Bile acids stimulate PKCα autophosphorylation and activation; role in the attenuation of prostaglandin E1-induced cAMP production in human dermal fibroblasts

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Abbreviations used: CDCA, chenodeoxycholic acid; cAMP, 3’-5’ cyclic adenosine monophosphate; FBS, fetal bovine serum; IBMX, 3-Isobutyl-1-methylxanthine; MEM, minimum essential medium; PGE1, prostaglandin E1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TCA, taurocholic acid; UDCA, ursodeoxycholic acid.

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ABSTRACT

The aim was to identify the specific PKC isoform(s) and their mechanism of activation responsible for the modulation of cAMP production by bile acids in human dermal fibroblasts. Stimulation of fibroblasts with 25-100 µM ofchenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) led to YFP-PKCα and YFP-PKCδ translocation in 30-60 min followed by a transient 24-48h down-regulation of the total PKCα, PKCδ and PKCε protein expression by 30-50%, without affecting that of PKCζ. Increased plasma membrane translocation of PKCα was associated with an increased PKCα phosphorylation, while increased PKCδ translocation to the perinuclear domain was associated with an increased accumulation of phospho-PKCδ Thr505 and Tyr311 in the nucleus. The PKCα specificity on the attenuation of cAMP production by CDCA was demonstrated using a combined with PKC down-regulation or inhibition, as well as PKC isoform DN mutants. Under these same conditions, neither PI3 Kinase, p38 MAP kinase, p42/44 MAP kinase, nor PKA inhibitors had any significant effect on the CDCA-induced cAMP production attenuation. CDCA concentrations as low as 10 µM stimulated PKCα autophosphorylation in vitro. This bile acid effect required phosphatidylserine and was completely abolished by the presence of Gö6976. CDCA at concentrations less than 50 µM enhanced the PKCα activation induced by PMA while greater CDCA concentrations reduced the PMA-induced PKCα activation. CDCA alone did not affect PKCα activity in vitro. In conclusion, while CDCA and UDCA activate different PKC isoforms, PKCα plays a major role in the bile acid-induced inhibition of cAMP synthesis in fibroblasts. This study emphasizes potential consequences of increased systemic bile acid concentrations, and cellular bile acid accumulation in extrahepatic tissues during cholestatic liver diseases.
INTRODUCTION

Previously, we have reported that dihydroxy bile acids were the most potent bile acids to inhibit stimulated 3', 5'-cyclic adenosine monophosphate (cAMP) production not only in hepatocytes (10; 12) but also in cells of non-hepatic origin such as human dermal fibroblasts (8). This bile acid inhibitory effect was shown to involve protein kinase C (PKC) activation (8; 10). However, to be effective, the bile acid had to cross the cell plasma membrane and cell permeabilization was necessary for most conjugated bile acids to acutely inhibit cAMP production in cells devoid of the bile acid transporter (8).

Under physiological conditions, the maximum systemic bile acid concentration is around 1-3 µM (1; 59). The level of unconjugated bile acids exhibits a diurnal variation, attaining a maximum concentration of 30-40% of the total serum bile acids after breakfast. However, in patients with the stagnant loop syndrome, serum unconjugated bile acid levels increase due to bacterial overgrowth in the small intestine. Furthermore, in cholestatic hepatobiliary disorders, bile acids accumulate in the systemic circulation, resulting in a 20- to 100-fold increase in serum bile acid concentration (37). Under these conditions, serum levels of unconjugated bile acids can increase dramatically, particularly if portal cirrhosis is present (37). Furthermore, there is considerable evidence both in man and in animal models that cholestasis is associated with increased deposition of bile acids in extrahepatic tissues including the skin (3; 15; 21; 25; 56). Hedenburg et al have reported that the bile acid concentration could be greater in these tissues than that measured in the serum (25). Elevated serum bile acid levels under cholestatic conditions have been associated with hepatotoxicity (22; 23), hepatic fibrosis (39), pruritus (51) cardiomyopathy (35) and vasodilation (6). In addition, most tissues outside of the enterohepatic circulation, including the skin, do not take up conjugated bile acids acutely (8). However, certain conjugated hydrophobic bile acids can, when present chronically, cross the plasma membrane. Therefore, under pathological conditions, tissues outside of the enterohepatic circulation can become in contact with bile acids, which in turn could play a role in the phenotypic alterations mentioned above.

PKC comprises a family of at least 12 related serine/threonine protein kinases that vary in tissue distribution and are differentially regulated and expressed (17; 46; 48). Several mechanisms have
been proposed to explain the activation of PKC by bile acids, including a bile acid-induced increase in diacylglycerol synthesis and stabilization, as well as a direct activation of the kinase (see (11) for review). However, the specific mechanism of PKC isoform activation by bile acids remains to be addressed.

PKC comprises a family of at least 12 related serine/threonine protein kinases. Based on structural properties and cofactor requirements, the family has been subclassified into the classic (c)PKC (PKCα, PKCβ1, PKCβ2 and PKCγ), novel (n)PKC (PKCδ, PKCe, PKCθ and PKCη) and atypical (a)PKC (PKCζ, PKCµ and PKCι) PKCs (41; 45; 58). Acute activation of most of the classic and novel PKC isoforms by various agents, including diacylglycerol and phorbol 12-myristate 13-acetate (PMA) results in the redistribution of these enzymes from the cytosol to specific membranes and compartments (4; 17). Indeed, translocation to membranes for at least the cPKC isoforms is generally considered a hallmark of activation and is frequently used as a marker of PKC isoform activation in intact cells (61). Chronic stimulation down-regulates PKC activity and protein expression due to an increase in PKC degradation with a PKC isoform-specific rate of cleavage (34; 67). Certain PKC isoforms have been implicated in the activation of various cellular functions, including cell proliferation and differentiation, as well as activation/deactivation of G-protein coupled receptors (GPCRs, see (18)). In human dermal fibroblasts, prostaglandin (PG) E1 stimulates cAMP production through its binding to the GPCR EP2 and/or EP4 receptors (see.(57) for review). The PGE1 response can be inhibited by over 50% and 90% following acute and chronic addition of bile acid, respectively (8). However, although PKC activation has been implicated in this bile acid inhibitory action, the PKC isoform(s) involved remain to be determined.

