Phosphoinositide 3-kinase-dependent Ras activation by tauroursodesoxycholate in rat liver

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Ursodesoxycholic acid, widely used for the treatment of cholestatic liver disease, causes choleric, anti-apoptotic and immunomodulatory effects. Here the effects on choleresis of its taurine conjugate tauroursodesoxycholate (TUDC), which is present in the enterohepatic circulation, were correlated with the activation of important elements of intracellular signal transduction in cultured rat hepatocytes and perfused rat liver. TUDC induced a time- and concentration-dependent activation of the small GTP-binding protein Ras and of phosphoinositide 3-kinase (PI 3-kinase) in cultured hepatocytes. Ras activation was dependent on PI 3-kinase activity, without the involvement of

protein kinase C- and genistein-sensitive tyrosine kinases. Ras activation by TUDC was followed by an activation of the mitogen-activated protein kinases extracellular-signal-regulated kinase-1 (Erk-1) and Erk-2. In perfused rat liver, PI 3-kinase inhibitors largely abolished the stimulatory effect of TUDC on taurocholate excretion, suggesting an important role for a PI 3-kinase/Ras/Erk pathway in the choleric effect of TUDC.

Key words: bile salt, cholestasis, hepatocytes, MAP kinases, signal transduction

INTRODUCTION

Tauroursodesoxycholate (TUDC), a choleric dihydroxy bile salt, prevents bile-salt-induced cholestasis [1,2] and exerts choleric effects by stimulating the excretion of other bile salts due to modulation of hepatocellular signalling pathways leading to increased canalicular transport capacity [3–10]. In addition, TUDC is thought to exert hepatoprotection [11–14]. This may explain the clinical use of ursodesoxycholic acid, which *in vivo* is rapidly conjugated with taurine or glycine [15], for treatment of cholestatic liver diseases such as primary biliary cirrhosis and primary sclerosing cholangitis [16–22]. A recent study investigating the possible mechanisms underlying the effects of TUDC showed that, in primary rat hepatocytes, TUDC caused an activation of the MAP (mitogen-activated protein) kinases extracellular-signal-regulated kinase-1 (Erk-1) and Erk-2 [10]. This MAP-kinase activation may be of relevance for the understanding of the physiological and therapeutic effects of TUDC in the liver [10]. The TUDC-induced MAP-kinase activation proceeds via a Raf-dependent pathway, as suggested by its inhibition by cAMP. Furthermore, a specific inhibitor of MEK (MAP kinase/Erk kinase), PD098059 [23–25], completely blocked TUDC-induced MAP-kinase activation [10].

MAP kinases mediate the hypo-osmotic and TUDC-induced stimulation of taurocholate (TC) excretion into bile [8,10]. TUDC [4,7] like hypo-osmotic cell swelling [3,9,26], causes a microtubule-dependent increase in the capacity of canalicular bile salt excretion and stimulation of exocytosis [3,26,27], presumably due to apical targeting of vesicles containing bile salt transporter molecules in response to MAP-kinase activation [4,8,10].

MAP kinases have a central control position in cellular signal transduction, and different upstream signalling events, often converging at the level of Ras/Raf, are known to participate in this signal transduction towards Erks. Ras is a small monomeric

GTP-binding protein that cycles between an inactive GDP-bound and an active GTP-bound form [8,10,28–30].

In the present study, the involvement of TUDC-induced signal elements upstream of the MAP kinases Erk-1 and Erk-2 were analysed. The results reveal TUDC as a potent activator of phosphoinositide 3-kinase (PI 3-kinase) and Ras, and suggest that TUDC signalling via the PI 3-kinase/Ras/Raf/MEK/Erk pathway mediates its choleric effect.

