Tauroursodeoxycholic acid exerts anticholestatic effects by a cooperative cPKCα-/PKA-dependent mechanism in rat liver

R Wimmer,1 S Hohenester,1 T Pusl,1 G U Denk,1 C Rust,1 U Beuers1,2

1 Department of Medicine II, Klinikum Grosshadern, University of Munich, Germany; 2 Department of Gastroenterology & Hepatology, Academic Medical Center, University of Amsterdam, The Netherlands

Correspondence to: Professor U Beuers, Department of Gastroenterology & Hepatology, Academic Medical Center, University of Amsterdam, PO Box 22700, NL-1100 DE Amsterdam, The Netherlands; u.h.beuers@amc.uva.nl

ABSTRACT

Objective: Ursodeoxycholic acid (UDCA) exerts anticholestatic effects in part by protein kinase C (PKC)-dependent mechanisms. Its taurine conjugate, TUDCA, is a cPKCα agonist. We tested whether protein kinase A (PKA) might contribute to the anticholestatic action of TUDCA via cooperative cPKCα-/PKA-dependent mechanisms in taurolithocholic acid (TLCA)-induced cholestasis.

Methods: In perfused rat liver, bile flow was determined gravimetrically, organic anion secretion spectrophotometrically, lactate dehydrogenase (LDH) release enzymatically, cAMP response-element binding protein (CREB) phosphorylation by immunoblotting, and cAMP by immunoassay. PKC/PKA inhibitors were tested radiochemically. In vitro phosphorylation of the conjugate export pump, Mrp2/Abcc2, was studied in rat hepatocytes and human Hep-G2 hepatoma cells.

Results: In livers treated with TLCA (10 µmol/l)+TUDCA (25 µmol/l), combined inhibition of cPKC by the cPKC-selective inhibitor Go6976 (100 nmol/l) or the non-selective PKC inhibitor staurosporine (10 nmol/l) and of PKA by H89 (100 nmol/l) reduced bile flow by 36% (p<0.05) and 48% (p<0.01), and secretion of the Mrp2/Abcc2 substrate, 2,4-dinitrophenyl-β-glucuronide, by 31% (p<0.05) and 41% (p<0.01), respectively; bile flow was unaffected in control livers or livers treated with TUDCA only or TLCA+taurocholic acid. Inhibition of cPKC or PKA alone did not affect the anticholestatic action of TUDCA. Hepatic cAMP levels and CREB phosphorylation as readout of PKA activity were unaffected by the bile acids tested, suggesting a permissive effect of PKA for the anticholestatic action of TUDCA. Rat and human hepatocellular Mrp2 were phosphorylated by phorbol ester pretreatment and recombinant cPKCs, nPKCs, and PKA, respectively, in a staurosporine-sensitive manner.

Conclusion: UDCA conjugates exert their anticholestatic action in bile acid-induced cholestasis in part via cooperative post-translational cPKCα-/PKA-dependent mechanisms. Hepatocellular Mrp2 may be one target of bile acid-induced kinase activation.

Ursodeoxycholic acid (UDCA) has been used for the treatment of jaundice in Chinese traditional medicine since the Tang dynasty (618–907 AD) in the form of dried black bear’s bile. Today, UDCA represents the only drug approved by the US Food and Drug Administration for the treatment of primary biliary cirrhosis (PBC), a model cholestatic liver disease.1 UDCA improves biliary secretion in PBC and a number of other cholestatic disorders, such as primary sclerosing cholangitis (PSC) or intrahepatic cholestasis of pregnancy (ICP).2 In early-stage PBC, UDCA delays progression to cirrhosis3 4 as well as development of complications,5 and normalises life expectancy.6 7 Several mechanisms of action of UDCA have been discussed, and stimulation of impaired hepatobiliary secretion, detoxification of bile, and anti-apoptotic effects are assumed to contribute to the beneficial effect of UDCA in cholestatic disorders.8