Therefore, the present study was designed to investigate the acute and chronic effect of different bile acids on specific PKC isoform activation and down-regulation in human dermal fibroblasts. Furthermore, the role of these PKC isoforms in the bile acid-mediated attenuation of PGE1-induced cAMP production was investigated. Finally, studies were also initiated to determine whether bile acids can stimulate PKCα trans- and/or autophosphorylation. The role of this phosphorylation in the activation of PKCα by bile acids was also investigated.
MATERIALS AND METHODS

Materials: Ursodeoxycholic acid (UDCA) was supplied by Tokyo Tanabe (Tokyo, Japan) and chenodeoxycholic acid (CDCA) was supplied by Dr. Falk Pharma GmbH (Freiburg, Germany). Taurocholic acid (TCA) was purchased from Steraloids (Wilton, NH). HeLa cells and cAMP antibody were a gift from Dr. A. Kumar (The George Washington Medical Center, Washington, DC) and Dr. T. Gettys, (Pennington Biomedical Research Center, (Baton Rouge, LA), respectively. Human recombinant PKC was purchased from Pan Vera LLC, (Madison, WI). PKCα-YFP and PKCδ-YFP plasmids were kindly provided by Dr. R. Kubitz (Heinrich-Heine University, Dusseldorf, Germany (31)), while the dominant negatives vectors, DN PKCα, DN PKCδ and DN PKCε were provided by Dr. Jae-Won Soh, (Inha University, Yonghyun-dong, Nam-gu Incheon, Korea (60)). Affinity purified polyclonal rabbit anti-PKCα antibody was from Gibco Life Technologies Inc. (Frederick, MD). Affinity purified polyclonal rabbit anti-PKCβ2, δ, ε, and ζ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-PKCα (Ser657) PKCδ (Thr505) and PKCδ (Tyr311) antibodies were from Upstate (Lake Placid, NY) and Cell Signaling (Beverly, MA), respectively. Rabbit HRP-labeled anti-mouse antibody was from Miles Scientific (Neperville, IL) and both rabbit anti-mouse and goat anti-rabbit Alexa fluor-labeled antibodies were from Molecular Probe. Hyperfilm and ECL detection kit were from Amersham (Arlington Heights, IL). Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (San Diego, CA). Monoclonal mouse anti-β-actin antibody, polyoxyethylene sorbitan monolaurate (Tween-20), Histone III-S, L-α-phosphatidyl-L-serine, and 1, 2-dioctanoyl-sn-glycerol (C8:0) were purchased from Sigma (St. Louis, MO). Trichloroacetic acid and Whatman G4 anion exchange filter paper were from Fisher (Pittsburgh, PA). Other chemicals were of the highest purity available.

Culture and incubation conditions for human dermal fibroblasts: Human dermal fibroblasts (GM03377C), obtained from forearm skin biopsy were purchased from the Coriell Institute for Medical Research (Camden, NJ). The fibroblasts (3-5 x 10⁴ cells/well in six-well plates) were cultured in DMEM supplemented with 1% L-glutamine, 2% essential and nonessential amino acids, 1% penicillin and streptomycin, and 10% fetal bovine serum (FBS). Except as otherwise indicated, the cells were incubated in the presence or absence of PMA (1µM), CDCA (100µM),
UDCA (100µM), and TCA (100µM) for a predetermined period of time. PGE1 was used at the concentrations of either 0.7 or 1 µM without significant differences in the cAMP production between those two concentrations.

For PKC expression level and activity determination, the incubation was stopped by washing the cells with ice cold buffer A (20mM Tris-HCl (pH 7.5) containing 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml leupeptin, and 0.1 mg/ml soybean trypsin inhibitor). Furthermore, except as otherwise mentioned, the fibroblasts were preincubated for 4h in a medium containing 0.1% FBS before the cellular cAMP level was determined by radioimmunoassay after stopping the reaction with 12% HClO₄. It is worthwhile to mention that all the results were expressed as either a ratio to β-actin or GAPDH or even per mg of protein to take into account any possible changes in cell proliferation or protein expression associated with the chronic bile acid treatment. Furthermore, the bile acids were used from a stock solution of 100 mM of the sodium salt and therefore, as otherwise indicated, PBS was used as control.

**Determination of PKC translocation by fluorescence microscopy:** Fibroblasts were seeded onto 35mm glass-bottom dishes (MatTek, Ashland MA) 24 hours after transfection with PKCα-YFP or PKCδ-YFP plasmids using the transfection kit for primary cells (AMAXA Biosystems, Gaithersburg, MD) according to manufacturer’s instructions. Transfection efficiency was always greater than 70% as measured by counting the ratio of the fluorescent cells to the total cell number, a similar protocol was used to transfec fibroblasts with the dominant negative PKCα, PKCδ, PKCe and PKCζ mutant, respectively. Before the experiments were performed, the cells were washed twice and the medium was replaced with serum-free and phenol red-free DMEM containing 10 mM HEPES. The cells were treated with 25-50 µM of either CDCA or UDCA and 100 nM PMA. During the course of the experiment, the culture dishes were kept on a heated microscope stage. Images were collected at 30 second to 2 min intervals using an Olympus IX-81 fluorescence microscope (60x objective). For tyrosine phosphorylated protein residues and Golgi apparatus detection and/or co-localization, the cells were seeded onto Poly-L-lysine coated coverslips in 35 mm dishes and starved for one hour before treatment with PMA or bile acid for 30-40 minutes at 37ºC. After treatment, the cells were washed with PBS, fixed in 3.7%
paraformaldehyde for 10 minutes and incubated in 2% BSA/PBS (blocking buffer) for 1 hour. Both the human anti-Golgin-97, and anti-phospho PKCδ primary antibodies were diluted in blocking buffer and incubated with the fixed cells overnight at 4°C, followed by a 45-minute incubation with an anti-rabbit-Alexa fluor 568 or with anti-mouse Alexa fluor 488 (Molecular Probes) secondary antibody. The cells were then washed and mounted on slides using Mowiol. The coverslips were processed and the data analyzed as described above. Both fluorescent and confocal microscopes were used to analyze the samples without any major difference in signal detection.