MATERIALS AND METHODS

Materials

Anti-Erk-1 and anti-Erk-2 antibodies were obtained from UBI (Lake Placid, NY, U.S.A.); the rat monoclonal anti-Ras antibody Y13-259 was provided by the Max-Planck-Institut für Molekulare Physiologie (Dortmund, Germany). The anti-phosphotyrosine (p-Tyr) antibody was obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany); genistein, daidzein and PD098059 were purchased from Calbiochem-Novabiochem GmbH (Bad Soden, Germany); wortmannin and LY294002 were from Biomol Research Laboratory Inc. (Hamburg, Germany); and cholera toxin was from Research Biochemicals Inc. (Natick, MA, U.S.A.). Gö6850 was a gift from Gödecke AG (Freiburg, Germany). Cell culture media and fetal calf serum were from Gibco Life Technologies (Gaithersburg, MD, U.S.A.). EGF (epidermal growth factor), TUDC, TC, Protein A-Sepharose and IGEAL CA-630 [(octylphenoxy)-polyethoxyethanol] were obtained from Sigma-Aldrich Chemie GmbH. ATP was from Roche Diagnostics GmbH (Mannheim, Germany), [γ -³²P]ATP was from Amersham Pharmacia Biotech (Uppsala, Sweden), and [³H]TC was from DuPont (Bad Homburg, Germany). All other chemicals were from E. Merck (Darmstadt, Germany) at the highest quality available.

Abbreviations used: EGF, epidermal growth factor; Erk, extracellular-signal-regulated kinase; GST, glutathione S-transferase; JNK, Jun kinase; MAP, mitogen-activated protein; MEK, MAP kinase/Erk kinase; PI 3-kinase, phosphoinositide 3-kinase; p-Tyr, phosphotyrosine; RBD, Ras-binding domain; TC, taurocholate; TUDC, tauroursodesoxycholate.

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Cell preparation and culture

Isolated hepatocytes were prepared from livers of male Wistar rats by a collagenase perfusion technique, as described [31]. Aliquots of 1.5×10^6 cells were plated on collagen-coated culture dishes (36 mm diameter) and maintained at 37 °C and a 5 % CO₂ atmosphere in bicarbonate-buffered Krebs–Henseleit medium (115 mmol/l NaCl, 25 mmol/l NaHCO₃, 5.9 mmol/l KCl, 1.18 mmol/l MgCl₂, 1.23 mmol/l NaH₂PO₄, 1.2 mmol/l Na₂SO₄ and 1.25 mmol/l CaCl₂), supplemented with 6 mmol/l glucose. After 2 h the medium was removed and the cells were washed twice. Subsequently the culture was continued for 24 h in 1.5 ml of Williams medium E (Sigma-Aldrich Chemie), supplemented with glutamine (2 mmol/l), penicillin (100 units/ml), streptomycin (0.1 mg/ml), insulin (0.1 µmol/l), dexamethasone (0.1 µmol/l) and 5 % (v/v) fetal calf serum. The viability of the hepatocytes was more than 95 %, as assessed by Trypan Blue exclusion. TUDC and TC were dissolved in medium; the inhibitors, depending on their solubility, were dissolved either in medium or in DMSO. No effect of medium or DMSO addition on the activation of MAP kinase was observed.

Detection of mobility-shifted Erk-1 and Erk-2

At the end of the incubation period, the medium was removed and the cells were immediately lysed at 4 °C using RIPA buffer (50 mmol/l Tris/HCl buffer, pH 7.2, containing 150 mmol/l NaCl, 40 mmol/l NaF, 5 mmol/l disodium salt of EDTA, 5 mmol/l EGTA, 1 mmol/l vanadate, 0.5 mmol/l PMSF, 0.1 % aprotinin, 1 % IGEPAL CA-630, 0.1 % sodium deoxycholate and 0.1 % SDS). The homogenized lysates were centrifuged at 20000 g at 4 °C, and the supernatant was added to an identical volume of gel loading buffer containing 200 mmol/l 1,4-dithiothreitol, and adjusted to pH 6.8. After heating to 95 °C for 5 min, the proteins were subjected to SDS/PAGE (50 µg of protein/lane; 9 % gel). Following SDS/PAGE, gels were equilibrated with transfer buffer (39 mmol/l glycine, 48 mmol/l Tris/HCl, 0.03 % SDS and 20 % methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia Biotech GmbH, Freiburg, Germany). Blots were blocked in TBST (20 mmol/l Tris/HCl, pH 7.5, 150 mmol/l NaCl and 0.1 % Tween 20) containing 5 % (w/v) BSA and then incubated overnight with 1:50000-diluted antiserum against Erk-1 or Erk-2. After washing with TBST and incubation with horseradish peroxidase-coupled anti-(rabbit IgG) antibody diluted 1:10000 at room temperature for 1 h, the blots were washed three times and developed using enhanced chemiluminescent detection (Amersham Pharmacia Biotech).

