Ca²⁺-dependent Protein Kinase C Isoforms Induce Cholestasis in Rat Liver

Ralf Kubitzz, Nirmalendu Sahaš, Thomas Kühlkamp, Supiya Dutta, Stephan vom Dahl, Matthias Wettstein, and Dieter Häussinger

From the Department of Gastroenterology, Hepatology, and Infectiology, Heinrich-Heine University, D-40225 Düsseldorf, Germany

Bile secretion is regulated by different signaling transduction pathways including protein kinase C (PKC). However, the role of different PKC isoforms for bile formation is still controversial. This study investigates the effects of PKC isoform selective activators and inhibitors on PKC translocation, bile secretion, bile acid uptake, and subcellular transporter localization in rat liver, isolated rat hepatocytes and in HepG2 cells. In rat liver activation of Ca²⁺-dependent cPKCs and Ca²⁺-independent PKCε by phorbol 12-myristate 13-acetate (PMA, 10nmol/liter) is associated with their translocation to the plasma membrane. PMA also induced translocation of the cloned rat PKCε fused to a yellow fluorescent protein (YFP), which was transfected into HepG2 cells. In the perfused liver, PMA induced marked cholestasis. The PKC inhibitors G66850 (1 µmol/liter) and G66976 (0.2 µmol/liter), a selective inhibitor of Ca²⁺-dependent PKC isoforms, diminished the PMA effect by 50 and 60%, respectively. Thymeleatoxin (Ttx), a selective activator of Ca²⁺-dependent cPKCs, did not translocate rat PKCε-YFP transfected in HepG2 cells. However, Ttx (0.5–10 nmol/liter) induced cholestasis similar to PMA and led to a retrieval of Bsep from the canalicular membrane in rat liver while taurocholate-uptake in isolated hepatocytes was not affected. G66976 completely blocked the cholestatic effect of Ttx but had no effect on tauroursodeoxycholate-induced cholestasis. The data identify Ca²⁺-dependent PKC isoforms as inducers of cholestasis. This is mainly due to inhibition of taurocholate excretion involving transporter retrieval from the canalicular membrane.

Cholestasis is a major clinical issue and results from dysregulation of transporter proteins in the sinusoidal (1) and the canalicular membranes (2). Long term down-regulation of these transport systems involves changes in mRNA and protein levels (3, 4) while short term regulation is achieved by transporter retrieval and possibly covalent modification of transporter proteins (5, 6). In some forms of cholestasis retrieval of transporter proteins precedes their expressionional down-regulation (7). Although many inducers of short term cholestasis are known (7–10), the underlying signal transduction mechanisms are incompletely understood.

Several studies related cholestasis to the action of protein kinase C (PKC)1 (11–13). The first PKC isoform was isolated by Nishizuka (14). Subsequently, 12 isoforms were characterized and grouped into classical, Ca²⁺-dependent (cPKC: α, βI, βII, γ), novel, Ca²⁺-independent (nPKC: ε, δ, θ, η), and atypical (aPKC: ξ, ι) PKCs. In rat hepatocytes expression of PKCα, βII, δ, ε and ζ was demonstrated (15, 16). Stimulation of the Ca²⁺-independent PKCε isoform by tauroliothiocholate was reported to decrease bile flow (12), while the therapeutically used bile acid tauroursodeoxycholate (TUDC) increases bile flow (17, 18). The choleretic effect of TUDC is mediated by MAP kinases of the Erk-type (18) and by the p38MAPK (17), while an anticholestatic effect of TUDC was attributed to the activation of the Ca²⁺-dependent PKCα (19, 20). Thus, controversy exists regarding the role of individual PKC isoforms in bile secretion. In this study, the effects of PKC isoforms on bile formation were studied. Ca²⁺-dependent PKCs were found to trigger cholestasis and (in contrast to previous data (19, 20)) were not involved in the choleretic response to TUDC.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate (PMA), G66976, and thymeleatoxin were from Calbiochem (Bad Soden, Germany), G66850 and the mouse anti MRP2/Mrp2 (M2III-6) antibody were from Alexis (Grünberg, Germany). The rabbit anti-rat Bsep antibody (K12) and the rabbit anti-rat Ntcp (K4) were generous gifts from Dr. B. Stieger and Prof. P. Meier-Abt (Kantonsspital Zürich, Switzerland). The anti-mouse ZO-1 antibody was from Biozol (Eching, Germany). Isoform-specific PKC antibodies were from BD Biosciences (Heidelberg, Germany). The plasmid MARCKS-GFP was generously provided by Prof. N. Saito (Kobe University, Japan) (21).

