatial and temporal resolution of most video-imaging techniques for Ca^{++}.

The role of sustained elevation of $[Ca^{++}]_i$ from extracellular sources in biliary secretion has not been previously determined. Thus, since Ca^{++} entry across the plasma membrane is markedly stimulated by the dihydroxy bile acid TUDCA in normal hepatocytes (10; Fig. 4), we examined the effects of Ca^{++} influx on vesicular exocytosis utilizing the perfused rat liver. TUDCA induced a sustained increase of biliary HRP excretion which differed from and exceeded the transient increase in HRP excretion previously described for the trihydroxy bile acid taurocholic acid (47). These data suggest that the TUDCA-induced Ca^{++} entry and sustained $[Ca^{++}]_i$ increase in hepatocytes may be functionally related to the sustained stimulation of vesicular exocytosis. The previously demonstrated transient effect of low physiologic taurocholic acid concentrations on HRP excretion in our experimental models also correlates with its lack of effect on $[Ca^{++}]_i$ and Ca^{++} influx in hepatocytes at low physiologic concentrations (10; see also 39, 40).

In a preliminary report, the Ca^{++} ionophore A23187 and the microsomal Ca^{++} ATPase inhibitor tBuBHQ did not stimulate HRP excretion into bile in the perfused rat liver (48). Both Ca^{++} agonists, however, caused marked rises in portal pressure and decreases in bile flow indicating potent effects on cells in addition to hepatocytes in the liver. Hemodynamic effects caused by these agonists, therefore, might obscure effects on hepatocellular exocytosis in this experimental model. In contrast to these Ca^{++} agonists, TUDCA at low physiologic concentrations might act as a specific hepatocellular Ca^{++} agonist after carrier-mediated uptake into the liver cell whereas it apparently does not affect other cell types within the liver at low physiologic concentrations as demonstrated by the lack of any hemodynamic effect in our experiments. This might be due to differences in membrane permeability between the hydrophilic bile acid TUDCA at low physiologic concentrations and lipophilic compounds such as A23187 or tBuBHQ.

Dihydroxy bile acids TUDCA and, to a lesser extent, TCDCA cause a sustained $[Ca^{++}]_i$ increase by (*a*) depleting IP_3-sensitive microsomal Ca^{++} stores independent of IP_3 and (*b*) mobilizing extracellular Ca^{++} via yet undefined mechanisms (10). It has recently been pointed out that the ionophoretic activity of TUDCA which has been demonstrated in vitro (49) is highly unlikely to play a role in TUDCA-induced Ca^{++} entry across the plasma membrane at low physiologic concen-

trations (10). Experiments in the present study using low extracellular Ca^{++} or the Ca^{++} channel blocker $NiCl_2$ suggest that dihydroxy bile acid-induced $[Ca^{++}]_i$ increases are the result of a two-step mechanism. First, the minor bile acid-induced transient $[Ca^{++}]_i$ increase observed under these conditions represents the release of Ca^{++} from IP_3-sensitive stores. Second, the sustained bile acid-induced $[Ca^{++}]_i$ increase, not seen in the presence of Ni^{++} or low Ca^{++} medium, depends on the influx of extracellular Ca^{++} via Ca^{++} channels whose characteristics remain to be defined.

In cholestatic hepatocytes from 24-h bile duct–ligated rats, a small transient bile acid–induced $[Ca^{++}]_i$ increase could be observed comparable to that seen in normal cells in the presence of low extracellular Ca^{++} or the Ca^{++} channel blocker $NiCl_2$, whereas the sustained rise in $[Ca^{++}]_i$ seen in normal cells was nearly completely blocked. In addition, phenylephrine-induced Ca^{++} signals were impaired in cholestatic cells and tended to be reduced in the presence of low extracellular Ca^{++}. These findings suggest that activation of Ca^{++} entry via receptor-operated Ca^{++} channels was also diminished. In contrast, responses of these cells to the microsomal Ca^{++}-ATPase inhibitor tBuBHQ were not significantly altered in comparison to normal cells indicating that the size of the microsomal Ca^{++} stores was not markedly affected by cholestasis in this bile duct ligation model. These data suggest that the impairment in bile acid- and phenylephrine-induced Ca^{++} signaling in these cholestatic cells is not related to depleted or diminished intracellular Ca^{++} stores and must be related to other as yet undefined mechanisms. Alternatively, impairment of the TUDCA-induced sustained rise in $[Ca^{++}]_i$ may be due to dilution of TUDCA in the bile acid–loaded cholestatic cell. A 3- to 5-fold increase of bile acid content has been reported in cholestatic rat livers after 3–10 d of bile duct ligation (50). However, a 10-fold increase of extracellular TUDCA, from 10 to 100 μM, had no additional effect on $[Ca^{++}]_i$ in cholestatic cells (see Results), whereas a marked and sustained $[Ca^{++}]_i$ increase was observed in normal cells at concentrations as low as 1 μM TUDCA (10). That receptor-operated Ca^{++} channels might be involved in dihydroxy bile acid-induced Ca^{++} entry is suggested by (*a*) similar effects of cholestasis and low Ca^{++} medium on dihydroxy bile acid- and phenylephrine-induced Ca^{++} signals, and (*b*) Ni^{++}, which inhibited TUDCA-induced sustained $[Ca^{++}]_i$ increase and is known to block receptor-operated Ca^{++} channels in hepatocytes (41, 51). These conclusions are also indirectly supported by the observation that increases in $[Ca^{++}]_i$ induced by tBuBHQ were not affected in cholestatic hepatocytes (Fig. 5). Previous studies suggest that tBuBHQ does not activate receptor-operated Ca^{++} channels in hepatocytes (45) yet tBuBHQ is capable of inducing a sustained $[Ca^{++}]_i$ increase when extracellular Ca^{++} is normal, but not in low Ca^{++} medium (45; Fig. 5). Thus, more than one form of Ca^{++} channel may exist in hepatocytes. Our findings suggest that cholestasis impairs Ca^{++} channels involved in dihydroxy bile acid–induced Ca^{++} entry, but not those involved in tBuBHQ-induced Ca^{++} entry.