Ras activation assay

The minimal Ras-binding domain (RBD) of Raf-1 was used as an activation-specific probe for Ras-GTP [32]. GST–RBD, a fusion protein comprising glutathione S-transferase (GST) and the RBD (amino acids 51–131) of Raf-1, was constructed and expressed in *Escherichia coli*, as previously described [30]. The bacterial pellet (50 g wet weight) was resuspended in PBS containing 2 mM 1,4-dithiothreitol, 0.1 µM aprotinin, 1 µM leupeptin and 20 µM Pefabloc. After sonication for 6 × 1 min on ice and subsequent centrifugation (50000 g for 60 min), the supernatant was chromatographed on a column (2.6 cm × 6 cm) of glutathione–Sephadex (Pharmacia Biotech). The column was washed with PBS and protein was eluted with 10 mM glutathione in PBS. Fractions containing GST–RBD were concentrated by ultrafiltration and applied in PBS to a HiLoad 26/60 Superdex 200 gel-filtration column (Pharmacia Biotech) for final puri-

fication. Purified GST–RBD (10 mg) was bound to glutathione–Sephadex (2 ml) by incubation for 30 min and washed three times with RIPA buffer.

GST–RBD (2 µg of protein), recoupled to glutathione–Sephadex beads in 4 µl of RIPA buffer, was added to the supernatant of a primary culture lysed in 100 µl of RIPA buffer and the sample was incubated at 4 °C for 30 min. Beads were collected by centrifugation (20000 g for 20 min), washed three times with RIPA buffer and resuspended in 10 µl of loading buffer. The protein samples were submitted to SDS/PAGE (15 % polyacrylamide gel) and immunoblotted. Ras was detected using the rat monoclonal pan-Ras antibody Y13-259 (16 h at 4 °C), followed by rabbit-anti-(rat IgG) antibody (2 h at room temperature) coupled to horseradish peroxidase. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (Amersham Buchler). Densitometric evaluation was performed with the E.A.S.Y. RH-system (Herolab, Wiesloch, Germany).

PI 3-kinase assay

The assay was performed according to [33]. Briefly, cell cultures were washed twice with ice-cold PBS, and lysed in a buffer containing 1 % Triton X-100, 150 mmol/l NaCl, 10 mmol/l Tris/HCl (pH 7.5), 1 mmol/l EDTA, 1 mmol/l EGTA, 20 mmol/l NaF, 0.2 mmol/l PMSF and 40 mmol/l 0.5 % Nonidet P-40. Soluble extracts were prepared by centrifugation at 12000 g for 15 min at 4 °C. The supernatants were removed and assayed for total protein by the Bradford method [33]. For immunoprecipitation, 500 µg of protein from each lysate was incubated with 2 µg of an antibody against p-Tyr for 2 h at 4 °C. Immune complexes were collected with the aid of Protein A–Sephadex, washed three times with kinase buffer (10 mmol/l Tris/HCl, pH 7.4, 150 mmol/l NaCl, 10 mmol/l MgCl₂ and 0.5 mmol/l 1,4-dithiothreitol), washed two times with a buffer containing 0.5 mmol/l LiCl and 100 mmol/l sodium vanadate in 0.1 mmol/l Tris/HCl (pH 7.5), and two times with a buffer containing 10 mmol/l Tris, 100 mmol/l NaCl, 1 mmol/l EDTA (pH 7.5) and 200 mmol/l sodium vanadate. PI 3-kinase assays were performed in a reaction mixture containing 50 mmol/l Hepes (pH 7.5), 200 mmol/l ATP, 50 mmol/l MgCl₂ and 1 mmol/l EGTA (pH 7.5). A 30 µl portion of assay buffer supplemented with 20 µg of sonicated PtdIns and 8 µCi of [γ -³²P]ATP was added to the immune complex. The reactions were allowed to proceed at 25 °C for 30 min, and were stopped by the addition of 100 µl of 1 M HCl to each sample. Lipid extracts were obtained by the addition of 180 µl of chloroform/methanol (1:1, v/v), intense mixing of the probes, and centrifugation (14000 g for 2 min) for phase separation. Aliquots of 40 µl of lower organic phase were spotted on to TLC plates. The plates were developed using chloroform/methanol/4 M NH₄OH (9:7:2, by vol.). The activity of PI 3-kinase was monitored by autoradiography.