UDCA conjugates, such as tauroursodeoxycholic acid (TUDCA), are potent signalling molecules both in hepatocytes and cholangiocytes.9 10 In hepatocytes, TUDCA has been shown to induce increases of cytosolic free calcium (Ca2+), and Ca2+ influx,11 12 to selectively activate Ca2+-dependent conventional protein kinase Cα (cPKCα),13 14 to stimulate an integrin-dependent dual signalling pathway leading to activation of mitogen-activated protein kinases (MAPKs), ERK1/2 and p38 MAPK,15 16 and to induce targeting and insertion of key apical transporters like the bile salt export pump, Bsep/Abcb11, and the conjugate export pump, Mrp2/Abcc2, into canalicular membranes of hepatocytes.17 18 In normal hepatocytes, MAPK-dependent mechanisms mediate, in part, the choleretic effect of TUDCA,19 20 whereas in experimental cholestasis, PKC-dependent mechanisms appear to contribute to the anticholestatic action of TUDCA.21 22

Taurolithocholic acid (TLCA) is the most potent cholestatic agent among the major human bile acids23 and has recently been shown to exert its cholestatic action at the hepatocyte level by phosphatidylinositol-3-kinase, and putatively nPKCζ-dependent mechanisms in isolated perfused rat livers (IPRLs) and isolated rat hepatocyte couples.24 Like TUDCA, TLCA is a potent signalling molecule which elevates hepatocellular [Ca2+]i, without stimulation of Ca2+ influx,25 selectively translocates nPKCs to canalicular membranes and activates membrane-bound PKC,26 and induces retrieval of key apical transporters such as the bile salt export pump, Bsep/Abcb11, from canalicular membranes of hepatocytes.21

Direct effects of bile acids such as TLCA or TUDCA on PKA activity in hepatocytes have not been disclosed although glucagon-induced cAMP formation was impaired by TUDCA in a staurosporine-sensitive fashion in hamster hepatocytes.27 PKC agonists at moderate concentrations and PKA are known to stimulate liver cell secretion.28 A cooperative PKC-/PKA-dependent mechanism has recently been described to potentiate chloride secretion via the Xenopus cystic fibrosis transmembrane conductance regulator, XCFTR,29 in Xenopus oocytes, and concomitant activation of cPKCα and PKA led to marked stimulation of insulin secretion.
in pancreatic beta cells by a convergent mechanism. Therefore, we tested the hypothesis that TUDCA may exert anticholestatic effects in the well-established model of TLCA-induced cholestasis by a cooperative cPKCα/PKA-dependent mechanism.

MATERIALS AND METHODS

Materials

Bile acids and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, Missouri, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was from ICN Biomedicals (Aurora, Ohio, USA). The nonselective PKC inhibitor staurosporine (ST), the cPKC inhibitor Go6976, the PKA inhibitor H89, and the recombinant catalytic subunits of cPKCα, nPKCε and PKA were from Calbiochem-Novabiochem (Nottingham, UK). The anti-MRP2/ABC2 antibody was from Alexis (Lausen, Switzerland). A rabbit anti-pCREB antibody and a monoclonal CREB antibody were from Cell Signalling (Danvers, Massachusetts, USA), and a monoclonal mouse anti-GAPDH antibody was from Abcam (Cambridge, UK). A goat-anti-rabbit-IgG-HRP-conjugate-antibody was from Bio-Rad Laboratories ( Hercules, USA). A cAMP enzyme immunoassay kit was from Amersham BioSciences (Arlington Heights, USA). A goat-anti-mouse-IgG-HRP antibody and a monoclonal mouse anti-phosphorylated CREB antibody were from Cell Signalling (Danvers, Massachusetts, USA). An anti-MRP2/ABCC2 antibody was from Bio-Rad Laboratories (Hercules, USA). A rabbit anti-MRP2/ABCC2 antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). A goat-anti-rabbit-IgG-HRP-conjugated-antibody was from Roche Diagnostics (Mannheim, Germany). A rabbit anti-β-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). An anti-MRP2/ABCC2 antibody was from Millipore (Bedford, Massachusetts, USA). A monoclonal mouse anti-GAPDH antibody was from Abcam (Cambridge, UK). A goat-anti-rabbit-IgG-HRP-conjugate-antibody was from Bio-Rad Laboratories (Hercules, USA). An anti-MRP2/ABCC2 antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). An anti-MRP2/ABCC2 antibody was from Millipore (Bedford, Massachusetts, USA). A monoclonal mouse anti-GAPDH antibody was from Abcam (Cambridge, UK). A goat-anti-rabbit-IgG-HRP-conjugate antibody was from Roche Diagnostics (Mannheim, Germany). Marker molecular weight standard was from Sigma (St. Louis, Missouri, USA). Hyperfilm ECL was from Amersham (Little Chalfont, UK). A chemiluminescence reagent was from NEN (Boston, Massachusetts, USA). A rabbit anti-pCREB antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). A goat-anti-rabbit-IgG-HRP-conjugate antibody was from Bio-Rad Laboratories (Hercules, USA). A monoclonal mouse anti-GAPDH antibody was from Abcam (Cambridge, UK). A goat-anti-rabbit-IgG-HRP-conjugate antibody was from Roche Diagnostics (Mannheim, Germany). Marker molecular weight standard was from Sigma (St. Louis, Missouri, USA). A rabbit anti-pCREB antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). A goat-anti-rabbit-IgG-HRP-conjugate antibody was from Bio-Rad Laboratories (Hercules, USA). A monoclonal mouse anti-GAPDH antibody was from Abcam (Cambridge, UK).

