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Ca²⁺-DEPENDENT PROTEIN KINASE C ISOFORMS ARE CRITICAL TO ESTRADIOL 17β-D-GLUCURONIDE-INDUCED CHOLESTASIS IN THE RAT

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Abstract

The endogenous estradiol metabolite estradiol 17β-D-glucuronide (E₂17G) induces an acute cholestasis in rat liver coincident with retrieval of the canalicular transporters Bsep (Abcc11) and Mrp2 (Abcc2) and their associated loss of function. We assessed the participation of Ca²⁺-dependent PKC isoforms (cPKC) in the cholestatic manifestations of E₂17G in the perfused rat liver (PRL) and in isolated rat hepatocyte couplets (IRHC). In the PRL, E₂17G (2 μmol/liver; intraportal, single injection) maximally decreased bile flow, total glutathione and [³H] taurocholate excretion by 61, 62, and 79%, respectively; incorporation of the specific cPKC inhibitor Gö6976 (500 nM) in the perfusate almost totally prevented these decreases. In dose-response studies using IRHC, E₂17G (3.75–800 μM) decreased the canalicular vacuolar accumulation of the Bsep substrate cholyl-lysylfluorescein with an IC₅₀ of 54.9 ± 7.9 μM. Gö6976 (1 μM) increased the IC₅₀ to 178.4 ± 23.1 μM, and similarly prevented the decrease in the canalicular vacuolar accumulation of the Mrp2 substrate, glutathione methylfluorescein. Prevention of these changes by Gö6976 coincided with complete protection against E₂17G-induced retrieval of Bsep and Mrp2 from the canalicular membrane, as detected both in the PRL and IRHC. E₂17G also increased paracellular permeability in IRHC, which was only partially prevented by Gö6976. The cPKC isoform PKCα, but not the Ca²⁺-independent PKC isoform, PKCε, translocated to the plasma membrane after E₂17G administration in primary cultured rat hepatocytes; Gö6976 completely prevented this translocation, thus indicating specific activation of cPKC. This is consistent with increased autophosphorylation of cPKC by E₂17G, as detected by western blotting. Our findings support a central role for cPKC isoforms in E₂17G-induced cholestasis, by inducing both transporter retrieval from the canalicular membrane and opening of the paracellular route.

Keywords

Mrp2; Bsep; transporter localization; perfused rat liver; isolated rat hepatocyte couplets

Bile formation represents a key liver function by which xenobiotics and endogenous metabolites such as cholesterol, bilirubin, and hormones are eliminated from the body (1,2). Efflux of solutes by ATP-dependent transporters at the canalicular membrane of hepatocytes provide the driving force for osmotic bile formation; among these transporters, the bile salt

export pump (Bsep; Abcc11) and the multidrug resistance-associated protein 2 (Mrp2; Abcc2), responsible for transporting bile salts and glutathione (GSH) and glucuronide conjugates, respectively, play a central role in this process (1,3).

Alterations of canalicular transporter expression, localization, or activity can lead to cholestasis (3). Short-term endocytic retrieval of Bsep and Mrp2 occurs in several experimental models of cholestasis (3,4). This mechanism likely impairs secretory function by reducing the total number of efflux transporters in the canalicular domain and therefore their transport capacity (V_{max}) within minutes. Stimulation of exocytic insertion of transporter-containing vesicles by administration of tauroursodeoxycholate or cyclic AMP prevents cholestasis, thus supporting this hypothesis (5).

The endogenous estradiol metabolite, estradiol 17 β -D-glucuronide (E₂17G) is a useful model to study the manifestations associated with estrogen-induced cholestasis. Indeed, E₂17G induces a dose-dependent, acute and reversible cholestasis by impairing both Bsep-mediated efflux of bile acids and the Mrp2-mediated efflux of GSH (6). As a likely mechanism, E₂17G causes a microtubule-independent, endocytic retrieval of both Bsep and Mrp2 (7,8); spontaneous exocytic re-insertion of these canalicular transporters in a microtubule-dependent fashion is linked to restoration of bile flow and canalicular transport activity (9).