Liver perfusions

Livers from male Wistar rats (120–180 g body weight), fed *ad libitum* with a standard diet, were perfused as described previously [34] in a non-recirculating system at 37 °C with bicarbonate-buffered Krebs/Henseleit medium, supplemented with 2.1 mmol/l L-lactate and 0.3 mmol/l pyruvate. The flow was adjusted such that the influent K⁺ concentration was 5.9 mmol/l. The perfusate was equilibrated with O₂/CO₂ (95:5); the perfusate flow rate was 3.5–4 ml·min⁻¹·g of liver⁻¹. Portal pressure was monitored continuously with a pressure transducer; tissue vi-

ability was routinely tested by measuring the release of lactate dehydrogenase during perfusion. The inhibitors used in the present study were without effect on tissue viability and perfusion pressure. Bile ducts were cannulated, and bile was collected in 2-min samples. Bile flow was determined gravimetrically. Probes of 100 $\mu\text{mol/l}$ [^3H]TC were added to the perfusion buffers to give a final radioactivity of approx. 37 kBq/l. Biliary TC excretion was calculated by measuring radioactivity in the bile samples and on the basis of the specific radioactivity in the influent perfusate. To wash out endogenously formed bile salts and to obtain steady-state TC excretion, livers were pre-perfused for 30–40 min before the experiments were started.

GTPase assay

The GTPase activity of Ras protein was determined using HPLC, as described previously [35,36]. Aliquots from a 100 $\mu\text{mol/l}$ Ras-GTP solution in the absence and in the presence of TUDC were taken at different time intervals and the reaction was stopped by freezing in liquid nitrogen. For detection of guanosine phosphates, the samples were applied to an RP-18 HPLC column (250 mm $\times$ 4 mm), and isocratic acetonitrile elution (7.5% for GTP analysis) was performed at a flow rate of 1.8 ml/min. The HPLC solution contained 10 mmol/l tetrabutylammonium bromide and 2 mmol/l NaNO_3 . The GTP content is expressed by the quotient $[\text{GTP}] / ([\text{GTP}] + [\text{GDP}])$.

Statistics

Results from different experiments were expressed as means $\pm$ S.E.M. (n = number of independent experiments). Results were compared using Student's t -test; $P < 0.05$ was considered statistically significant.

RESULTS

Activation of Ras by TUDC in cultured rat hepatocytes

To examine whether the bile salt-induced activation of MAP kinases which is found with TUDC, and to a much lesser extent with TC [10], is accompanied by Ras activation, rat hepatocytes were exposed to different concentrations of TUDC and TC. Whereas TC, even at a concentration of 500 $\mu\text{mol/l}$, did not induce a significant effect, TUDC produced a strong activation of approx. 8.2 ± 3.5 -fold ($n = 9$; Figure 1, Table 1). Regardless of the variability of this activation, ranging from 15-fold (Figure 1A) to 4-fold (Figure 2) in all experiments ($n = 9$), pronounced Ras activation was observed. Ras activation by TUDC was concentration-dependent, and detectable at a concentration of 100 $\mu\text{mol/l}$. Because TUDC at concentrations up to 500 $\mu\text{mol/l}$ did not affect the viability of hepatocytes [10], this concentration was used to establish the time-dependency of Ras activation (Figure 1B). TUDC-induced activation of Ras was maximal after 10 min and no longer detectable after 30 min (Figure 1B). Experiments performed with TC under identical conditions showed no detectable Ras activation, whereas EGF, which is known to stimulate the Ras/Raf-dependent MAP-kinase pathway in hepatocytes [37], caused the expected Ras activation (Figure 1B).