Protein kinase inhibitors

The effect of inhibitors on activity of cPKCα, nPKCε, and PKA was tested in vitro at levels of at least 5- to 10-fold above their IC50 for cPKCα, as indicated in the literature, using myelin basic protein (MBP) as substrate to apply concentrations in the IPRLs high enough to be effective, but not too high to cause cholestatic effects. The results showed that cPKCα was more effectively blocked by the nonselective PKC inhibitor staurosporine (10 nmol/l) than by the cPKC-specific inhibitor Go6976 (10–100 nmol/l) (table 1). nPKCε was inhibited by staurosporine (10 nmol/l), but not by Go6976 (10–100 nmol/l). In addition, the selective PKA inhibitor, H89 (100 nmol/l), and staurosporine (10 nmol/l), but not Go6976 blocked PKA effectively (table 1).

Animals

Male Sprague–Dawley rats (208 (SD 22) g) were obtained from Charles River (Sulzfeld, Germany) and were subjected to a 12 h day–night rhythm with free access to rodent chow and water. Isolated rat liver perfusions were performed as described in detail previously. Bile flow was determined gravimetrically in pre pared tubes.

Perfusion protocol

Rat livers were perfused for a total of 115 min in a non-recirculating fashion with Krebs–Ringer bicarbonate solution (pH 7.4, 37°C). Twenty-five minutes after starting the perfusion, the nonselective PKC inhibitor staurosporine (final concentration in the portal vein, 10 nmol/l), the cPKC inhibitor Go6976 (100 nmol/l), and/or the PKA inhibitor H89 (100 nmol/l) were infused continuously into the perfusion medium. Forty-five minutes after start of the perfusion, bile acids (TUDCA, TLCA, TCA, TUDCA+TLCA, TCA+TLCA), dibutyryl cAMP (dbcAMP) or the carrier DMSO only (control, 0.1%, v/v) were added continuously to the buffer by using an infusion pump to reach

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>cPKCα</th>
<th>nPKCε</th>
<th>PKA</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O standard</td>
<td>309 (74)</td>
<td>77</td>
<td>4.4 (1.7)</td>
</tr>
<tr>
<td>DMSO control</td>
<td>294 (46)</td>
<td>71 (11)</td>
<td>4.7 (1.5)</td>
</tr>
<tr>
<td>ST</td>
<td>23 (3)**</td>
<td>7 (1)**</td>
<td>0.5 (0.6)*</td>
</tr>
<tr>
<td>Go6976</td>
<td>163 (52)</td>
<td>61 (10)</td>
<td>4.3 (1.5)</td>
</tr>
<tr>
<td>H89</td>
<td>217 (53)</td>
<td>44 (8)</td>
<td>0.2 (0.1)**</td>
</tr>
<tr>
<td>ST+H89</td>
<td>23 (4)**</td>
<td>6 (1)**</td>
<td>0.1 (0.1)**</td>
</tr>
<tr>
<td>Go6976+H89</td>
<td>107 (20)**</td>
<td>44 (5)</td>
<td>0.4 (0.2)**</td>
</tr>
</tbody>
</table>