Bile duct ligation represents a well-defined and widely studied experimental model of cholestasis that shares morphologic, biochemical and functional features with clinical cholestatic disorders (17, 52–54) including chronic cholestatic liver diseases such as primary biliary cirrhosis and primary sclerosing cholangitis. In these diseases bile duct destruction occurs via yet unexplained immunologic mechanisms and produces progressive liver cell damage possibly as a consequence of intrahepatic accumulation of bile due to obstruction and damage of bile ductules and ducts (55, 56). UDCA treatment is now regarded as beneficial treatment in these and other forms of cholestasis although the mechanism of its beneficial effect remains unknown (1–9). Thus the observation that TUDCA enhances biliary exocytosis in the normal isolated perfused rat liver is of interest.

Biliary HRP in our experimental model may be of three origins: (*a*) a paracellular pathway, (*b*) a transcytotic vesicular pathway, and (*c*) a lysosomal pathway (19). The paracellular pathway may play a major role in the loading period and the wash period of the perfusion experiments described above, but not thereafter. In the cholestatic liver, paracellular loss of biliary HRP into hepatovenous effluate via disrupted tight junctions may also be considered. 1. However, TUDCA stimulates bile flow in cholestatic liver by an amount comparable to that in normal liver, suggesting that the relative amount of biliary TUDCA lost into hepatovenous effluate is very low. 2. Because the molecular weight of TUDCA is 100-fold lower than that of HRP, paracellular loss of biliary HRP seems unlikely to occur at significant rates. 3. Lysosomal enzyme excretion was not stimulated by TUDCA treatment, also confirming that TUDCA specifically stimulates vesicular exocytosis, the last step of the transcytotic vesicular pathway. In contrast, biliary excretion of the lysosomal marker enzyme *N*-acetyl-β-D-glucosaminidase was markedly impaired by TUDCA independently of extracellular Ca^{++} (Table I). This may indicate a membrane-stabilizing effect of TUDCA at low physiologic concentrations. Whether this unexpected effect is specific for TUDCA or common to different bile acids is presently unknown.

Vesicular exocytosis represents a major secretory mechanism not only for high molecular weight proteins, lipids and more lipophilic organic anions (16) but also for bile acids in bile acid–loaded hepatocytes (14, 15). In addition, vesicular transport may be crucial for the targeting and insertion of apical membrane transport proteins like the Cl^-/HCO_3^- exchanger into the canalicular membrane (57). In experimental cholestasis, vesicular exocytosis is impaired as demonstrated by accumulation of vesicles in the hepatocellular pericanalicular zone and impaired secretion of secretory IgA (17, 18). It may be speculated that impairment of bile acid–induced Ca^{++} entry across the plasma membrane as demonstrated in this study may be partly responsible for the disturbed excretory function observed in cholestatic hepatocytes and the cholestatic liver.

Taken together with previous observations, (*a*) TUDCA, but not taurocholic acid induces a sustained $[Ca^{++}]_i$ increase at low physiologic levels (10), (*b*) TUDCA, but not taurocholic acid stimulates sustained, Ca^{++}-dependent vesicular exocytosis at physiologic levels, and (*c*) long-term administration of UDCA in patients with cholestatic liver diseases leads to gradual improvement of cholestatic features (1–3) and to replacement of the least potent Ca^{++} mobilizing bile acid conjugates of cholic acid by the most potent Ca^{++} mobilizing conjugates of UDCA (8, 58, 59). We speculate that the Ca^{++} mobilizing effect of UDCA conjugates may gradually (re-)activate the impaired process of hepatocellular vesicular exocytosis in cholestatic liver. This may contribute to the improvement of liver structure and function observed during long-term administration of UDCA in chronic cholestatic liver disease.

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