**Determination of PKC and β-actin expression by immunoblotting techniques:** Total cellular homogenates were prepared from cultured fibroblasts as previously described (10). Protein samples (20-30 µg) of the respective cellular fractions were separated by SDS-PAGE according to the method of Laemmli (32), using a mini gel apparatus (Bio-Rad, Richmond, CA) and transferred to nitrocellulose membranes using the Bio-Rad Trans-blot semi-dry transfer apparatus according to the manufacturer’s directions. The protein-containing nitrocellulose membranes were blocked in 10% BSA and further incubated overnight at 4°C with specific antibodies (anti-β-actin (1:3000) or anti-PKCα, PKCβ2, PKCδ, PKCe, and PKCζ (1:1000)). The nitrocellulose membranes were next incubated for 1 hour at room temperature in the respective secondary HRP-labeled antibody. To demonstrate the specificity of each antibody, comparable blots were processed under similar conditions and incubated overnight with the respective antibody, which had been pre-incubated with the specific peptide against which it was raised. Furthermore, hamster brain and liver tissues were also used as control. The immunoreactive proteins were visualized by enhanced chemiluminescence. After exposure to the nitrocellulose membrane, the Hyperfilm was analyzed by densitometric scanning using photoimaging (Molecular Dynamics, Sunnyvale, CA). While every attempt was made to normalize the conditions to compensate for the differences in antibody affinity from batch to batch, or titer from one blot to another, or for differences in gel protein loading, the immunoreactive signals were always normalized against that of β-actin.

**Determination of PKC and GAPDH mRNA expression by RT-PCR:** Total RNA was extracted from fibroblasts cultured in 6-well plates using RNA Bee™ (Tel-Test Inc., Friendswood, TX).
Oligo(dT) and Superscript III were used for transcription containing 2-4 ug of RNA. Reverse transcription was conducted at 50°C for 60 min following RNAse H treatment for 30 minutes. 5-10 ng of DNA was used for PCR reactions with Taq DNA polymerase. PCR reactions were conducted at 94°C for 3 min for denaturation, followed by 94°C for 45 sec, 55°C 30 sec, 72°C 40-60 sec, for either 27-30 cycles for GAPDH or 33-36 cycles for human PKCα and PKCδ followed by a final extension at 72°C for 6 min. The reaction products were analyzed by electrophoresis on 1.5% agarose gels. The gels were analyzed by densitometric scanning as described above. The following primers were used for hPKCα (#X52479; F: 5'-CTT CAG ACA AAG ACC GAC GAC-3' +725/+745; R: 5'-CAT GAC GAA GTA CAG CCG ATC-3', +1278/+1258 for 554bp), hPKCδ (#L07860; F: 5'-GTC ATC CAG ATT GTG CTA ATG CG-3', +260/+282; R-5' TCT TGT GGA TGG C AG CGT TCA-3'; +653/+635 for 394bp, and hGAPDH (#M33197; F: 5'-CCA TGA CAA C TT TGG TAT CGT GG-3' +555/+577; R: 5'-CAG GTC CAC CAC TGA CAC GTT-3' +798/+778 for 244bp).

**Determination of PKC phosphorylation and kinase activity:** Phosphorylation was determined by incubating human recombinant PKCα (0.1-1ng) in HEPES buffer (pH 7.4) containing 0.3% Triton-X100, phosphatidylserine (PS) and diolein and sonicated 30 sec at 4°C, as well as 100 μM \[^{32}P\]ATP. The PKCα was incubated at 30°C for 30 min in the presence of the indicated agents and the reaction was stopped by boiling for 3 min and the proteins were separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and exposed to a phosphor screen for up to 3 days and analyzed by densitometric scanning. PKCα detection by Western blotting was used to assess the respective loading of the wells after sufficient decay of the radioactive \(^{32}\)P. The results were expressed as the ratio of phospholabeled PKCα to the immunoblotted PKCα.

Total kinase activity in cell homogenates and recombinant PKC activity were assayed using mixed micelles prepared by sonication of phosphatidylserine (PS) and diolein in Tris-HCl (pH 7.5) solution containing Triton X-100 (0.1%). Activity was determined by measuring the rate of \[^{32}\)P\] phosphate incorporation from \[^{32}\)P\]-ATP into Histone III-S for the indicated period of time at 30°C as previously described (13). Briefly, the reaction was started by adding 25-50 μg of protein sample or 0.1 ng of recombinant PKC to a final reaction mixture containing Histone III-S (0.2 μg/μl), Diolein
(1 ng/µl), PS (50 ng/µl), Triton (0.02%), and CaCl₂ (400 µM) or EGTA (5mM). Diolein was omitted when indicated. The incubations were terminated by precipitating Histone III-S with trichloroacetic acid (25%). The precipitate was then spotted onto Whatman paper and washed four times with trichloroacetic acid and the radioactivity determined in a LS 3801 β-counter (Beckman, Palo Alto, CA).

**Cyclic AMP determination:** Cells were incubated alone, with the respective bile acid and/or with PGE1 for the designated period of time. Cellular cAMP production was measured in HClO₄ extracts, by radioimmunoassay as previously described (8; 10) using the method of Gettys, et al. (20). The results were expressed as % of the maximum obtained by incubating the cells with PGE1 alone.

**Statistical analyses:** Except as otherwise indicated, the results were expressed as mean ± SE. The statistical significance was determined by either Student’s t-test or ANOVA when more than two groups were compared.
RESULTS

Study of PKC translocation following acute exposure of human dermal fibroblasts to either bile acids or PMA.

In transfected fibroblasts and under control conditions, PKCα was distributed evenly in the cytoplasm. After treatment with PMA, PKCα rapidly translocated to the plasma membrane with a maximum translocation in 10-15 min (Fig. 1A). This was confirmed by measuring the disappearance of PKCα from the cytosol (Fig. 1B). This disappearance was selected rather than the membrane accumulation because we noticed, as have many other authors, that the cell membrane conformation changes over time, thus making accurate quantification impossible. Similarly, albeit slower, PKCα translocation was observed in response to treatment with the bile acids CDCA and UDCA at concentrations as low as 25 µM (Fig. 1A). The disappearance of PKCα was quicker upon UDCA versus CDCA stimulation (Fig. 1B). However, ~40 minutes incubation with either bile acid is required to induce a maximum PKCα translocation to the plasma membrane and disappearance from the cytosol (Fig. 1A and 1B).

The association between PKCα membrane translocation and activation was further evidenced by measuring phosphorylation of PKCα by CDCA over time (Fig. 1C and 1D). The results indicated a 1.2 to 1.9 fold increase in phospho-Ser657 PKCα induced by CDCA over the 150 min tested without reaching a plateau. It is worthwhile to mention that, under similar conditions and as reported above, the translocation of PKCα to the plasma membrane was maximum in 25-60 min (Fig. 1A and 1B vs 1C and 1D). This could suggest that PKCα translocation to the plasma membrane precedes its phosphorylation or that PKCα translocates to other organelles beside the plasma membrane.