Pharmacological characterization of TUDC-induced activation of Ras and Erk-1/Erk-2

In order to identify TUDC-activated signalling components upstream of Ras, inhibitor studies were performed. None of the tyrosine kinase inhibitor genistein, its inactive analogue daidzein, or the inhibitor of G-protein function cholera toxin prevented

Figure 1 Activation of Ras by TUDC and TC

Rat hepatocytes were maintained in primary culture for 24 h and subsequently exposed to bile salts. Ras-GTP was removed from the supernatant of lysed cultures with the aid of GST-RBD coupled to glutathione-Sepharose beads. Antigen-antibody complexes were visualized using enhanced chemiluminescence detection (see the Materials and methods section). Densitometric estimation is related to the corresponding controls from untreated cells and is indicated below each band. The immunoblots are representative of three experiments with similar results. (A) Concentration-dependence of Ras activation. Primary rat hepatocytes were treated with the indicated concentrations of TUDC or TC for 10 min. (B) Time course of Ras activation. Rat hepatocytes were exposed to TUDC (500 $\mu\text{mol/l}$), TC (500 $\mu\text{mol/l}$) or EGF (10 ng/ml) for the indicated time periods.

Table 1 Effects of inhibitors of protein kinase C, tyrosine kinase, G-protein function and PI 3-kinase on TUDC-induced Ras activation

Densitometric estimation is related to the corresponding controls from untreated cells (see the Materials and methods section). Data are given as means $\pm$ S.E.M., for the numbers of different experiments (n) shown. Significant difference compared with TUDC alone: * $P < 0.05$.

Compounds	<i>n</i>	Relative Ras activation
TUDC	9	8.2 ± 3.5
+ Gö6850	3	6.8 ± 2.8
+ genistein	3	8.2 ± 2.7
+ cholera toxin	3	5.7 ± 1.6
+ wortmannin	5	$1.2 \pm 0.2^*$
+ LY294002	5	$0.9 \pm 0.3^*$

the activation of Ras by TUDC in rat hepatocytes ($n = 3$; Figure 2A, Table 1). In the presence of Gö6850, a potent inhibitor of most protein kinase C isoforms [38], Ras activation by TUDC was not significantly affected ($n = 3$; Figure 2A, Table 1). These results are in accordance with previous findings that activation of Erk-1 and Erk-2 by TUDC was likewise not affected by these inhibitors [10], and support the conclusion that TUDC-induced Ras activation occurs upstream of MAP-kinase activation [39].

Sensitivity of TUDC signalling to PI 3-kinase

In order to examine a possible involvement of PI 3-kinase in TUDC-induced signalling, two structurally different inhibitors, wortmannin and LY294002 [40], were tested. Preincubation of hepatocyte cultures with wortmannin (100 nmol/l) for 10 min largely suppressed TUDC-induced activation of both Ras and the MAP kinases Erk-1 and Erk-2 (Figure 2B, Table 1). Expressed

Figure 2 Pharmacological characterization of TUDC-induced activation of Ras and Erk-1/Erk-2