Recent evidence indicates that activation of protein kinase C (PKC) is associated with impairment of the secretory function of hepatocytes, and ultimately leads to cholestasis (10–12). Thymeleatoxin-mediated selective activation of the classical Ca²⁺-dependent PKC isoforms (cPKC), of which PKC α and β_{II} are expressed in hepatocytes (13), is cholestatic in perfused rat liver (PRL) (14). Similarly, oxidative stress-induced impairment of bile salt secretory function in isolated rat hepatocyte couplets (IRHC) is dependent on activation of PKC α (15). Interestingly, this PKC α activation is also associated with retrieval of Bsep and the associated loss of its secretory function (14,15).

In the present study, we determined whether cPKC isoforms mediate the cholestasis induced by E₂17G in the *in situ* PRL and in IRHC. Our results unambiguously demonstrate that cPKC isoforms are critical to the impairment of localization and function of Bsep and Mrp2 induced by E₂17G.

Experimental Procedures

Materials

L-15 culture medium, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, E₂17G, collagenase, methylbutane (isopentane), bovine serum albumin (BSA), Phorbol-12-myristate-13-acetate (PMA) and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, MO). Gö6976 was from Calbiochem (San Diego, CA) and 5-chloromethylfluorescein diacetate (CMFDA) from Molecular Probes (Carlsbad, CA). Cholyl-l-lysylfluorescein (CLF) was a generous gift of Dr. Charles O. Mills (Queen Elizabeth Hospital, Birmingham, UK). [³H]taurocholate (3.0 Ci/mmol) was from Perkin Elmer Life and Analytical Sciences (Boston, MA). All other chemicals were of analytical grade purity, and used as supplied.

Rat Liver Perfusion

Female, Sprague-Dawley rats (180–210 g) (Harlan Industries, Indianapolis, IN) were anesthetized with urethane (1,000 mg/kg, i.p.) and the bile duct cannulated with PE-10 tubing (Intramedic, Clay Adams). Livers were perfused *in situ* via the portal vein in a non-recirculating single-pass design with Krebs-Ringer bicarbonate at 37°C, equilibrated with

5% CO₂/95% O₂, at a constant flow rate of 30 ml/min (~4 ml/min *per g* liver). [³H]taurocholate (2 μCi/l; 0.7 μmol/l) was added to the perfusion medium for bile salt secretion studies. After a 20-min equilibration, the selective cPKC inhibitor Gö6976 (16) (500 nM final concentration) or its solvent (DMSO, 370 μl/l) was added to the reservoir. Fifteen minutes later, a 5-min basal bile sample was collected, followed by administration of E₂17G (2 μmol/liver, intraportal single injection over a 1-min period) or its solvent (DMSO: 10% BSA in saline, 4:96), and bile collected at 5-min intervals for an additional 60 min. Experiments were considered valid only if initial bile flow (after equilibration) was greater than 30 μl/min/kg (~0.9 μl/min/g liver). Viability of the liver was monitored by LDH activity in the perfusate outflow; experiments exhibiting activities over 20 U/l were considered invalid.

Transport activity of Mrp2 and Bsep was evaluated by measuring biliary GSH and [³H]taurocholate excretion, respectively. Total GSH content was measured by the recycling method of Tietze (17). [³H]Taurocholate was determined by liquid scintillation spectroscopy.

Confocal laser scanning microscopy and image analysis

A separate group of PRL was used for confocal immunofluorescence microscopy of Mrp2, Bsep, ZO-1, and occludin localization. In these experiments, perfusion was stopped 20 min after E₂17G, a liver lobe excised and frozen immediately in isopentane precooled in liquid nitrogen, and stored at -80°C. Liver samples were sectioned and fixed as described previously (8,9). Mrp2 was labeled using a monoclonal antibody to human MRP2 (M2 III-6, Alexis Biochemicals, Carlsbad, CA). Bsep was labeled with a polyclonal antibody to mouse Bsep (Kamiya Biomedical, Seattle, WA). ZO-1 and occludin were labeled with polyclonal and monoclonal antibodies directed to human ZO-1 and occludin, respectively (Zymed Laboratories Inc., South San Francisco, CA). Immunostaining was completed by treatment of the preparations with appropriate Cy2- or Cy3-conjugated donkey anti-IgGs (Jackson ImmunoResearch Laboratory, Inc. West Grove PA). All confocal studies were performed in a True Confocal Scanner Leica TCS SP II microscope (Heidelberg, Germany). To ensure comparable staining and image capture performance for the different groups belonging to the same experimental protocol, liver slices were prepared on the same day, mounted on the same glass slide in a single well, and subjected to the staining procedure and confocal microscopy analysis simultaneously.