The cellular trafficking of PKCδ upon PMA and bile acid stimulation was also investigated. Under basal conditions using YFP-PKCδ transfected fibroblasts, PKCδ was present both in the cytoplasm and in the perinuclear region, presumably Golgi (Fig. 2A). Upon stimulation with PMA, PKCδ translocated out of the perinuclear region for the first 5-7 min and then accumulated mainly in the perinuclear region with a maximum accumulation in around 30 min (Fig. 2A and 2B). Upon stimulation with either CDCA or UDCA, PKCδ progressively
accumulated in the perinuclear region to a maximum of around 2 fold in 25-30 min (Fig. 2A and 2B). However, none of the bile acids tested induced an initial loss in perinuclear PKCδ (Fig. 2B) and while the stimulatory effect of CDCA was rapid and required less than 5 min to reach a noticeable increase, that of UDCA was delayed by 15-20 min (Fig. 2B). Furthermore, since PKCδ can be phosphorylated at Thr505 and Tyr311 and that this phosphorylation affects both the activity and the localization of the kinase, these parameters were studied by confocal microscopy using the endogenous PKCδ. A specific anti-Golgin-97 antibody was used for the Golgi co-localization studies. Under basal condition, there was a small amount of PKCδ already phosphorylated on either of these sites and under these conditions, the phospho-PKCδ was located in the nucleus and cytosol. However, upon stimulation with CDCA and UDCA, the phosphorylated PKCδ Thr505 and Tyr311 (Fig. 2D) accumulated almost exclusively in the nucleus with little or no co-localization with the Golgi (data not shown). Nuclear PKCδ Tyr311 accumulation is increased by around 2 fold by PMA and by around 1.6-1.8 fold by CDCA and UDCA, respectively (Fig. 2E). The accumulation of at least phospho PKCδ Thr505 in the nucleus upon stimulation has also been recently reported by Weidlocha et al., to occur in 3T3 fibroblasts (66). However, it is not clear from this study whether the bile acids stimulate the translocation to the nucleus of phosphorylated PKCδ and/or stimulate the phosphorylation of PKCδ in the nucleus.

Study of PKC isoform down-regulation following chronic exposure of human dermal fibroblasts to either bile acids or PMA.

The major PKC isoforms detected in fibroblasts were PKCα, PKCδ, PKCe and PKCζ (Fig. 3A). Although the protein expression of both PKCβ1 and PKCβ2 was investigated, these isoforms were not detectable in dermal fibroblasts, which is in keeping with what was previously reported by Racchi et al (50).

Among the members of the Ca^{2+}-dependent PKC subfamily, PKCα was detected as an ~80 kDa protein. Incubation of the cells with either 1 µM PMA, 100 µM UDCA, or 100 µM CDCA, but not 100 µM TCA (data not shown) for 24h resulted in the down-regulation of the total expression of PKCα by 99 ± 1%, 37 ± 4%, and 45 ± 4% of control respectively (n=4; p<0.05;
Fig. 3A and 3B). Similar results were observed when UDCA and CDCA were used at a concentration of 50 \( \mu \text{M} \) (data not shown). As far as the novel PKC subfamily was concerned, PKC\( \delta \) was identified as a doublet of 85/80 kDa, which was validated using a PKC\( \delta \) peptide against which the antibody was raised. PMA, UDCA and CDCA down-regulated the total expression of PKC\( \delta \) by 90 \( \pm \) 5\%, 48 \( \pm \) 8\%, and 34 \( \pm \) 7\% respectively (\( n=3; \ p<0.05; \) Fig. 3A and 3B). PKC\( \varepsilon \) was the other novel PKC isoform detected in fibroblasts. This isoform has a molecular weight of 92 kDa, and its total expression was decreased by 95\% \( \pm \) 8\%, 30 \( \pm \) 12\%, and 28 \( \pm \) 7\% following incubation of the cells with PMA, UDCA and CDCA, respectively, for 24 hours (\( n=3; \ p<0.05; \) Fig 3A and 3B). Furthermore, the atypical PKC\( \zeta \) was detected as an ~82 kDa protein and was not significantly down-regulated after 24h incubation of the cells with either PMA, UDCA, or CDCA (\( n=3; \) Fig. 3A and 3B).

Since under pathological conditions, tissues could be exposed to bile acids for a prolonged period of time, we compared the effect of 1\( \mu \text{M} \) PMA and 100 \( \mu \text{M} \) UDCA or CDCA on PKC\( \alpha \) and PKC\( \delta \) total expression following incubation of the cells with these respective agents for 24h and 48h (Fig. 3C and Fig.3D). While the effect of both PMA and UDCA on PKC\( \alpha \) protein expression remained significant, that of CDCA was transient and completely disappeared after 48h incubation (Fig. 3C). Under these conditions, the PKC\( \delta \) down-regulation by the different agents tested was transient and was not significantly different from control after 48h incubation with UDCA and CDCA (Fig. 3D).

One possible mechanism responsible for the alteration of PKC protein expression over time could be due to either alteration of PKC\( \alpha \) and PKC\( \delta \) protein synthesis and/or degradation. Therefore, to address the possible change in protein synthesis, the mRNA expression level of both PKC\( \alpha \) (Fig. 4A and 4B) and PKC\( \delta \) (Fig. 4A and 4C) was studied over time. Little effect on PKC\( \alpha \) was observed by any of the agents tested, while a PMA-dependent increase in PKC\( \delta \) mRNA by over 2 fold was observed at 8h (Fig. 4A, 4B and 4C). Furthermore, PMA stimulated both PKC\( \alpha \) and PKC\( \delta \) mRNA synthesis by 2 to 2.5 fold up to 48h. CDCA significantly increased PKC\( \alpha \) mRNA level by 2.5 fold only after 48h but stimulated PKC\( \delta \) mRNA level by 80-90\% after 24h and 48h. UDCA did not significantly affect PKC\( \alpha \) mRNA expression level at
any time point tested but significantly increased PKCδ mRNA level by ~25% at 24h (Fig. 4B and 4C).