Table 1: Effect of the protein kinase C (PKC) inhibitors staurosporine (ST, 10 nmol/l) and Go6976 (100 nmol/l), and the PKA inhibitor H89 (100 nmol/l) on activity of recombinant cPKCα, nPKCε and PKA in vitro using myelin basic protein as kinase substrate

PKC and PKA inhibitors were tested at concentrations at least 5- to 10-fold above their respective IC50 for cPKCα and PKA, respectively, at which they did not affect bile flow and organic anion secretion in isolated perfused rat livers. For details see Materials and methods. Results are given as mean (SD) of protein kinase activity of four independent experiments (% of H2O standard).

*p<0.05, **p<0.01 vs DMSO control (0.1%, v/v), ANOVA post hoc test (Tukey).

DMSO, dimethylsulfoxide.
final portal venous concentrations of 25 μmol/l (TUDCA, TCA) or 10 μmol/l (TLCA, dbcAMP). Sixty-five minutes after start of the perfusion, 1-chloro-2,4-dinitrobenzene (CDNB; 30 μmol/l), the precursor of the model Mrp2/Abcc2 substrate, 2,4-dinitrophenyl-S-glutathionine (GS-DNP), was infused for 10 min. At this concentration, saturation of the biliary GS-DNP secretion was observed in the perfused rat liver.20

Biliary secretion of 2,4-dinitrophenyl-S-glutathione

The model Mrp2/Abcc2 substrate, GS-DNP, was determined in bile spectrophotometrically as described previously.20

Hepatovenous efflux of lactate dehydrogenase

Lactate dehydrogenase (LDH) was determined as an indicator of liver cell damage in the hepatovenous effuante by use of a standard enzymatic test.20

cAMP in liver tissue

cAMP was extracted from liver tissue with a liquid phase extraction method conforming to the supplier’s protocol and was determined by an enzyme immunoassay.

pCREB in liver tissue

An aliquot of shock-frozen liver tissue (−80°C) was homogenised, and proteins were separated by western blotting. pCREB and GAPDH were identified by use of specific antibodies. pCREB and GAPDH were semiquantified by densitometry and pCREB was expressed as a ratio pCREB/GAPDH.

Rat hepatocyte isolation and in vitro phosphorylation of Mrp2/Abcc2

Rat hepatocytes were isolated and cultured on collagen-coated wells as described previously.20 A sample of 5 × 10⁶ cells/well was incubated for 2 h after plating for 4 h with ½P-orthophosphoric acid (74 MBq) and were then treated for 30 min with DMSO (0.1%, v/v), 100 nmol/l phorbol 12-myristate 13-acetate (PMA), 10 nmol/l staurosporine, 25 μmol/l TUDCA, or 10 μmol/l TLCA. Cells were then washed three times with phosphate-buffered saline (PBS).

Immunoprecipitation of rat Mrp2/Abcc2 or human Mrp2/Abcc2

Rat Mrp2/Abcc2 and human MRp2/ABCC2 were immunoprecipitated from rat hepatocytes and human HepG2 hepatoma cells, respectively, as published previously.21 Efficacy of Mrp2/Abcc2 immunoprecipitation was tested by western blotting.

Phosphorylation of human MRp2/ABCC2 in vitro

Phosphorylation was performed using a phosphorylation buffer containing 10 μmol/l ATP and per sample 370 kBq [γ³²P]ATP, and 400 U activated PKA or 90 ng activated cPKCα or/and 110 ng activated nPKCs. MBP was used as a phosphorylation control. Samples were immunoblotted and autoradiography of the gels was performed for 18 h. The bands corresponding to ³²P-MRP2/ABCC2 were excised and radioactivity was counted in a scintillation counter.

Statistics

Data are expressed as mean (SD). Results were compared between different groups using ANOVA post hoc test (Tukey). Comparison of two groups only was performed using an unpaired two-tailed Student t test. A value of p<0.05 was considered statistically significant.
tissue was unaffected by the bile acids used (table 2) suggesting that PKA activity, in line with previous findings, is rather permissive for the anticholestatic action of TUDCA.