Assessment of Bsep and Mrp2 endocytic internalization in confocal images was performed using the Image J 1.34m software (NIH), as described (9). Immunodetection of the tight junctional-associated proteins ZO-1 and occludin was performed to visualize the border of bile canalicular structures in samples stained for Mrp2 or Bsep, respectively.

Isolation and culture of rat hepatocytes

Isolated hepatocytes were obtained by collagenase perfusion and mechanical disruption of rat liver, as described (18). To obtain a preparation enriched in IRHC, livers were perfused according to the two-step collagenase perfusion procedure and further enriched by centrifugal elutriation (19). Viability of final preparations (assessed by trypan blue exclusion) was greater than 90%.

Localization and function of Bsep and Mrp2 in IRHC

IRHC were plated in 24-well plastic plates at a density of 0.5×10^5 units/ml in L-15 culture medium, and cultured for 5 h to allow restoration of couplet polarity. IRHC were exposed to DMSO (control group) or E₂17G (3.75–800 μM), for 20 min. To evaluate the role of cPKC

isoforms, Gö6976 (1 μ M) was added to the culture medium 15 min before the addition of E₂17G, and maintained throughout the period of exposure to E₂17G (20 min).

The canalicular vacuolar accumulation of the fluorescent cholyl-l-lysylfluorescein (CLF) was used to quantify Bsep transport; similarly, canalicular accumulation of glutathione methylfluorescein (GS-MF), derived from CMFDA, was used to quantitate Mrp2 transport (7). CLF is a bile salt analogue transported selectively by Bsep (20) whereas CMFDA is a lipophilic compound taken up by passive diffusion across the basolateral membrane and converted into GS-MF by the action of both intracellular esterases and glutathione *S*-transferases. For transport studies, cells were washed twice with L-15, and exposed to 2 μ M CLF (21,22) or 2.5 μ M CMFDA (21,23) for 15 min. Finally, cells were washed twice with L-15, and canalicular transport activity for both substrates assessed by fluorescence microscopy (21) under an inverted microscope (Zeiss Axiovert 25) and images captured with a digital camera (Zeiss AxioCam MRc5). Quantitation was determined as the percentage of IRHC in the images displaying visible green fluorescence in their canalicular vacuoles from a total analysis of at least 200 couplets *per* preparation.

For intracellular localization of Bsep and Mrp2, IRHC were cultured on glass coverslips (12 mm diameter) placed in 24-well plates, treated with E₂17G and/or Gö6976 as described above, and then fixed and stained as reported (21). Briefly, cells were incubated with the specific antibodies to Bsep or Mrp2 (1:200) for 2 h, followed by incubation with Cy2-conjugated donkey anti-IgG (1:100) for 40 min. Densitometric analysis of images (taken with a Zeiss Axiovert 25 fluorescence microscope) was made along a line perpendicular to the canalicular vacuole using the Image J 1.34m software, as described (9).

Evaluation of paracellular permeability in IRHC

We assessed the permeability of tight junctions by quantitating the retention of CLF in the canalicular vacuoles (24). CLF was added for 15 min to the culture media (L-15) to allow it to accumulate in canalicular vacuoles, and then removed from the incubation media by washing twice with L-15 at 37 °C. IRHC were then incubated with Gö6976 (1 μ M) for 15 min before the addition of E₂17G (50 μ M); exposure to E₂17G and/or Gö6976 was maintained for an additional 40 min. Cells were then washed with cold (4 °C) culture medium, and the proportion of IRHC displaying CLF-associated green fluorescence in their canalicular vacuoles assessed as indicated above.