Rat Liver Perfusion—Livers of male Wistar rats (150 g) were perfused as described previously (18) in the presence of 100µmol/liter of taurocholate. Bile was collected every 2 min. Liver viability was assessed from the measurements of portal pressure, pH, and LDL- and glucose-release. After 30 min of perfusion, inhibitors or agonists were added to the medium as indicated. At various time points liver specimens were excised and snap frozen for cryosections or Western blots as described in Refs. 7 and 9.

Isolation and Culture of Rat Hepatocytes—Rat hepatocytes were

1 The abbreviations used are: PKC, protein kinase C; Bsep, bile salt export pump; Mrp2, multidrug resistance-associated protein 2; Ntcp, sodium taurocholate cotransporting polypeptide; cPKC, classical Ca²⁺-dependent protein kinase C; nPKC, novel Ca²⁺-independent protein kinase C; aPKC, atypical protein kinase C; MARCKS, myristoylated alanine-rich C kinase substrate; PMA, phorbol 12-myristate 13-acetate; Ttx, thymeleatoxin; ZO-1, zona occludens protein 1 associated with the tight junctions; YFP, yellow fluorescent protein; TC, taurocholate; TUDC, tauroursodeoxycholate.
prepared as described previously (18). Hepatocytes were cultured for 24 h in Dulbecco’s modified Eagle’s medium (containing 10% fetal bovine serum, 6 mmol/liter glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 100 mmol/liter dexamethasone, and 100 mmol/liter insulin). FBS was omitted 12 h before experiments were started.

**Western Blot Analysis and Densitometry**—Liver specimens were minced in homogenization buffer containing Tris (20 mmol/liter, pH 7.4), sucrose (250 mmol/liter), EDTA (5 mmol/liter), MgCl₂ (1 mmol/liter), and protease inhibitors according to Ref. 12. Samples were ultracentrifuged for 60 min at 150,000 × g. The pellets were enriched in cell membrane-bound proteins while supernatants contained cytosolic proteins. Equal protein amounts of the membranous or cytosolic fractions were separated by SDS-PAGE. PKC isoforms were detected by specific monoclonal antibodies. Densitometry was performed with 1D Image Analysis Software (PerkinElmer Optoelectronics, Wiesbaden, Germany). The amount of membrane-bound (mb) PKC isoforms is expressed as the intensity ratio \( I_{\text{mb}}/I_{\text{cytosol}} \) or as the x-fold change compared with control. The total amount of PKC in cultured rat hepatocytes was determined in whole cell lysates.

**Uptake Studies in Isolated Hepatocytes**—Rat hepatocytes cultured for 12 h in fetal calf serum-free medium were treated with PMA, Ttx, or Me₂SO (control) for 30 min and were then incubated for 2–10 min in fresh medium containing 150 µmol/liter taurocholate (TC) and 450 cpm/fmol [³H]TC. Uptake was stopped by removing the medium and washing the cells three times with ice-cold phosphate-buffered saline. Cells were lysed in a buffer containing 0.2 mol/liter NaOH and 0.05% SDS. Radioactivity of supernatants and cell lysates were measured in a liquid scintillation counter (Packard instruments, Frankfurt, Germany). Unspecific binding of taurocholate was determined by incubating the cells in ice-cold incubation medium for 5 s. These values were subtracted from the uptake rates. Protein concentrations in cell lysates were determined with the Bradford method. TC uptake is expressed as pmol/mg protein. Uptake and unspecific binding was measured in three dishes per condition per cell preparation.