**Hepatovenous LDH release**

The cholestatic effect of TLCA is associated with a serious cytotoxic action as demonstrated by a 32-fold increase of hepatovenous LDH release as a readout of liver cell damage. TLCA-induced LDH release was attenuated by addition of TUDCA, but markedly increased after combined inhibition of PKC and PKA in livers treated with TLCA+TUDCA – but not after inhibition of either PKC or PKA alone – in parallel with the inhibition of the anticholestatic action of TUDCA (table 3). In contrast, LDH release was unaffected by combined inhibition of PKC and PKA in controls and livers treated with TUDCA or

---

**Figure 1** Bile flow is stimulated by tauroursodeoxycholic acid (TUDCA) via a cooperative c protein kinase C/protein kinase A (cPKC/-PKA)-dependent mechanism in tauroliothocholic acid (TLCA)-induced cholestasis, but not under control conditions. (A) Bile flow was markedly impaired by TLCA (10 μmol/l) in isolated perfused rat livers. TUDCA (25 μmol/l) reversed TLCA-induced cholestasis. The nonselective PKC antagonist staurosporine (10 nmol/l), but not the selective PKA inhibitor H89 (100 nmol/l), when given alone, tended to impair the anticholestatic effect of TUDCA whereas combined administration of staurosporine and the selective PKA inhibitor H89 (100 nmol/l) significantly impaired the anticholestatic effect of TUDCA. (B) The selective cPKC inhibitor Gö6976 (100 nmol/l), when given alone, did not impair the anticholestatic effect of TUDCA in TLCA-induced cholestasis. Combined administration of Gö6976 and H89 led to a significant impairment of the anticholestatic effect of TUDCA in TLCA-induced cholestasis. (C) Combined administration of the nonselective PKC inhibitor staurosporine (10 nmol/l) and the PKA inhibitor, H89 (100 nmol/l), did neither affect bile flow of DMSO controls nor of livers treated with TUDCA or TLCA alone (see also fig 2). All results are given as means (SD) of four to five experiments, each. For statistical evaluation, see fig 2. Results for TLCA in panels B and C, and for TLCA + TUDCA in panel B are identical to those in panel A and are provided for clarity.
Hepatology

Figure 2  Bile flow and organic anion secretion are stimulated by tauroursodeoxycholic acid (TUDCA) via a cooperative c protein kinase C-/protein kinase A (cPKC-/PKA)-dependent mechanism in tauroliothocholic acid (TLCA)-induced cholestasis. (A) Total bile flow during the last 50 min after addition of inhibitors, the bile acids, TLCA, TUDCA and cholic acid (TCA), and the 2,4-dinitro-3-glutathione (GS-DNP) precursor, 1-chloro-2,4-dinitrobenzene (CDNB), for the experiments shown in fig 1A,B. (B) Biliary GS-DNP secretion during the last 50 min after addition of inhibitors, the bile acids, TLCA, TUDCA and TCA, and the GS-DNP precursor CDNB. All results are given as means (SD) of four to five experiments, each. *p<0.05, **p<0.01, ANOVA post hoc test.

Table 3  The cytoprotective effect of tauroursodeoxycholic acid in tauroliothocholic acid-induced cholestasis is impaired by combined inhibition of c protein kinase C and protein kinase A

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>Inhibitor</th>
<th>LDH release</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>ST-H89</td>
<td>100</td>
</tr>
<tr>
<td>TUDCA</td>
<td>ST-H89</td>
<td>173</td>
</tr>
<tr>
<td>TUDCA</td>
<td>ST-H89</td>
<td>103</td>
</tr>
<tr>
<td>TLCA</td>
<td>ST-H89</td>
<td>100</td>
</tr>
<tr>
<td>TCA</td>
<td>ST-H89</td>
<td>84</td>
</tr>
<tr>
<td>TCA+TUDCA</td>
<td>ST-H89</td>
<td>100</td>
</tr>
<tr>
<td>TCA+TUDCA</td>
<td>ST-H89</td>
<td>336</td>
</tr>
<tr>
<td>TCA+TUDCA</td>
<td>G66976</td>
<td>190</td>
</tr>
<tr>
<td>TUDCA+TCA</td>
<td>H89</td>
<td>184</td>
</tr>
<tr>
<td>TUDCA+TCA</td>
<td>H89</td>
<td>300</td>
</tr>
<tr>
<td>TUDCA+TCA</td>
<td>G66976+H89</td>
<td>100</td>
</tr>
<tr>
<td>TUDCA+TCA</td>
<td>ST-H89</td>
<td>100</td>
</tr>
</tbody>
</table>