Immunoblotting of PKC α and PKC ϵ

Isolated rat hepatocytes were cultured on 9-cm glass Petri dishes at a density of 1×10^6 cells/ml. After culture for 5-h, cells were exposed to E₂17G (50 μ M) for 5, 10, 15 or 20 min, washed with cold 0.3 M sucrose, resuspended in 0.3 M sucrose plus protease inhibitors (25 μ g/ml leupeptin, 5 μ g/ml pepstatin A and 0.1 mM PMSF), and disrupted by sonication. In separate experiments, we tested the effect of Gö6976 (1 μ M) by exposing the cells for 15 min to the inhibitor, previous to the incubation with E₂17G (50 or 200 μ M, 10 min) or its solvent; Gö6976 was maintained throughout the period of exposure to E₂17G. Cytosolic (supernatant) and total membrane (pellet) enriched fractions were obtained by ultracentrifugation for 60 min at $100,000 \times g$ (25) after elimination of nuclei and cell fragments by centrifugation for 10 min at $500 \times g$, and total protein determined (26), using BSA as a standard. Western blotting in total membrane and cytosolic fractions used an amount of protein that gave a densitometric signal in the linear range for the antibodies used. Proteins were separated by electrophoresis on a 10%-SDS-polyacrylamide gel; membrane and cytosolic fractions from the same experiment were loaded in the same gel. After electrotransfer, nitrocellulose membranes (Sigma Chemical Co) were incubated overnight with monoclonal antibodies to human PKC α or PKC ϵ (BD Biosciences Pharmingen;

1:1000) followed by incubation with an alkaline phosphatase-linked secondary antibody (Bio-Rad, 1:5000) for 1 h, and further detection with a color development solution (Bio-Rad). Densitometry was performed with the Gel-Pro Analyzer (Media Cybernetics) software. To estimate the amount of PKC associated with both cytosolic and membrane fractions, the relative intensity of each band was divided by the μg of protein loaded in that lane, and then multiplied by the total amount of protein recovered in the corresponding fraction. The proportion of membrane-bound PKC isoforms was expressed as the amount in membranes (A_{membrane}) relative to the total cellular amount, according to: $A_{\text{membrane}} / (A_{\text{membrane}} + A_{\text{cytosol}})$. In separate experiments, we compared the effect of treatment of hepatocytes for 10 min with E_217G ($50 \mu\text{M}$) or PMA ($1 \mu\text{M}$), as a positive control, on PKC ϵ translocation to membranes.

Phosphorylation of PKC at several aminoacidic residues is necessary for acquisition of their full catalytic activity (27). PKC α undergoes phosphorylation at Thr497, Thr638 and Ser660, with Thr638 being an autophosphorylation site. The degree of phosphorylation of PKC provides an estimate of the extent of its activation (28). Isolated rat hepatocytes were exposed to E_217G ($50 \mu\text{M}$) or PMA ($1 \mu\text{M}$) as a positive control, for 10 min, washed with cold 0.3 M sucrose, resuspended in sodium vanadate buffer (Cell Lysis Buffer, Cell Signaling Technology, Inc) plus protease inhibitors ($25 \mu\text{g}/\text{ml}$ leupeptin, $5 \mu\text{g}/\text{ml}$ pepstatin A and 0.1 mM PMSF), and disrupted by sonication followed by centrifugation for 10 min at $500 \times g$. The phosphorylated cPKC was quantitated by immunoblotting of the supernatants, using a specific phospho-PKC $\alpha/\beta/\text{II}$ (Thr638/641) antibody (Cell Signaling Technology, Inc.).

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA, followed by Newman Keuls test. Densitometric profiles of Bsep and Mrp2 were compared using the Mann-Whitney test. The four-parameter dose-response curves were compared using GraphPad Prism software (F test). Values of $p < 0.05$ were considered as statistically significant.

Results

Inhibition of cPKC isoforms prevents E_217G -induced impairment of biliary secretory function

A single dose of E_217G induced an acute 61% decrease in bile flow within 10 min that did not recover during the 60-min perfusion period (Fig. 1A), consistent with the 62% and 79% decreases induced by E_217G in the biliary excretion of total GSH and [^3H]taurocholate, respectively (Fig. 1B and C). The selective cPKC isoform inhibitor, Gö6976, prevented the impairment of both bile flow and total GSH excretion, but provided only partial protection against the initial decrease in [^3H]taurocholate secretion, followed by a total recovery of taurocholate secretion 20 min after E_217G administration.