**Determination of total kinase activity in cultured human dermal fibroblasts following chronic exposure of the cells to either bile acids or PMA.**

Next, we measured total kinase activity as an indirect assessment of the down-regulation of PKC expression level by either bile acids or PMA. The phosphorylation of histone IIIS in cell homogenate in the presence of DAG and phosphatidylserine and 24h after incubation of the fibroblasts with the different bile acids or PMA was investigated. As reported in Figure 5, 24h incubation of the cells with TCA did not affect the total kinase activity. However, the total kinase activity decreased from 46 ± 2 to 0.5 ± 0.5 pmol/mg protein/min with PMA, and to 31 ± 2 pmol/mg protein/min, and 27 ± 8 pmol/mg protein/min, after UDCA and CDCA exposure, respectively. As compared to control, the reduction in the total kinase activity following 24h incubation of the cells with PMA and bile acids were approx. 99%, and 32% (n=3; p<0.05; Fig. 5), respectively.

**Role of PKC down-regulation and inhibition in the bile acid-induced attenuation of cAMP production.**

We have previously reported that CDCA was the most potent of the bile acids tested to attenuate stimulated cAMP production in dermal fibroblasts with a maximum inhibitory effect observed with 25-50 µM (8). Therefore, we preincubated the cells with 1µM PMA for 24h to study the effect of PKC down-regulation in CDCA-induced inhibition of stimulated cAMP production. Down-regulation of the PKCs by PMA did not significantly affect the basal cellular cAMP level (Fig. 6A). Furthermore, CDCA did not significantly alter the basal cAMP level, as previously reported (8). However, the respective significant 20% and 45% inhibitory effect observed after 2h incubation with 10 and 25 µM CDCA on 0.7 µM PGE1-induced cAMP production was completely abolished after 24h PMA treatment (Fig. 6A). These results suggest, therefore, that the PKC(s) involved in the regulation of cAMP production by bile acids are PMA-sensitive.
To further determine the PKC isoform(s) involved in the bile acid inhibitory effect we tested the general PKC inhibitor calphostin C (1 µM). Preincubation of the cells with this inhibitor for 60 min completely abrogated the inhibitory effect of 25 µM CDCA and significantly reduced that of 50 µM CDCA (Fig. 6B). Similar results were observed with 10 µM of the PKCα/PKCβ-specific inhibitor, Gö6976 (Table 1). The lack of complete inhibition of the PKC response with 50 µM CDCA by calphostin C may be due to a partial UV-induced activation of this inhibitor. Therefore, the results may represent an underestimation of the action of calphostin C.

**Comparative effect of various PKC isoform dominant-negative mutants on CDCA-induced decreased cAMP production.**

To determine the role of the different PKC isoforms in the regulation of cAMP production by CDCA, the cells were transfected with the respective PKCα, PKCδ, PKCζ and PKCε dominant negative mutant and the regulation of PGE1-induced cAMP production by 50 µM CDCA was studied (Fig. 7). Under these conditions, CDCA alone had no effect on the basal cAMP production level. However, the inhibitory effect of CDCA was completely abolished only when the cells were transfected with PKCα DN mutant (Fig. 7). The inhibitory effect of CDCA was enhanced to the same extent and by around 3 fold when the cells were transfected with PKCδ and PKCζ DN mutants. Transfection of the cells with PKCε DN mutant had no effect on the regulation of cAMP production by CDCA (Fig. 7).

**Comparative effect of various protein kinase inhibitors on CDCA-induced decreased cAMP production.**

Different protein kinases, including MAP kinase and PI3 kinase have also been reported to be regulated by bile acids (55; 65). Therefore, the role of these kinases in the CDCA-induced inhibition of stimulated cAMP production was studied. However, none of the inhibitors of other protein kinases, including P38 MAP kinase (SB202190), ME Kinase (UO126), P42/44 MAP kinase (PD98059), PI3 kinase (LY294002) and cAMP-dependent protein kinase (H89) affected CDCA-induced activation of cAMP production (Table 1). This suggests that under the conditions studied, there is a direct PKC-specific effect on the cAMP synthesis pathway modulated by bile acid.
**Potential mechanism(s) of activation of PKCα by bile acids.**

Based on the results from the above studies, we next focused our work on the ability of bile acid to directly regulate PKCα activity. Previous work by Newton’s laboratory reported that several PKCs, including PKCα, present at least three phosphorylation sites in the carboxyl terminus of the enzyme and that phosphorylation of these sites is required to induce PKC maturation and full activation (42). Furthermore, results described in Fig 1A and 1B, suggested that bile acids stimulate PKCα phosphorylation at Ser657. Therefore, experiments were designed to study PKCα cross- and/or autophosphorylation in the presence of CDCA and PMA.

Recombinant PKCα was incubated with increasing concentrations (2.5-250 µM) of CDCA in the presence and absence of phosphatidylserine (PS) and phosphorylation was determined using $[^{32}\text{P}]$-ATP. The PKC protein level was determined by Western blotting once the radioactivity had significantly decayed. The results reported in Fig. 8A and 8B, indicate that CDCA, at concentrations up to 250 µM stimulated PKCα phosphorylation only when incubated in the presence of PS. Within the concentrations tested, the effect of CDCA was dose-dependent (Fig. 8A and 8B). The bile acid effect was significant at a concentration as low as 10 µM, while 250 µM CDCA stimulated PKCα phosphorylation by up to 3 fold and to the same or greater extent as that induced by 1µM PMA (Fig. 8A and 8B). Finally, preincubation of PKC with 1 µM Gö6976 resulted in the abrogation of both the PMA- and bile acid-induced PKCα phosphorylation (Fig. 8C), thus supporting the specificity of the effect. These results suggest that CDCA can stimulate the cross and/or autophosphorylation of at least the PKCα isoform.