Hepatovascular lactate dehydrogenase (LDH) release as a readout of hepatocellular damage was determined during steady state at 85 min in perfused rats. All results are given as means (SD) of four to five experiments, each. *p<0.05 vs respective bile acid control, ANOVA post hoc test. DMSO, dimethylsulfoxide.

DISCUSSION

The present study demonstrates that the short-term anticholestatic effect of TUDCA in the established experimental model of TLCA-induced cholestasis is mediated mainly by a cooperative cPKC-/PKA-dependent mechanism.

We and others have previously observed that TUDCA at low micromolar concentrations stimulates Ca\(^{2+}\) entry into hepatocytes independent of inositol-1,4,5-trisphosphate.\(^{11-13}\) Selectively translocates Ca\(^{2+}\)-dependent cPKC\(_{\alpha}\) to hepatocyte membranes.\(^{14, 20, 34}\) Stimulates formation of 5,6,1,2-diacylglycerol (DAG)\(^{14}\), activates membrane-bound PKC\(^{14,34}\) and stimulates impaired biliary secretion of organic anions and bile acids in TLCA-induced cholestasis by PKC-dependent mechanisms in isolated perfused rat liver (IPRL)\(^{20}\) and isolated rat hepatocyte co-couples,\(^{32}\) respectively. Similar effects of TUDCA on [Ca\(^{2+}\)]\(_{i}\) and Ca\(^{2+}\)-dependent cPKC\(_{\alpha}\) have been observed in cholangiocytes.\(^{35}\) The present study not only confirms a role of PKC, but, by use of the cPKC-specific inhibitor G66976 (fig 1B and 2A), specifically shows that a Ca\(^{2+}\)-dependent PKC isoform mediates the anticholestatic effect of TUDCA. Among the known cPKC isoforms, cPKC\(_{\alpha}\), cPKC\(_{\beta}\)-I, cPKC\(_{\beta}\)-II and cPKC\(_{\gamma}\), only cPKC\(_{\alpha}\) and cPKC\(_{\beta}\)-II have been detected in hepatocytes. We were not able to disclose translocation of cPKC\(_{\beta}\)-II by TUDCA to hepatocyte membranes in IPRL, IRHC and Ntcp-transfected HepG2 hepatoma cells (data not shown; see also Beuers et al\(^{d}\)). Thus, cPKC\(_{\alpha}\) is most likely the PKC isoform involved in the anticholestatic action of TUDCA in experimental cholestasis as suggested previously.\(^{14, 20, 30}\)

incubation with cPKC\(_{\alpha}\) and nPKC\(_{\alpha}\) tended to induce a more pronounced phosphorylation than incubation with cPKC\(_{\alpha}\) or nPKC\(_{\alpha}\) alone in line with the assumption that different PKC isoforms induce phosphorylation of the MRP2 complex at different sites.

Phosphorylation of rat Mrp2 by the nonselective PKC agonist, phorbol 12-myristate 13-acetate

Rat Mrp2 was immunoprecipitated from freshly isolated rat hepatocytes in short-term culture after incubation of hepatocytes with \(^{32}\)P-orthophosphoric acid for 4 h and with DMSO (0.1%), PMA (100 nmol/l), PMA+staurosporine (10 nmol/l), TUDCA (25 nmol/l), and TLCA (10 nmol/l) for 30 min. The results show that Mrp2 was phosphorylated under control conditions. The PMA-induced increase in phosphorylation was reversed by staurosporine. Bile acids at low micromolar concentrations did not affect total phosphorylation of Mrp2 (fig 3A).