The functional status of Bsep and Mrp2 was evaluated in IRHC by monitoring efflux of Mrp2 (GS-MF) and Bsep (CLF) substrates into the canalicular vacuole (Fig. 2). In dose-response studies, Gö6976 significantly increased the IC_{50} of E_217G from $54.9 \pm 7.9 \mu\text{M}$ (E_217G alone) to $178.4 \pm 23.1 \mu\text{M}$ (E_217G + Gö6976). The minimal and maximal percent inhibition of CLF accumulation and the slope of the dose-response curve as derived from non-linear fit of the dose-response curves were not modified by cPKC inhibition (Fig. 2). Similarly, canalicular accumulation of GS-MF in the presence of $50 \mu\text{M}$ E_217G , was inhibited $40 \pm 3\%$; this inhibition was reduced to only $20 \pm 2\%$ in the presence of Gö6976.

Inhibition of cPKC isoforms prevents E₂17G-induced retrieval of Bsep and Mrp2

E₂17G-induced endocytic retrieval of both Bsep and Mrp2 from the canalicular membrane to intracellular, vesicular compartments decreases their transport activity, and thereby bile flow (7–9). Figure 3 shows confocal images of Bsep (green) and occludin (red), or Mrp2 (red) and ZO-1 (green) 20 min after E₂17G administration. In E₂17G-treated livers, both Bsep and Mrp2 were detected in intracellular structures (white arrows), consistent with their endocytic retrieval from the canalicular membrane. Consistent with our earlier studies (9), this pattern of internalization was evident only in some canalicular structures and coexisted with preserved canalicular localization of the transporters at other sites. We analyzed profiles of distribution of transporter-associated fluorescence along a line perpendicular to the canaliculus (Fig. 4). E₂17G-treated livers showed a wider profile, consistent with increased fluorescence at a greater distance from the canalicular membrane, indicative of retrieval of these transporters into the intracellular compartment. In livers perfused with E₂17G+Gö6976, the distribution of both Bsep and Mrp2 was almost identical to that in control livers. These data demonstrate that cPKC inhibition protected against E₂17G-induced retrieval of Mrp2 and Bsep. Analysis of the distribution profiles of the tight junctional proteins ZO-1 and occludin (Fig. 4) demonstrates a conserved width of the canaliculi in all the experimental groups, thus eliminating the possible influence of changes in this parameter on the distribution profiles of Bsep and Mrp2.

Similar results were obtained in IRHC exposed to E₂17G in the presence or absence of Gö6976 (Fig. 5). Both in control cells and in those incubated with E₂17G + Gö6976, Bsep and Mrp2 appeared to be localized within the canalicular space, whereas in cells treated only with E₂17G, Bsep and Mrp2 exhibited a fuzzy staining pattern, compatible with their internalization. These patterns of distribution were confirmed by densitometric analysis of the images, which demonstrated an E₂17G-induced redistribution of both Bsep and Mrp2 over a greater distance from the canalicular vacuoles; Gö6976 prevented this redistribution. When given alone, Gö6976 did not cause any difference in these patterns of distribution relative to DMSO (data not shown).

Inhibition of cPKC isoforms partially prevents E₂17G-induced increased paracellular permeability in IRHC

We assessed the importance of cPKC activation in increasing the permeability of the paracellular pathway by quantitating the ability of IRHC to retain CLF already accumulated in the canalicular vacuoles. Addition of E₂17G alone significantly diminished retention of CLF in canalicular vacuoles (Fig. 2B); in the presence of Gö6976, the effect of E₂17G was only partially, but yet significantly blocked, so that retention of CLF was still significantly less than in control IRHC. Incubation with Gö6976 alone did not affect this measure (data not shown). These data imply that E₂17G does increase paracellular permeability, but that this effect is only partially dependent on cPKC.

E₂17G selectively activates cPKC isoforms

PKC activation is associated with translocation of the protein from the cytosol to cellular membranes. Figure 6A shows representative immunoblots of the PKC α and PKC ϵ in both cytosolic and membrane fractions of primary cultured rat hepatocytes at various times after addition of E₂17G (50 μ M). Calculation of the percentage of each PKC isoform in the membrane fraction showed that E₂17G increased membrane-bound PKC α by 60% within 5 min; this increase persisted for 15 min, and returned to control values by 20 min after E₂17G (Fig. 6A). The proportion of PKC ϵ associated with the membrane fraction was not modified by E₂17G (Fig. 6A). The selective translocation to membranes of PKC α induced by 50 and 200 μ M E₂17G, was prevented by the specific cPKC inhibitor Gö6976 (Fig. 6B). Treatment with Gö6976 alone did not affect the normal pattern of distribution of PKC α (data not

shown). To further assess the selective effect of E₂17G on PKC translocation to membranes, we compared the effects of a 10-min treatment with E₂17G and the diacylglycerol analog PMA on the proportion of PKC ϵ associated with membranes (Fig. 6C); only PMA induced a significant increase (30 %, $p<0.05$) in PKC ϵ associated with membranes.