**Cloning of the Rat PKCs and Human PKCa and Transfection into HepG2 Cells**—In order to clone the rat PKcs or the human PKca, respectively, reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) and oligo(dT) as a primer. The templates were mRNA from rat brain and from HepG2 cells, respectively. For the subsequent PCR the forward primers were 5′-ATCCGCTA-GCGAATTCGAGGAGGCAG-3′ and 5′-ATCCGCTAGCGGAGGC- AAGAGGTGGTGTTG-3′, respectively. They contained an NheI restriction site and started in front of the 5′-untranslated region of rat PKc and human PKca, respectively (according to the sequence with the accession numbers M18331 and NM002731, respectively). The reverse primers were 5′-CGCCGTACCCAGGCATCAGGTCCTCA- CC-3′ and 5′-CCGCGGATCCATCTGACCTGCAAGC-3′, respectively, where the stop codons were replaced by KpnI restriction sites. The pGEM-T PCR products were cloned into the pGEM-T vector (Promega, Mannheim, Germany). After propagation in Escherichia coli inserting were excised with NheI and KpnI and were ligated into the vector pEYP-N1 (Clontech, Palo Alto, CA) in frame to the N terminus of the enhanced yellow fluorescent protein. The sequences of the resulting plasmids (PKc-YFP and PKcα-YFP) were confirmed by sequencing.

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium: NutriXim F12 with 10% fetal calf serum. PKc-YFP, PKcα-YFP, or MARCKS-GFP were transfected into HepG2 cells with LipofectAMINE 2000 according to the manufacturer’s guidelines (Invitrogen). 24–48 h later cells were analyzed by confocal microscopy.

**Confocal Laser Scanning Microscopy—**Immunostaining and confocal laser-scanning microscopy were performed as described in Ref. 7 using a Leica TCS-NT confocal laserscanning system mounted on an inverted microscope. Identical settings were used to compare liver sections from different experimental conditions. Cross-talk of fluorochromes was excluded by the use of the acousto optical tunable filter. The entire depth of a section was scanned in 0.5-µm steps. The resulting stacks of pictures were mounted as single projections.

HepG2 cells transfected with PKcα-YFP, PKcα-YFP, or MARCKS-GFP were cultured on 12-mm glass cover slips. 24–48 h after transfection coverslips were placed in a bath holder at 37 °C and covered with medium. Cells were selected according to the expression of PKc-YFP, PKcα-YFP, or MARCKS-GFP. Densitometric adjustments of the microscope (excitation at 488 nm, emission at >510 nm), confocal pictures of HepG2 cells were taken in regular intervals (10 s to 1 min). After 2 min, PMA (100 nmol/liter) or Ttx (100 nmol/liter) were added to the medium, and cells were scanned for up to 60 min. Go6976 (1 µmol/liter) or

**RESULTS**

**Phorbolester-induced Cholestasis—**PMA (10 nmol/liter) induced cholestasis in perfused rat liver and reduced bile secretion by 77% from 3.25 ± 0.22 to 0.75 ± 0.36 µg/liver/min (n = 4) after 60 min in the presence of 100 µmol/liter taurocholate (Fig. 1). Taurocholate excretion was similarly reduced by 78% from 201 ± 16 to 45 ± 21 nmol/g liver/min (n = 4) (Fig. 1) indicating that taurocholate was the major driving force for bile secretion in the experimental setup. After 60 min of LDH release was 144 ± 19 (n = 3) and 100 ± 13 milliunits/g liver/min (n = 4) in the absence and presence of PMA, respectively, suggesting that PMA perfusion does not cause cell damage to the perfused livers.

Go6976 (1 µmol/liter) were added 30 min before microscopy was started. In the case of PKcα PMA was applied to the medium at the end of Ttx treatment to finally prove translocation of PKcα-YFP.

**Statistics—**Data were reproduced from at least three perfusions or cell preparations. Values are given as means ± S.D. (Western blots, uptakes) or S.E. (perfusions). The two-sided Student’s t test was used for statistical analysis, with a p < 0.05 considered to be statistically significant.
Calcium-dependent PKCs Mediate Cholestasis—Ttx, a diterpene-derivate of mezerein from the plant Thymelea hirsuta, is a selective activator of Ca$^{2+}$-dependent PKC isoforms (27, 28). In the perfused rat liver, Ttx at a concentration of 0.5 nmol/liter induced cholestasis (Fig. 3) and reduced bile flow and taurocholate excretion by ~80%. The cholestatic effect was dose-dependent (Table I) resulting in an almost complete cholestasis at a Ttx concentration of 10 nmol/liter, while no signs of liver damage were detectable as assessed by LDH release (Table I). Ttx at 0.5 nmol/liter induced a minor increase in portal pressure (Fig. 3), which was more pronounced with increasing concentrations, therefore further experiments were performed with low Ttx concentrations (0.5 nmol/liter). Initial glucose output was 1.8 ± 0.2 nmol/g liver/min (n = 4) in controls and declined during perfusion. It was not significantly affected by Ttx (Fig. 3). The pH was not affected by Ttx treatment. At the beginning it was 7.44 ± 0.02 and 7.45 ± 0.01 in control and Ttx livers, respectively, and increased to 7.49 ± 0.03 and 7.51 ± 0.01, respectively, after 60 min.