Phosphorylation of human MRP2 by cPKC\(_{\alpha}\), nPKC\(_{\alpha}\) and PKA

Human MRP2\(^{29}\) was immunoprecipitated from HepG2 hepatoma cells and MRP2 phosphorylation was studied in vitro using recombinant activated PKC\(_{\alpha}\), PKC\(_{\alpha}\) or PKA. The results show that the MRP2 complex after immunoprecipitation was phosphorylated by cPKC\(_{\alpha}\), nPKC\(_{\alpha}\) and PKA (fig 3B). Combined phosphorylation of the MRP2 complex after immunoprecipitation was studied in vitro using recombinant activated PKC\(_{\alpha}\), PKC\(_{\alpha}\) or PKA. The results show that the MRP2 complex after immunoprecipitation was phosphorylated by cPKC\(_{\alpha}\), nPKC\(_{\alpha}\) and PKA (fig 3B). Combined phosphorylation with cPKC\(_{\alpha}\) and nPKC\(_{\alpha}\) tended to induce a more pronounced phosphorylation than incubation with cPKC\(_{\alpha}\) or nPKC\(_{\alpha}\) alone in line with the assumption that different PKC isoforms induce phosphorylation of the MRP2 complex at different sites.

Phosphorylation of rat Mrp2 by the nonselective PKC agonist, phorbol 12-myristate 13-acetate

Rat Mrp2 was immunoprecipitated from freshly isolated rat hepatocytes in short-term culture after incubation of hepatocytes with \(^{32}\)P-orthophosphoric acid for 4 h and with DMSO (0.1%), PMA (100 nmol/l), PMA+staurosporine (10 nmol/l), TUDCA (25 nmol/l), and TLCA (10 nmol/l) for 30 min. The results show that Mrp2 was phosphorylated under control conditions. The PMA-induced increase in phosphorylation was reversed by staurosporine. Bile acids at low micromolar concentrations did not affect total phosphorylation of Mrp2 (fig 3A).

Phosphorylation of human MRP2 by cPKC\(_{\alpha}\), nPKC\(_{\alpha}\) and PKA

Human MRP2\(^{29}\) was immunoprecipitated from HepG2 hepatoma cells and MRP2 phosphorylation was studied in vitro using recombinant activated PKC\(_{\alpha}\), PKC\(_{\alpha}\) or PKA. The results show that the MRP2 complex after immunoprecipitation was phosphorylated by cPKC\(_{\alpha}\), nPKC\(_{\alpha}\) and PKA (fig 3B). Combined
A role of cPKCα as a mediator of the choleretic effect of TUDCA in IPRL was recently questioned and the cPKC agonist thymeleatoxin was shown to induce cholestasis in IPRL. In contrast to thymeleatoxin, TUDCA interacts with various constitutive pathways mediating the choleretic effect of TUDCA in IPRL. We were not able to show a role of MAPK for the anticholestatic action of TUDCA in IPRL when either Erk1/2 or p38MAPK or Erk1/2 and p38MAPK concomitantly were inhibited. Thus, different signalling pathways mediate the choleretic and the anticholestatic action of TUDCA in hepatocytes.

A cooperative stimulation of secretory activity by PKC/PKA as assumed in the present model for the anticholestatic action of TUDCA has previously been described in different cell types. Apical carriers and their anchoring proteins are potential targets of a concerted action of PKC and PKA. Interestingly, Mrp2/Abcc2 can be phosphorylated in vitro in rat hepatocytes by PKC agonists or staurosporine-sensitive way, but not by bile acids at low micromolar concentrations. Rat hepatocytes were preloaded with orthophosphoric acid and then treated for 4 h with dimethyl sulfoxide (DMSO) (0.1%, v/v), 100 nmol/l PMA, 100 nmol/l PMA + 10 nmol/l staurosporine, 25 μmol/l tauroursodeoxycholic acid (TUDCA) or 10 μmol/l taurolithocholic acid (TLCA). Cells were then homogenised, Mrp2/Abcc2 was immunoprecipitated and immunoblotted as described in Materials and methods. A pilot experiment is shown. (B) Human Mrp2/Abcc2 was phosphorylated in vitro by recombinant cPKCα, nPKCε, and PKA in a staurosporine-sensitive way. Mrp2/Abcc2 was immunoprecipitated from human HepG2 hepatoma cells and was exposed to recombinant cPKCα, nPKCε, and PKA for 2 h in the absence or presence of the potent PKC inhibitor staurosporine (ST, 10 nmol/l). Mrp2/Abcc2 phosphorylation in the presence of ATP was determined radiochemically as described in Materials and methods. Results are given as means (SD) of three independent preparations.