Activation of cPKC isoforms by E₂17G was further assessed by detecting autophosphorylation of PKC α and β II at positions Thr638 and Thr641, respectively (Fig. 6D). There was a significant increase in the total cellular content of phosphorylated PKC α and β II in response to treatment with both E₂17G and PMA (86% and 112% over control, respectively, $p<0.05$).

Discussion

The present work provides important insights regarding the signaling pathways involved in cholestasis mediated by E₂17G, that contribute to estrogen-induced cholestasis. The present data in both the PRL and IRHC clearly implicate cPKC isoforms in the inhibition of bile formation and Mrp2- and Bsep-mediated canalicular transport.

In the perfused liver, the selective cPKC inhibitor Gö6976, completely prevented the E₂17G-induced decrease in bile flow and GSH excretion, and partially prevented the impairment in [³H]taurocholate excretion. Further support for the role of cPKC came from dose-response studies of canalicular accumulation of CLF in hepatocyte couplets, where Gö6976 induced a parallel shift to the right of the dose-response curve, leading to a 3 fold increase in the IC₅₀ of E₂17G. Further, both in perfused liver and hepatocyte couplets, E₂17G activated a cPKC-dependent signaling that was responsible for retrieval of Bsep and Mrp2. Analysis of the fluorescence associated with Bsep and Mrp2 supports the conclusion that cPKC inhibition almost completely prevented their E₂17G-induced retrieval.

The data also demonstrated that E₂17G selectively activated PKC α , which is the main cPKC present in rat hepatocytes (13). PKC α activation was transient, with a maximum occurring between 5 and 10 min after E₂17G administration, and returning to basal values after 20 min. The novel, Ca²⁺-independent PKC ϵ , which is activated by oxidative stress to induce Mrp2 retrieval (29), was not activated by E₂17G at these time periods, further indicating that E₂17G activates specific isoforms of PKC. Interestingly, activation of cPKC at 5 min, as detected in cultured hepatocytes, preceded maximal impairment of bile flow *in vivo* and in the perfused liver (7,8,30), as well as retrieval of both Mrp2 and Bsep *in vivo* (7,8), which occur between 10 and 20 min after E₂17G administration. While exposure of hepatocytes to E₂17G in culture could lead to intracellular effects more rapidly than could exposure of the liver via the blood supply, the data are consistent with a role for cPKC activation in initiating transporter retrieval and inhibition of bile flow. Activation of PKC by phorbol esters, the Ca²⁺-elevating hormone vasopressin, or the cholestatic bile salt tauroolithocholate is correlated with their ability to induce cholestasis (10,12,31,32). Moreover, activation of PKC α by the pro-oxidant compound *t*-butylhydroperoxide (15) or by the selective PKC α activator thymeleatoxin (14) induces cholestasis associated with retrieval of Bsep. Our data on E₂17G-induced cholestasis are consistent with these findings.

The intracellular targets for cPKC following its activation by E₂17G are unknown. However, multiple lines of evidence have long indicated that activation of PKC can regulate the permeability of epithelial tight junctions, (33,34). PKC α participates in the disassembly of the tight junctions, while nPKC ϵ plays a role in tight junction formation in some epithelial tissues (35). Tight junctions represent the apical portion of the junctional complex, and serve as a gatekeeper of the paracellular pathway, regulating the passage of water and ions that, in liver, is a critical determinant of bile flow (36). PKC activation increases tight junction