Determination of Ras activation was performed as indicated in the legend to Figure 1. Activation of Erk-1 and Erk-2 was determined by mobility-shift assays. The supernatants of the lysed cultures were subjected to SDS/PAGE and analysed by immunoblotting (see the Materials and methods section). Antigen-antibody complexes were visualized using enhanced chemiluminescence detection. Densitometric estimation was performed as described in the legend to Figure 1. **(A)** Insensitivity to inhibitors of tyrosine kinase, G-protein function and protein kinase C. Rat hepatocytes cultured for 24 h were treated with TUDC (500 $\mu\text{mol/l}$) for 10 min in control cells or in cells pretreated with cholera toxin (CTX; 5 $\mu\text{g/ml}$) for 8 h, genistein (100 $\mu\text{mol/l}$) for 20 min, daidzein (100 $\mu\text{mol/l}$) for 20 min or Gö6850 (1 $\mu\text{mol/l}$) for 20 min. Representative results, from a series of three independent experiments, are shown. **(B)** TUDC-induced activation of Ras and of Erk-1/Erk-2 is sensitive to inhibition of the PI 3-kinase. Rat hepatocytes cultured for 24 h were treated with TUDC (500 $\mu\text{mol/l}$) for 10 min after preincubation with wortmannin (100 nmol/l) or LY294002 (50 $\mu\text{mol/l}$) for 20 min, and compared with cells treated with the inhibitors alone. Representative results, from a series of five independent experiments, are shown.

as intensity of activation related to the control in the absence of TUDC, the presence of wortmannin caused a significant decrease in the TUDC-induced activation of Ras, from 8.2 ± 3.5 -fold to 2.3 ± 1.3 -fold ($n = 5$). The activation of the MAP kinases declined for Erk-1 from 3.6 ± 1.1 -fold in the absence of wortmannin to 1.2 ± 0.2 -fold ($n = 3$) in its presence, and for Erk-2 from 8.2 ± 1.2 to 2.0 ± 1.6 -fold ($n = 3$). An even more complete inhibition of TUDC-induced Ras, Erk-1 and Erk-2 activation was observed in the presence of LY294002 (50 $\mu\text{mol/l}$, $n = 5$; Figure 2B, Table 1). These findings indicate that PI 3-kinase is involved in TUDC-induced activation of Ras and MAP kinase.

In order to substantiate the involvement of PI 3-kinase in TUDC signalling, the activity of PI 3-kinase was assayed directly. Exposure of rat hepatocytes to TUDC (500 $\mu\text{mol/l}$) resulted in an approx. 2.8 ± 0.9 -fold ($n = 3$) activation of PI 3-kinase within 5 min. TUDC-induced PI 3-kinase activation thus preceded TUDC-induced Erk activation (compare Figures 2B and 3).

Involvement of PI 3-kinase in the stimulation of bile salt excretion by TUDC in the perfused rat liver

TUDC at concentrations of 10–50 $\mu\text{mol/l}$ has been shown to increase the capacity for TC excretion into bile [3,4]. Upstream inhibition of the TUDC-induced activation of MAP kinase by increasing the intracellular cAMP level or inhibition of MEK fully abolished the TUDC-induced stimulation of TC excretion into bile in the perfused rat liver. This suggested an important role for MAP-kinase activation in the TUDC-induced increase in

Figure 3 Time course of TUDC-induced PI 3-kinase activation in cultured hepatocytes

PI 3-kinase activity was determined in rat hepatocytes maintained in primary culture for 24 h and subsequently exposed to TUDC (500 $\mu\text{mol/l}$). The cell lysates were subjected to immunoprecipitation with an antibody against p-Tyr, followed by the determination of PI 3-kinase activity (see the Materials and methods section). [^{32}P]Phosphatidylinositol 3-phosphate (PIP) was quantified by autoradiography and used as an indication of total PI 3-kinase activity. Hepatocytes were exposed to TUDC for the indicated time periods. Representative results, from a series of three independent experiments, are shown. The corresponding controls were not exposed to TUDC.