Identification of key serine/threonine phosphorylation sites required for apical insertion and retrieval of Mrp2/Abcc2 or BSEP/ABCB11 – as demonstrated for the sodium taurocholate co-transporting polypeptide, Ntcp/Slc10a1, in the basolateral membrane – will be a prerequisite to further unravel the cholestatic and anticholestatic post-translational effects of bile acids in hepatocytes.

In conclusion, the well-known anticholestatic effect of TUDCA is largely blocked by selective pharmacological intervention in the present study, indicating that TUDCA exerts its anticholestatic effect via a cPKCα-mediated, in a lipid raft-dependent way. Type II InsP3 are essential for hepatocellular Ca++ wave formation and, possibly, Ca++/cPKCα/PKA interaction. Thus, more sophisticated methodological approaches will be needed to further elucidate the possibly permissive role of PKA in the anticholestatic action of TUDCA in the pericanalicular zone of hepatocytes.

Figure 3 The conjugate export pump, Mrp2/Abcc2, is phosphorylated by protein kinase C (PKC) and protein kinase A (PKA) in a staurosporine-sensitive way. (A) Rat hepatocyte total Mrp2/Abcc2 phosphorylation was stimulated by phorbol 12-myristate 13-acetate (PMA), in a staurosporine-sensitive way, but not by bile acids at low micromolar concentrations. Rat hepatocytes were preloaded with orthophosphoric acid and then treated for 4 h with dimethyl sulfoxide (DMSO) (0.1%, v/v), 100 nmol/l PMA, 100 nmol/l PMA + 10 nmol/l staurosporine, 25 μmol/l tauroursodeoxycholic acid (TUDCA) or 10 μmol/l taurolithocholic acid (TLCA). Cells were then homogenised, Mrp2/Abcc2 was immunoprecipitated and immunoblotted as described in Materials and methods. A pilot experiment is shown. (B) Human Mrp2/ABCC2 was phosphorylated in vitro by recombinant cPKCα, nPKCε, and PKA in a staurosporine-sensitive way. Mrp2/ABCC2 was immunoprecipitated from human HepG2 hepatoma cells and was exposed to recombinant cPKCα, nPKCε, and PKA for 2 h in the absence or presence of the potent PKC inhibitor staurosporine (ST, 10 nmol/l). Mrp2/ABCC2 phosphorylation in the presence of ATP was determined radiochemically as described in Materials and methods. Results are given as means (SD) of three independent preparations.
Hepatology

post-translational anticholestatic effect mainly by a cooperative cPKCy/-PKA-dependent mechanism in the experimental model of TLCA-induced cholestasis.

Funding: This work was supported by a grant from the Deutsche Forshungsgemeinschaft (DFG Be 1242/5-5).

Competing interests: None.

REFERENCES


Tauroursodeoxycholic acid exerts anticholestatic effects by a cooperative cPKCα-/PKA-dependent mechanism in rat liver

R Wimmer, S Hohenester, T Pusl, et al.

Gut 2008 57: 1448-1454 originally published online June 26, 2008
doi: 10.1136/gut.2007.140871

Updated information and services can be found at:
http://gut.bmj.com/content/57/10/1448.full.html

These include:
- References
  This article cites 45 articles, 9 of which can be accessed free at:
  http://gut.bmj.com/content/57/10/1448.full.html#ref-list-1
  Article cited in:
  http://gut.bmj.com/content/57/10/1448.full.html#related-urls
- Email alerting service
  Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.
- Topic Collections
  Articles on similar topics can be found in the following collections
  Pancreas and biliary tract (1814 articles)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/