However, this increase in phosphorylation did not result in a direct or significant increase in PKCα activity by CDCA as determined *in vitro* using Histone-IIIS as a substrate (Fig. 9). Indeed, CDCA concentrations up to 250 µM did not significantly increase the phosphorylation of Histone-IIIS by PKCα when tested alone or under the conditions leading to PKCα phosphorylation. In these experiments, the period of incubation of PKCα with PMA and/or
CDCA was reduced from 30 to 10 min to decrease the background and therefore, increase the sensitivity of the assay. However, the activation of PKCα by PMA was linear during the period tested from 5 to at least 30 min (data not shown). Furthermore, PMA stimulated PKCα activation in a dose-dependent manner with a maximum effect of a 6-7 fold increase in activity observed with 1μM. Furthermore, CDCA potentiated 10 nM PMA-induced Histone-IIIS phosphorylation in a dose-dependent manner with a maximum increased activity of approx. 60% observed at concentrations ranging from 5-25 μM (Fig. 9). However, greater CDCA concentrations resulted in a dose-dependent reduction in the PMA-induced PKCα activation.
DISCUSSION

Results from the present study clearly underline a role for PKC, rather than either PKA, PI3K or MAPK, in the bile acid-induced inhibition of stimulated cAMP production in human dermal fibroblasts. PKCs are a multifunctional protein kinase family whose members have been implicated in different cellular functions, including glucose metabolism (13), cellular contraction (24), proliferation and differentiation ((26), see (18) for review). One additional action of PKC is to modulate the glucagon-associated responses by bile acids (10). The direct PKC involvement in the bile acid-induced modulation of cAMP production is clearly shown in the present study. Indeed, the almost complete cellular depletion of PKCα and the partial depletion of PKCδ and PKCε protein expression following 24h pretreatment of the cells with PMA, at least partially abolished the inhibitory effect of both PMA and CDCA on cAMP production induced by PGE1. Furthermore, the present data rule out any possible direct destabilization effect of the bile acids on the GPCR, as previously suggested by Jones and Garrison (30). Indeed, while a destabilizing effect on the interaction of the β and γ subunits of various G proteins was detected with 0.5% (~12 mM) of cholic acid, no effect was observed at concentrations below 0.1% (~2.5 mM) (30). Therefore, the concentrations used in the present study in the 20-100 µM range are 25-100 fold lower that those reported to induce any destabilizing effects.

The present study demonstrates that bile acids, at concentrations reachable in the systemic circulation, at least under cholestatic conditions, modulate specific PKC isoform expression and activity in cells of non-hepatic origin. In dermal fibroblasts, CDCA and UDCA significantly stimulated PKCα and PKCδ translocation and phosphorylation while long-term bile acid stimulation resulted in partial down-regulation of these PKCs, as well as of PKCε total protein expression. Two different hypotheses can be proposed to explain the increased accumulation of phosphor PKCδ in the nucleus. This could be the result of either the bile acid stimulating the translocation of the cytosolic and already phosphorylated PKCδ to the nucleus and nuclear membrane or the bile acids that have been reported to be detectable in the nucleus stimulate the phosphorylation of the PKCδ present in the nucleus. However, since PKCδ can be activated in the absence of the phosphorylation, the increased phosphorylation on Thr505 and Tyr311 could
be important for the stability of the PKC isoform or in the localization., i.e., nucleus vs Golgi (see (61) for review).

The observed increased PKC mRNA levels could be the result of a feedback loop due to the increased bile acid-induced PKC down-regulation at least as far as PMA and CDCA effects are concerned. Moreover, under these conditions, PKCζ expression was not affected by either bile acids or PMA, as previously reported by others with PMA (5; 52). Borner et al. have nevertheless reported that 12-O-tetradecanoyl phorbol 13-acetate activated and subsequently down-regulated this PKCζ isoform in R6 rat embryo fibroblasts (7) and this was also observed by us in human embryonic kidney cells (data not shown). This latter finding was unexpected because PKCζ lacks the binding site for DAG and PMA (28; 49) and would suggest that in these models, the PKCζ down-regulation is indirect and involves mechanisms which may be either species and/or cell-specific.

The modulation of PKC was bile acid-and PKC-isoform specific. Both CDCA and UDCA stimulated PKCα, PKCδ and PKCε to a similar extent as demonstrated by either or both translocation and down-regulation. However, although the differential mechanism is not clear, the effect of CDCA on PKCα was more transient than that of either UDCA or PMA and disappeared almost completely by 48h. Interestingly, the down-regulation of PKCδ by the different agents tested, including PMA, UDCA and CDCA was short lived since it was not significantly different from control after 48h incubation and with little correlation between protein and mRNA expression levels. The activation of PKCs, and of PKCα in particular, by TCA remains controversial. While TCA has been shown to activate PKCα in primary cultured hepatocytes (63), this was not observed in isolated hepatocytes (5). This was also not observed in cultured fibroblasts in the present study, as indicated by an absence of degradation of this isoform, as well as by the absence of any change in PKC activity after 24h incubation with TCA. While the present findings are supported by previous results (5), and since the bile acid has to cross the plasma membrane to be effective (15), it remains to be clarified whether the lack of effect of TCA in the present study is due, at least in part, to the absence of a bile acid transporter, preventing TCA from accessing the specific PKCs.
Previous studies from this laboratory have implicated the classic PKC isoforms (α, and β₂) in the modulation of glucagon-induced cAMP synthesis by bile acids (10). Furthermore, the inhibition of stimulated cAMP production by both bile acids and PMA in fibroblasts (8), as well as the inhibition of the bile acid inhibitory effect by staurosporine in both hepatocytes and fibroblasts (8; 10) suggests a similar regulatory mechanism in this model as that reported in hepatocytes. Taken together, and since fibroblasts are devoid of PKCβ ((50), and present study), these results would support a solely PKCα-dependent mechanism. Furthermore, the indolocarbazole Gö6976 derived from staurosporine has been shown to be a potent PKC inhibitor competing with ATP for its binding sites (38; 47). A report by Martiny-Baron et al. showed that Gö6976 displays greater selectivity for the classic PKCα and PKCβ1 over the novel PKCδ and PKCɛ (38). The IC₅₀ for inhibition of PKCα was in the nM range and was around 3 fold less than that necessary for PKCβ1 inhibition and required several hundred µM to inhibit the novel PKCs. (38). Therefore, the fact that Gö6976 significantly reduced or abolished the bile acid effect on cAMP production further supports the predominant involvement of PKCα as the major mediator of the bile acid action on the regulation of cAMP production. Furthermore, a primary role for PKCα is further supported by experiments using the respective PKC isoform DN mutants. Under these conditions, inhibition of PKCα abolished the CDCA-induced inhibition of cAMP production. It is also worthwhile to mention the possible opposing effects of PKCα vs PKCδ and PKCζ. Indeed, the inhibitory effect of CDCA was enhanced when either of the two latter PKC isoforms were inhibited. Together these results strongly support a key role for PKCα in the regulation of cAMP production by bile acids.