permeability in IRHC (24,32), an event that contributes in part to cholestasis by leakage of osmotically active solutes from the canalicular compartment. We recently demonstrated that E₂17G causes disruption of tight junctions, as indicated by increased paracellular permeability to horseradish peroxidase, and by loss of the “fence” function between canalicular and sinusoidal membranes, as indicated by redistribution of the canalicular transporter Mrp2 to the basolateral domain (37). Increased paracellular permeability occurred simultaneously with endocytic retrieval of Mrp2 *in vivo*, suggesting a causal relationship. Kubitz *et al.* (12) have similarly noted a PKC-dependent distribution of Mrp2 from the canalicular to the basolateral membrane in human HepG2 cells. In the current studies, Gö6976 only partially prevented the E₂17G-induced increase in paracellular permeability in hepatocyte couplets, implying that cPKC-independent factors also contribute to the disruption of the tight junctions observed in E₂17G-induced cholestasis. Further work is needed to determine the extent or nature of the link between endocytosis of Mrp2 or Bsep and increased tight-junctional permeability.

Direct phosphorylation of the retrieved transporters Bsep and Mrp2 may be also associated with PKC activation. The ATP-binding cassette transporter, Mdr1, the closest Bsep homologue, is phosphorylated by PKC at 3 serine residues in the C-terminal, “linker” region that binds to the actin-associated protein, HAX-1 (38). There is also evidence that HAX-1 participates in clathrin-mediated endocytosis of Bsep from the apical membrane in MDCK II cells (39). Whether this phosphorylation occurs also for Bsep, and whether these changes improve Bsep binding to HAX-1 so as to become more readily endocytosed remains to be ascertained.

In summary, the results indicate a key role for cPKC in the endocytic internalization of the canalicular transporters Bsep and Mrp2 in E₂17G-induced cholestasis. Further studies are needed to ascertain the specific target/s modulated directly or indirectly by cPKC that lead to their internalization.

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The abbreviations used are

Bsep	bile salt export pump
Mrp2	multidrug resistance-associated protein 2
E₂17G	estradiol 17β-D-glucuronide
PKC	protein kinase C
cPKC	Ca ²⁺ -dependent protein kinase C isoforms
IRHC	isolated rat hepatocyte couplets
PRL	perfused rat liver
CMFDA	5-chloromethylfluorescein diacetate
PMA	Phorbol-12-myristate-13-acetate
CLF	cholyl-lysylfluorescein
GSH	glutathione

GS-MF glutathione methylfluorescein

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Fig. 1. G66976 protects against E₂17G-induced inhibition of bile flow and biliary secretion of GSH and taurocholate in the perfused rat liver

Rat livers were perfused as described in Methods and the effects of E₂17G on bile secretory function determined in the presence and absence of the PKC α inhibitor, G66976 (500 nM), or its solvent (DMSO). Panels A, B and C show the effect of the treatments on bile flow, total GSH excretion and [3H]taurocholate excretion, respectively. G66976 alone did not induce any changes in these parameters (data not shown). Results are expressed as mean $\pm$ S.E.M, n = 4. ^aSignificantly different from E₂17G; ^bsignificantly different from control.

Fig. 2. G66976 partially prevents E₂17G-induced impairment of the canaliculic accumulation of CLF (A) and E₂17G-induced increase in paracellular permeability in isolated rat hepatocyte couplets (B)

A- IRHC were preincubated with G66976 (1 μM) for 15 min and then exposed to E₂17G (3.75–800 μM) for an additional 20-min. Canaliculic accumulation of CLF was determined as the percentage of couplets displaying visible fluorescence in their canaliculic vacuoles from a total of at least 200 couplets *per* preparation and expressed as a percentage inhibition of canaliculic accumulation, where 0 correspond to controls. Data are shown as mean ± S.E.M, n = 3.

B- Effects of E₂17G on paracellular permeability and the protection provided by G66976 were determined as described in Methods. Results are expressed as mean ± S.E.M, n = 4. ^aSignificantly different from control; ^bsignificantly different from E₂17G.

Fig. 3. Confocal images of E₂17G-induced retrieval of Mrp2 and Bsep and protection by Gö6976 Representative confocal images of immunostained liver samples displaying a co-staining of Bsep (green) and occludin (red) (left panels), and Mrp2 (red) and ZO-1 (green) (right panels). In control livers, both Bsep and Mrp2 were mainly confined to the canalicular space delineated by the tight junction-associated proteins occludin or ZO-1, respectively. Following E₂17G (2 μ mol/liver), some canaliculi show intracellular fluorescence associated with Bsep or Mrp2 (arrows) at a greater distance from the canalicular membrane, consistent with their endocytic retrieval. Gö6976 prevented the internalization of canalicular transporters, as illustrated by a control-like pattern of Bsep and Mrp2 distribution. Gö6976 itself did not induce any changes in transporters localization (images not shown). White bar = 10 μ m.