G66976 (200 nmol/liter) abolished the cholestatic effect of Ttx (Fig. 3). This finding strongly suggests that Ttx-induced cholestasis is mediated by a Ca$^{2+}$-dependent PKC isoform. G66976 itself had no significant effect (Fig. 3).

In Ttx-treated livers (1 nmol/liter), cPKCa but not nPKCe translocated to the membrane fraction (Fig. 4A), in line with selective activation of cPKCs by Ttx. At 10-fold higher Ttx concentrations selectivity was still observed. The amount of membrane-bound cPKCa was 2.2 ± 0.3-fold increased in Ttx-perfused livers, while no significant changes were observed in nPKCe. In control perfusions, no significant changes in translocation of either cPKC or nPKC were observed.

Ca$^{2+}$-dependent PKC isoforms induce cholestasis. Ttx, 0.5 nmol/liter (Δ) reduced bile flow (100 μmol/liter taurocholate present in the perfusate) and taurocholate excretion compared with control (○). The slight increases in portal pressure and glucose output induced by Ttx were not statistically significant. G66976 completely blocked the effects of Ttx (○ = G66976 + Ttx). G66976 itself (○) had no significant effect on bile flow. Means ± S.E., n = 3 perfusions.

The slight increases in portal pressure and glucose output induced by Ttx were not statistically significant. G66976 completely blocked the effects of Ttx (○ = G66976 + Ttx). G66976 itself (○) had no significant effect on bile flow. Means ± S.E., n = 3 perfusions.
In contrast, the ratio of membrane-bound to total nPKC (control, 0.47 ± 0.03; Ttx: 0.55 ± 0.06; Ttx: 0.47 ± 0.01) or of nPKCe (control: 0.66 ± 0.02; Ttx: 0.72 ± 0.07) were detectable (not shown). In the membrane fraction of Ttx-treated livers a second band of higher electrophoretic mobility was detected by the cPKC antibody (Fig. 4B). In order to provide further evidence for the specificity of Ttx compared with PMA, the Ca\(^{2+}\)-independent PKCs from rat and the Ca\(^{2+}\)-dependent human PKC\(\varepsilon\) were cloned as described in the methods section and were fused to the N terminus of the yellow fluorescent protein (YFP). Human HepG2 cells were transfected with PKCe-YFP or PKCe-YFP and were analyzed 24 h later. In many unstimulated cells PKCe-YFP already localized to the cell membrane to some extent (e.g., Fig. 5A). However, PMA (100 nmol/liter) always induced a strong and rapid translocation of PKCe-YFP to the cell membrane within 1–2 min (Fig. 5A and movie PKCe-YFP under Supplementary Materials). Activation of the Ca\(^{2+}\)-dependent human PKC\(\varepsilon\) as indicated by its translocation to the cell membrane was observed for both PKC activators, PMA (Fig. 5C) and Ttx (Fig. 5D). It was noted that PKCe translocated faster in response to PMA compared with Ttx and that PKCe translocated faster than PKC\(\alpha\) in response to PMA. Taken together, the data are in line with a selective activation of cPKCs but not nPKCs by Ttx.

**Differential Inhibition of PKC Isoforms by Go6850 and Go6976**—In order to investigate the efficacy of the two PKC inhibitors Go6850 and Go6976 in living cells, translocation of myristoylated alanine-rich C kinase substrates (MARCKS) was studied in transfected HepG2 cells. In unstimulated cells MARCKS is localized at the cell membrane because of its myristoylation at the N terminus and its basic residues in the
effector domain (29). Phosphorylation of multiple residues in the effector domain in response to PKC activation introduces negative charges into MARCKS and eventually leads to translocation of MARCKS into the cytosol (21). Therefore subcellular localization of MARCKS was used as an indicator of PKC activity.