Figure 4 Effects of the PI 3-kinase inhibitor LY294002 and the protein kinase C inhibitor Gö6850 on TUDC-induced TC excretion in perfused rat liver

Livers were perfused in the presence of 100 $\mu\text{mol/l}$ [^3H]TC. After a pre-perfusion period of 60 min, TUDC (20 $\mu\text{mol/l}$) was infused for 30 min (●). If present, infusion of LY294002 (2 mmol/l; □) or Gö6850 (50 nmol/l; ○) was started at 30 min of perfusion time. Data are given as means $\pm$ S.E.M. from 3–4 different experiments.

bile salt excretion [10]. In line with this, blockade of the TUDC-induced activation of Ras and Erk by inhibitors of PI 3-kinase, such as LY294002 and wortmannin, also largely blunted the stimulation of TC excretion by TUDC in the perfused rat liver (Figure 4; and results not shown). These inhibitors of PI 3-kinase had no effect on the steady-state excretion of [^3H]TC (Figure 4). Gö6850, an inhibitor of most isoforms of protein kinase C, had no effect on the stimulation of TC excretion from perfused rat liver by TUDC (Figure 4). In separate perfusion experiments, in the presence of Gö6850 the average increase in portal pressure during a 20 min infusion of PMA at 50 nM was 2.9 ± 0.4 cmH $_2$ O ($n = 4$), as compared with 7.8 ± 0.4 cmH $_2$ O ($n = 3$) in control experiments without Gö6850, indicating an effective inhibition of protein kinase C-dependent haemodynamic effects of PMA at this concentration.

DISCUSSION

The present study identifies TUDC as a potent activator of PI 3-kinase and of Ras, an important molecular switch in cell signalling, which is also activated by growth factors. Ras activation plays a role in the regulation of cell function, proliferation and also malignant transformation. The interaction of Ras with Raf in cell signalling has been studied thoroughly [41,42]. Ras proteins have a low intrinsic GTPase activity, which is enhanced by GTPase-activating proteins [43]. In order to assess whether Ras activation by TUDC involves direct effects of TUDC on the GTPase activity, the GTPase activity of Ras was determined in the absence and the presence of TUDC [43]. No effect of TUDC on the GTPase activity of Ras was detectable (results not shown), suggesting that Ras activation by TUDC is not the result of a direct interaction between Ras and TUDC. However, it is mediated in a PI 3-kinase-dependent way, whereas G-proteins, tyrosine kinases and protein kinase C are apparently not involved. Because TUDC induced an activation of PI 3-

kinase, it is unlikely that TUDC signalling towards Ras only requires basal PI 3-kinase activity. The primary molecular target of TUDC upstream of PI 3-kinase remains to be identified, since no direct effect of TUDC on PI 3-kinase activity was detectable *in vitro* (results not shown). Other bile salts, such as TC or the taurine conjugates of chenodesoxycholic acid, and desoxycholic acid or glycochenodesoxycholate [44], can activate Erk-1 and Erk-2 [10]. However, compared with TUDC the effect was small and was not investigated further. In addition, activation of PI 3-kinase by TC has been described [45].

TUDC-induced Ras activation is transient, occurs within 10 min and may trigger Erk activation via the Ras/Raf/MEK pathway. TUDC induced activation of PI 3-kinase, and this TUDC-induced Erk activation was sensitive not only to inhibitors of PI 3-kinase, which abolish TUDC-induced Ras activation, but also to the MEK inhibitor PD098059 and to cAMP [10], which inhibits the signalling pathway via protein kinase A activation at the level of Ras/Raf [46,47]. Thus TUDC signalling via the PI 3-kinase/Ras/Raf MEK pathway towards Erks apparently mediates the choleretic effect of TUDC, because the same inhibition pattern is observed for both TUDC-induced Erk activation in cultured hepatocytes and TUDC-induced stimulation of TC excretion in the perfused rat liver [10]. Although this inhibition pattern implies the importance of Erk activation in the choleretic effect of TUDC, the possibility is not excluded that additional, as yet unknown, signalling inputs are required in order to trigger cholestasis. Microtubules are involved in the TUDC signalling downstream of Erks, as suggested by the colchicine-sensitivity of TUDC-stimulated TC excretion in the perfused rat liver [4] and the fact that colchicine had no effect on TUDC-induced Erk activation [48]. The molecular link between TUDC-induced Erk activation and the involvement of microtubules remains to be disclosed. Possible targets are microtubule-associated proteins such as Tau [49,50], or protein kinases such as the MAP-kinase signal-integrating protein kinase MNK1 [28]. The involvement of microtubules in vesicular transport could well explain the choleretic effect of TUDC via short-term regulation of biliary excretion by insertion/retrieval of canalicular transport ATPases, as shown for MRP2 (multidrug resistance protein 2) in response to hypo-osmotic stimulation [51].