A large body of work has focused on the regulation of PKC activity by bile acids. Bile acids have been considered as non-PMA-type PKC activators, since they do not bind to the PKC DAG/PMA binding sites (27). However, the mechanism of regulation of PKC activation by bile acids is not clear. Increased DAG synthesis has been proposed as a possible mechanism for bile acid-induced PKC activation (5; 52). It has also been proposed that the activation of PKC by bile acids is rather due to the bile acids mimicking the effect of phosphatidylserine and acting as a PKC cofactor (19; 27; 64). Recently, another study has proposed that, in addition to stimulating
DAG synthesis, bile acids could stabilize DAG in the plasma membrane (52). Finally, bile acids could activate cPKCs through their effects on cellular PLC activation (33) and calcium mobilization (2; 9; 16). The present study suggests that bile acids stimulate PKC trans- or autophosphorylation, possibly increasing sensitization of PKC for activation and deactivation.

The requirement for post-translational phosphorylation of PKC as a necessary step to produce an activatable kinase is generally accepted (see (43; 61) for review). Phosphorylation of the activation loop at least at Thr497 but also at Thr495 is necessary for activation of PKCα, while mutation of Thr497 with a neutral amino acid results in an unactivatable kinase (14). Conversely, mutation of PKCα with a glutamic acid residue in position 495 constitutively activated this kinase (14). Furthermore, autophosphorylation of the turn (Thr638) and hydrophobic (Ser657) motifs play a role in controlling the maturation, localization and stability of PKCα (40). In addition, Stensman et al demonstrated that the autophosphorylation of the turn and hydrophobic motifs helped in controlling the intrinsic conformation of the kinase and, through this process, modulated the sensitivity of PKCα for DAG (62). This latter observation could explain, at least in part, the results in the present study that the increased phosphorylation of PKCα by CDCA, while not leading to a direct activation of this kinase increases the affinity and, in turn, the activation of PKCα by PMA. Sando et al have observed a correlation between autophosphorylation and activation of PKC with a lipid-dependent normally distributed activation pattern (54). These authors proposed that the decreased activity observed at high lipid concentrations was due to the dilution of PKC dimers or higher aggregates with greater kinase activity. However, in contrast to the present study, the decrease in PKC activity was associated with a decreased autophosphorylation (54).

The results that specific unconjugated and conjugated bile acids can profoundly alter dermal fibroblasts’ cellular signaling mechanisms have major physiological and pathophysiological significance. There is considerable evidence in man and in animal models, that cholestasis is associated with increased deposition of bile acids in the skin (25; 44). Furthermore, we have recently shown, in 2 hamster models of hepatic failure, namely bile duct-ligation, and functional hepatectomy, that bile acids were targeted to several tissues outside of the enterohepatic
circulation, most notably, the skin (15). Thus, one could imagine that in the event of hepatobiliary disorders, which would result in cholestasis, similar effects may be observed in vivo, as those observed in situ, in the present study. Furthermore, under conditions of impaired liver function and decreased bile secretion, such as those found in patients with portal cirrhosis (37), and in infants with extrahepatic biliary atresia and neonatal hepatitis (29), serum concentrations of unconjugated CDCA could reach a level greater than 20 µM, shown in the present study to significantly reduce the PGE1 signaling response. High serum concentrations of bile acids could affect signaling mechanism(s) in numerous other tissues and cells outside the enterohepatic circulation. Indeed, in several other studies, bile acids have been suggested to be responsible for the attenuation of the β-adrenergic-induced cAMP production in cardiomyocytes during cirrhosis (35; 36). PKCα has been reported to stimulate BCL2 and the inactivation of PKCα resulted in BCL2 degradation and an increased apoptosis (53). Furthermore, cAMP stimulation has been reported to affect both cell proliferation and cyclooxygenase 2 protein expression in dermal fibroblasts. Preliminary data from our laboratory suggest that bile acids and CDCA in particular can modulate the cAMP-dependent regulation of cell proliferation. In addition, CDCA can modulate COX-2 expression in a PKC-dependent manner (Data not shown). Therefore, not only activation but also down regulation of specific PKC isoforms could mediate, at least in part, the toxic effect associated to chronic bile acid stimulation.

In conclusion, this study is the first to suggest that while dihydroxy bile acids activate different PKC isoforms, PKCα is at least in part, involved in the bile acid-induced inhibition of cAMP synthesis in cells of non-hepatic origin. The activation of this kinase by bile acids is accompanied by an increased trans- and/or autophosphorylation. In keeping with the significant roles of cAMP and PKC in the regulation of vital cellular functions, this study emphasizes potential consequences of increased systemic bile acid concentrations, and cellular bile acid accumulation, not only in the liver but also in extrahepatic tissues, in cholestatic liver diseases.
ACKNOWLEDGMENTS

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REFERENCES


Table 1: Comparative effect of various protein kinase inhibitors. Cells were pre-incubated at 37°C with PBS (Control), Gö6976 (PKC inhibitor), SB202190 (P38 MAP kinase inhibitor), LY294002 (PI3 kinase inhibitor), UO126 (MEK inhibitor), PD98059 (P42/44 MAP kinase inhibitor) and H89 (cAMP-dependent protein kinase inhibitor). After 30 min to 1h, PBS (vehicle) or either 25 or 50 μM CDCA was added for 2h before the cellular cAMP level was determined. In the presence of PGE1. The results were expressed as % of control determined in the sole presence of PGE1. The cAMP production induced by PGE1- was not significantly affected by any of the agents tested. In the period of up to 20 min tested. ND, not determined; *, Significantly different vs. PBS alone, P<0.05; **, Significantly different vs respective control, P<0.05.

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<th>CDCA 25 μM</th>
<th>CDCA 50 μM</th>
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<tr>
<td><strong>PGE1 (0.7 μM)</strong> +</td>
<td>106 ± 5**</td>
<td>83 ± 10**</td>
</tr>
<tr>
<td>Control</td>
<td>69 ± 7*</td>
<td>44 ± 6*</td>
</tr>
<tr>
<td>Gö6976 10 μM</td>
<td>106 ± 5**</td>
<td>83 ± 10**</td>
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<tr>
<td>SB202190 25 μM</td>
<td>60 ± 7</td>
<td>45 ± 4</td>
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<td>LY294002 20 μM</td>
<td>58 ± 4</td>
<td>46 ± 2</td>
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<tr>
<td>UO126 20 μM</td>
<td>67 ± 5</td>
<td>41 ± 4</td>
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<tr>
<td>PD98059 25 μM</td>
<td>60 ± 5</td>
<td>42 ± 8</td>
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<td>H89 1 μM</td>
<td>ND</td>
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FIGURE LEGENDS