In transfected HepG2 cells, both, PMA (Fig. 6A) and Ttx (Fig. 6B) induced translocation of MARCKS-GFP to the cytosol, indicating PKC activation by these compounds. Go6850 inhibited PMA- (Fig. 6C) and Ttx- (Fig. 6D)-induced MARCKS translocation into the cytosol. E and F. Go6976 (1 μmol/liter) largely inhibited the Ttx- (F) but not the PMA- (E) induced MARCKS translocation into the cytosol. G, comprehensive summary of the differential effects of the used activators and inhibitors on MARCKS activation/translocation. A–F, representative cells are shown from at least three independent experiments.

Fig. 6. PKC-dependent MARCKS translocation is differentially inhibited by Go6976 and Go6850. A and B, PMA (100 nmol/liter, A) and Ttx (100 nmol/liter, B) induced translocation of MARCKS-GFP from the cell membrane into the cytosol in transfected HepG2 cells. C and D, Go6850 (1 μmol/liter) inhibited the PMA- (C) and Ttx (D)-induced MARCKS translocation into the cytosol. E and F. Go6976 (1 μmol/liter) largely inhibited the Ttx- (F) but not the PMA- (E) induced MARCKS translocation into the cytosol. G, comprehensive summary of the differential effects of the used activators and inhibitors on MARCKS activation/translocation. A–F, representative cells are shown from at least three independent experiments.

TUDC-induced Choleresis Is Independent of cPKCs—Tauroursodeoxycholate (TUDC, 20 μmol/liter) increased taurocholate excretion from 220 ± 8 to 280 ± 9 nmol/g liver/min (Fig. 7) as described earlier (17, 18). When Go6976 (200 nmol/liter) was added 20 min before TUDC, it did not affect the TUDC-dependent increase in TC excretion (Fig. 7). This suggests that Ca²⁺-dependent but not Ca²⁺-independent PKCs. The differential effects of PKC activators and inhibitors used in this study are summarized in Fig. 6G.

TC Uptake Is Not Impaired by PKC Activation in Isolated Hepatocytes—Taurocholate is taken up by the basolateral sodium taurocholate cotransporting polypeptide (Ntcp) and by organic anion-transporting polypeptides (Oatp) (30). It is secreted by the canalicular bile salt export pump (Bsep). In order to differentiate whether PKC stimulation affects the transporter at the sinusoidal or the canalicular membrane, uptake of taurocholate was determined in isolated rat hepatocytes. 12 to 24 h after cell isolation, Ntcp was localized at the basolateral and canalicular proteins such as Mrp2 at the apical membrane (Fig. 8A), demonstrating polarized localization of the transporters.

Cells cultured for 12 h were incubated 1 to 8 h with Ttx (100 nmol/liter) or PMA (100 nmol/liter). After treatment total amounts of cPKCα were determined in whole cell lysates by...
Western blots. Expressional down-regulation indirectly verified activation of cPKCα (Fig. 8B) by Ttx and PMA.

The $K_m$ of Ntcp for TC transport is $\sim 25$ μmol (31, 32). In order to saturate transport TC-uptake was measured in the presence of 150 μmol/liter of TC. TC uptake was almost linear during the first 10 min (Fig. 8D). When cells were preincubated for 30 min with 1, 10, or 100 nmol/liter of Ttx or PMA, no inhibition of the initial TC uptake was observed compared with controls (Fig. 8D). Likewise shorter preincubation (2 min) with Ttx or PMA (100 nmol/liter each) did not decrease the initial rate of TC uptake (not shown). These data suggest that cPKCs in cultured hepatocytes are responsive to PMA or Ttx treatment, but do not affect TC uptake across the sinusoidal membrane.

Effects of PMA and Ttx on Mrp2 and Bsep Localization—It was demonstrated recently that cholestasis can result from retrieval of canalicular transporter proteins into subapical vesicles (7, 9, 10, 33–35). Here, cryosections of livers were immunostained and imaged by confocal laser scanning microscopy. In control livers immunoreactivity to Bsep was almost completely confined to the canaliculi (Fig. 9A). In contrast, in PMA-treated livers (10 nmol/liter, 60 min) small Bsep-containing vesicle-like structures appeared in close vicinity to the canaliculi, and the canalicular shape became fuzzy (Fig. 9B). Similarly results were obtained for Mrp2-immunoreactivity (not shown). This transporter retrieval of canalicular transporter proteins might contribute to the PMA-induced cholestasis.