Although EGF induced activation of MAP kinase, which was also sensitive to the PI 3-kinase inhibitors LY294002 and wortmannin, it did not affect TC excretion in the perfused rat liver (results not shown). Possibly, EGF-induced MAP-kinase activation in rat hepatocytes leads to downstream targets other than TC excretion. Possible explanations for this difference in the activation of downstream targets include differences in the kinetics and intensity of MAP-kinase activation, involvement of different MAP-kinase modules in response to EGF and TUDC, or the involvement of different co-signalling events for cholestasis.

Like TUDC, hypo-osmotic swelling of hepatocytes triggers a choleretic response due to Erk activation [8,9]. However, TUDC and hypo-osmotic signalling towards Erks differ in their upstream events. TUDC signalling involves PI 3-kinase, whereas hypo-osmotic Erk activation is a G-protein- and tyrosine kinase-dependent process. Interestingly, both TUDC and hypo-osmotic signalling towards Erks were not affected by Gö6850 ([10]; Figure 2A), a broad-spectrum inhibitor of protein kinase C isoforms. Although this does not rule out protein kinase C activation by TUDC, as reported by others [5–7], the present and previous [10] results on the importance of Erk signalling in the TUDC-induced stimulation of bile salt excretion do not support the view that potential protein kinase C activation plays a significant role in this process. In line with this, stimulation of TC

excretion by TUDC in perfused rat liver was insensitive to Gö6850 (Figure 4). Ras and Erk activation were also induced by TC; however, it was less effective than TUDC. Nonetheless, these findings again support the previous view [10] of a feed-forward regulation of canalicular bile salt excretion by bile salts.

Erk activation by TUDC has been demonstrated at concentrations as low as 10–50 $\mu\text{mol/l}$ [10], whereas Ras activation required TUDC concentrations of $\geq 100 \mu\text{mol/l}$. This difference is most likely due to differing sensitivities of the detection methods for Ras and Erk activation, and further amplification of the signal within the signalling cascade. Therapeutic plasma levels of TUDC following administration of ursodesoxycholic acid in human plasma have been reported [52] to be in the range 7–16.5 $\mu\text{mol/l}$, and these concentrations are close to those required for activation of Ras and Erk in cultured hepatocytes. The activity of Ntcp, a basolateral Na^+ -dependent bile salt carrier, is rapidly down-regulated during culture of hepatocytes [53,54], and previous studies have suggested that Erk activation by TUDC requires its uptake into hepatocytes [10]. Erk activation decreased with an increase in the cultivation time of hepatocytes, and became almost undetectable when cells were cultured for 3 days. Thus hepatocytes in culture may even be less sensitive towards TUDC-induced Ras and Erk activation. In line with this, stimulation of TC excretion in the isolated perfused liver was maximal at TUDC concentrations of 10–50 $\mu\text{mol/l}$. Here 24 h-cultured hepatocytes were used to minimize the stress of the cell preparation, whereas they still exhibit appreciable bile salt uptake at this time point.

In addition to its stimulatory effect on the excretion of other bile salts, TUDC exerts anti-apoptotic and immunomodulatory effects [55]. In some cell types, such as neurons [56], the balance between Erk and Jun kinase (JNK) activity was suggested to strongly influence whether cell injury will trigger apoptosis or activation of a cell repair programme. Thus TUDC-induced Ras and Erk activation may inhibit apoptosis by inducing a shift in the Erk/JNK activity ratio [56]. Further studies are required to settle this issue.

This work was supported by the Forschungskommission of the University of Düsseldorf and the Fonds der Chemischen Industrie, Frankfurt, Germany. We thank Professor Dr Alfred Wittinghofer for helpful discussions, and Reza Amadian for carrying out the GTPase assay.

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Received 18 November 1999/18 April 2000; accepted 23 May 2000