Fig. 4. Densitometric analysis of localization of Bsep, occludin, Mrp2 and ZO-1 in perfused livers Profiles of Bsep (left, *top*) and Mrp2 (right, *top*) associated fluorescence in confocal images shown in Figure 3. Graphs represent the intensity of fluorescence associated with the transporters along an 8 μm line (from $-4 \mu\text{m}$ to $+4 \mu\text{m}$ of the canaliculus center) perpendicular to the canaliculus. In control livers, transporter-associated fluorescence was concentrated in the canaliculus space (from $-1 \mu\text{m}$ to $+1 \mu\text{m}$). E₂17G-induced retrieval of transporters from the canaliculus membrane ($p < 0.0001$ vs control) was detected as a decrease in the fluorescence intensity in the canaliculus area together with an increased fluorescence at a greater distance from the canaliculus (from $\pm 1 \mu\text{m}$ to $\pm 2 \mu\text{m}$). Distribution profiles of livers treated with E₂17G+Gö6976 showed a significantly decreased Mrp2 internalization ($p < 0.01$ vs E₂17G, $p < 0.0001$ vs control). Bsep distribution did not differ from controls in E₂17G+Gö6976 group ($p < 0.0001$ vs E₂17G). $n = 20$ – 50 canaliculi *per* preparation, 3 independent preparations. Statistical analysis of the distribution profiles of tight junction associated proteins, used to demarcate limits of the canaliculi, occludin (left, *bottom*) and ZO-1 (right, *bottom*) (for Bsep and Mrp2, respectively) showed no changes in the normal distribution by any of the treatments.

Fig. 5. Gö6976 prevents E₂17G-induced retrieval of Bsep and Mrp2 in isolated rat hepatocyte couplets

Isolated rat hepatocyte couplets were preincubated with Gö6976 (1 μM) and then exposed to E₂17G (50 μM), and fixed and immunostained as described in Experimental Procedures. Graphs depict quantitative analysis of transporter-associated fluorescence along a line (8 μm) perpendicular to the canalicular vacuole. Statistical analysis of the profiles revealed a significant internalization of Bsep and Mrp2 under E₂17G treatment ($p < 0.0001$ vs control), which was completely abolished by Gö6976 ($p < 0.0001$ vs E₂17G). Results are expressed as mean $\pm$ S.E.M. $n = 15$ – 25 canalicular vacuoles *per* preparation, 3 independent preparations.

Fig. 6. Specific PKC α activation by E₂17G in primary cultured hepatocytes

A Immunoblots of PKC α and PKC ϵ in membrane (60 μ g protein) and cytosolic (40 μ g protein) fractions. Primary cultured hepatocytes were treated with E₂17G (50 μ M) for 5, 10, 15 or 20 min, and PKC isoform distribution evaluated. The bar graph shows membrane bound PKC α and PKC ϵ expressed as percentage of total cellular content. **B** – Immunoblots of PKC α in membrane and cytosolic fractions. Primary cultured hepatocytes were treated with E₂17G (50 or 200 μ M) for 10 min, in the presence or absence of G66976 (1 μ M). The bar graph shows membrane bound PKC α expressed as percentage of total cellular content. **C** – Representative immunoblot of PKC ϵ in membrane and cytosolic fractions of primary cultured hepatocytes treated for 10 min with E₂17G (50 μ M) or the PKC activator PMA (1 μ M). The bar graph shows membrane bound PKC ϵ expressed as percentage of total cellular content. **D** – Representative immunoblots and quantitation of the phosphorylated forms of cPKC in total cellular extracts from E₂17G- (50 μ M, 10 min) and PMA- (1 μ M, 10 min) treated primary cultured rat hepatocytes. Results are expressed as mean $\pm$ S.E.M., n = 3. ^aSignificantly different from control, ^bsignificantly different from E₂17G 50 μ M, ^csignificantly different from E₂17G 200 μ M.