Thymeleatoxin also induced Bsep retrieval in rat livers. While in control livers (Fig. 9C) most Bsep immunoreactivity was localized within the canalicular domain delineated by the two lines of ZO-1, in Ttx livers (10 nmol/liter, 60 min) some pericanalicular Bsep staining was observed (Fig. 9D). When G6976 was administered 20 min prior to Ttx, the effect of Ttx on Bsep retrieval was reversed (Fig. 9E). It has to be noted that some amount of Bsep remains in the canalicular domain in Ttx-treated livers for up to 60 min although cholestasis is almost complete. These findings suggest that retrieval of transporter proteins from the canalicular membrane in response to selective stimulation of Ca$^{2+}$-dependent PKC isoforms participate in the induction of cholestasis.

**DISCUSSION**

Although many inducers of cholestasis are known (for review see Refs. 3 and 36), the corresponding signal transduction cascades are incompletely understood (37). PKCs were found to be involved in cholestasis, but the role of individual PKC isoforms in cholestasis is still controversial (8, 12, 37, 38). Other
to PMA, which translocated both isoforms (Fig. 2). The selectivity of Ttx is underlined by the finding that PMA but not Ttx-induced translocation of the transfected rat PKCε in HepG2 cells, while both PKC activators induce translocation of human PKCa.

Furthermore, complete inhibition of the Ttx-induced cholestasis was achieved by Go6976. The specificity of Go6976 toward Ca2+-dependent PKC isoforms compared with Go6850 was confirmed in this study. The subcellular localization of MARCKS was used to monitor PKC activity: MARCKS binds non-covalently to the cell membrane because of the myristate chain at its N terminus and because of electrostatic interactions of positively charged basic residues in the effector domain of MARCKS with negative charges of acidic membrane lipids (29, 43–45). Phosphorylation of the basic residues by different PKC isoforms reverses the electrostatic interaction and cause translocation of MARCKS into the cytosol (21). Here, HepG2 cells were transfected with a MARCKS-GFP fusion protein. Go6976 inhibited Ttx- but not PMA-induced MARCKS translocation to the cytosol. Apparently, activation of Ca2+-independent PKC isoforms by PMA is unaffected by Go6976 and is sufficient for MARCKS translocation. Taken together, several lines of evidence suggest that Ca2+-dependent PKCs induce cholestasis in the perfused rat liver.

Ca2+-independent PKC isoforms such as nPKCδ or nPKCs might contribute to cholestasis induced by PMA but not by Ttx. While PMA activates cPKCs and nPKCs, inhibition of the PMA-induced cholestasis by the selective cPKC-inhibitor Go6976 was incomplete (Fig. 1) indicating an additional effect of the Go6976-insensitive nPKCs. This would be in line with the finding that Ca2+-independent nPKCs contributes to the cholestatic effect of taurolithocholate (12).

Along with translocation of PKCa a band of higher electrophoretic mobility was regularly observed in Ttx-treated livers (Fig. 4). PKCs are phosphorylated at three pseudosubstrate sites (26). Dephosphorylation of PKCa occurs after activation of PKCa and precedes its inactivation and degradation (46). Therefore, the observed second band in Ttx-treated livers might represent the dephosphorylated PKCa.

Limited proteolysis at an arginine within the pseudosubstrate sequence resulting in decreased molecular mass was described for PKCβ after its activation (47). Cleavage at an arginine within the pseudosubstrate sequence of PKCa would result in a loss of a 3-kDa fragment. Therefore this might be another explanation for the appearance of a second band after Ttx stimulation. Both, dephosphorylation or limited proteolysis, are indicators of PKC activation (46, 47).

Other forms of cholestasis such as vasopressin-induced cholestasis involve reduced bile acid uptake by Ntcp (48, 49). This possibility was investigated by uptake studies in isolated rat hepatocytes. Because hepatocytes transiently lose their cell polarity after isolation (9), cells were allowed to regain cell polarity during a 12-h culture period (Fig. 5A).

In the presence of 150 μmol/liter of taurocholate the initial TC uptake was 2.8 ± 1.4 mmol/mg protein/min (mean ± S.D., n = 3) in line with results by others (32). PMA and Ttx activated cPKCa as demonstrated indirectly by their expressionional down-regulation (50), but both substances failed to reduce taurocholate uptake. These results are in line with previous findings, that PMA or vasopressin may inhibit the cAMP-induced TC uptake but not the “basal” taurocholate uptake rate (49).

Retrieval of Bsep from the canalicular membrane upon PMA or Ttx stimulation (Fig. 9) might well contribute to cholestasis in rat liver as described for Mrp2 (13). The functional significance of transporter retrieval is suggested by a tight association between transporter localization and bile secretion (7, 9,

---

**Fig. 9.** PMA treatment alters the localization of canalicular protein. A and B, immunoreactivity to Bsep in control livers (A) is confined to the canaliculi. In contrast, in PMA-treated livers (B) Bsep was found in vesicle-like structures. C, in control livers (60 min MeSO4) most of the Bsep immunoreactivity (red) was localized within the canalicular membrane delineated by the tight junction protein ZO-1 (green). D, in contrast, in Ttx-treated livers some Bsep (red) was found close to the canaliculi but inside the cells (arrows), as shown in relation to ZO-1 (green). E, Go6976 inhibited the appearance of Bsep (red) inside the cells. (C–E, left pictured: Bsep in red; middle pictures: ZO-1 in green; right pictures: overlay. The direct overlay of green and red results in yellow. Bars, 10 μm).
33, 34). In this study, transporter retrieval is shown to accompany Ttx-induced cholestasis, but it cannot be decided whether transporter retrieval causes reduced bile acid secretion or whether it only follows transporter inactivation in the canalicular membrane. The latter possibility is supported by the finding that significant amounts of Bsep are still present in the canalicular membrane, even when Ttx cholestasis is almost complete. However, the data show that PMA and Ttx affect bile acid secretion rather than bile acid uptake.

Phosphorylation of mouse-Bsep by PKCa along with stimulation of bile acid secretion was reported to occur in transfected SF9 cells (6). The regulation of Bsep by PKCa remains to be determined. Furthermore, the Ttx-induced cholestasis might be mediated by PKCδ, the other Ca2+-dependent PKC isoform expressed in rat liver. PKCa might also regulate other targets such as tight junctions, which may result in cholestasis after loss of their integrity (51).

Tauroursodeoxycholate (TUDC) has choleretic properties and is used in the treatment of cholestatic liver disease. TUDC was shown to induce translocation of cPKCa from the cytosol to the plasma membrane in isolated rat hepatocytes (20), and it was suggested that the choleretic effect of TUDC depends on cPKCa (20), although functional measurements were not performed. In this study no inhibition of the TUDC-induced choleresis was achieved by G60976 (Fig. 7) at a concentration sufficient to inhibit the PMA- and Txx-dependent cPKCa by TUDC (20) does not mediate its choleretic effect. Signaling by cPKCa is apparently overridden by the choleretic signaling of Erk (18) and the p38MAPK (17). Interestingly, inhibition of Erk-type MAP kinases by PD098059 not only inhibited the TUDC-induced choleresis but produced a slow cholestatic response by itself (18), which might be attributed to the remaining activation of PKCa toward cholestasis.

Taken together it is concluded, that (I) Ca2+-dependent PKCa isoforms induce cholestasis, (II) that this involves retrieval of Bsep and (III) that stimulation of Ca2+-dependent PKCa by TUDC (as shown by others, Refs. 19 and 20) does not explain the choleretic action of this bile acid.

Acknowledgments—We thank Nicole Eichhorst, Claudia Holneicher, and Markus Mroz for expert technical assistance.

REFERENCES

Ca$_{2+}$-dependent Protein Kinase C Isoforms Induce Cholestasis in Rat Liver
Ralf Kubitz, Nirmalendu Saha, Thomas Kühlkamp, Supiya Dutta, Stephan vom Dahl, Matthias Wettstein and Dieter Häussinger

doi: 10.1074/jbc.M306242200 originally published online December 16, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306242200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/01/30/M306242200.DC1

This article cites 51 references, 17 of which can be accessed free at
http://www.jbc.org/content/279/11/10323.full.html#ref-list-1