Figure 1: PKCα translocation and phosphorylation following acute exposure to either bile acids or PMA: A) Cultured human dermal fibroblasts seeded onto glass bottom dishes were transiently transfected with PKCα-YFP using the transfection kit for primary cells (AMAXA Biosystems, Gaithersburg, MD) according to manufacturer’s instructions. 24-48 hours after transfection, the cells were serum starved for one hour, placed on a heated stage and treated with 100 nM PMA, 50 µM CDCA or 50 µM UDCA. Images were collected every 0.5 - 2 minutes using a fluorescence microscope (60x objective). Data shown is representative of three independent experiments. B) The disappearance of PKCα from the cytosol was measured over time using Slidebook Digital Microscopy Software, C) Fibroblasts were stimulated with 100 µM CDCA for up to 150 min. Total cellular proteins (10-20mg/ml) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with specific antibodies against phosphor Ser657-PKCα, and β-actin followed by HRP-labeled secondary antibody. D) The results were quantified by densitometric scanning using photoimaging and normalized against the respective β-actin optical density signals. The results are expressed as % of control and are the mean +/- SE of 3 different experiments.

*, Significantly different from respective control, p<0.05.

Results for Fig 1B were analyzed by two way ANOVA and Bonferonni post test.
PMA, Significantly different from control after 5 min, p<0.001
CDCA, Significantly different from control after 33 min, p<0.001
UDCA, Significantly different from control after 16 min, p<0.001

Figure 2: PKCδ translocation and phosphorylation following acute exposure to either bile acids or PMA: A) Cultured human dermal fibroblasts seeded onto glass bottom dishes were transiently transfected with PKCδ-YFP and processed as described in the legend of Fig. 1. Data shown is representative of three independent experiments. B) The PKCδ localization to the Golgi was measured over time. C) Fibroblasts transfected with PKCδ-YFP were seeded onto poly-L-lysine coated coverslips. 24-48 hours after transfection, the cells were serum-starved for one hour and treated with 100 nM PMA, 50 µM CDCA or 50 µM UDCA for 30 minutes at 37ºC. The cells were fixed with 3.7% paraformaldehyde and incubated with human anti-Golgin-97
antibodies overnight, followed by incubation with Alexa Fluor 350 secondary antibody. The images collected using a confocal microscope are representative of at least three independent experiments. D) Fibroblasts were incubated as described above and stimulated with PMA, CDCA or UDCA for 30 min. The localization of the phosphor PKCδ was studied using either anti-PhosphoPKCδ Thr505 or anti-PhosphoPKCδ Tyr311. E) The results were analyzed and quantified using the Slidebook Digital Microscopy software.

Results for Fig 2B were analyzed by two-way ANOVA and Bonferroni Post tests.

- PMA, Significantly different from control between 2-18 min, p<0.001
- CDCA, Significantly different from control after 4 min, p<0.001
- UDCA, Significantly different from control after 24 min, p<0.001

Data in Figure 2E are representative of several regions of several cells from a single experiment for PKCδ Thr 505 and PKCδ Tyr311, respectively.

Figure 3: Total PKC expression following 24h and 48h exposure to either bile acids or PMA: A) Cultured human dermal fibroblasts were incubated for 24 h in the absence (CTL) and presence of 1µM PMA, 50 µM UDCA or 50 µM CDCA. Total cellular proteins (10-20µg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with specific polyclonal antibodies against PKCα, PKCδ, PKCε, PKζ, as well as β-actin, followed by HRP-labeled secondary antibody. B) The results were quantified by densitometric scanning using photoimaging and normalized against the respective β-actin optical density signals. The results are expressed as % of control and are the mean +/- SE of a least 3 experiments performed in duplicate. C) Cultured human dermal fibroblasts were incubated for 24h and 48h, respectively, in the presence of 1µM phorbol 12-myristate 13-acetate (PMA), 100 µM ursodeoxycholic acid (UDCA), or 100 µM chenodeoxycholic acid (CDCA). The total cellular proteins were analyzed as described above for PKCα (C) and PKCδ (D). The results are the mean +/- SE of at least 3 experiments performed in duplicate.

*, Significantly different from respective control, p<0.05.

Figure 4: Total PKCα and PKCδ mRNA in human dermal fibroblasts exposed to either bile acids or PMA: A) The cells were incubated for up to 48h in the presence of 1µM phorbol
12-myristate 13-acetate (PMA), 100 μM ursodeoxycholic acid (UDCA), or 100 μM chenodeoxycholic acid (CDCA). PKCα and PKCδ mRNA levels were determined by RT-PCR according to the method described under Materials and Methods at 8h, 24h and 48h. The results for PKCα (B) and PKCδ (C) were analyzed as described above and normalized to GAPDH mRNA expression and expressed as a % of control. The results are the mean +/- SE of 5 experiments. *, Significantly different from respective control, p<0.05.

**Figure 5: Total kinase activity in cultured human dermal fibroblasts following 24h exposure of the cells to either bile acids or PMA:** The cells were incubated as described in the legend of Fig. 2 in the presence of either 1μM PMA, 100 μM UDCA or 100 μM CDCA for 24h and the cells were kept frozen at -80°C until the activity was determined. The cells were lysed on ice using a Sonicator W380 (Heat Systems Ultrasonics Inc, Farmingdale, NY). The reaction was started by adding 25-50 μg of protein sample to a final reaction mixture containing either 30 mM Tris-HCl (pH 7.5), Histone III-S (0.2 μg/μl), Diolein (1 ng/μl), Phosphatidylserine (50 ng/μl), Triton (0.02%), [γ³²P]-ATP (~50,000dpm/tube) and 400 μM CaCl₂. The incubations were terminated by precipitating Histone III-S with a solution of trichloroacetic acid (0.25%) onto a Whatman filter paper and the radioactivity determined by liquid scintillation spectrometry in an LS 3801 β-counter (Beckman, Palo Alto, CA). The results are the mean +/- SE of 3 experiments performed in duplicate.

*, Significantly different from control, p<0.05.

**Figure 6: Effect of PKC down-regulation and inhibition on the bile acid-induced reduction of stimulated cAMP production:** A) Cultured human dermal fibroblasts were incubated for 24h in the presence of either 1 μM PMA or PBS used to dissolve PMA. The cells were then incubated for 2h either in the absence (CTL) or presence of increasing concentrations (10 and 25 μM) of chenodeoxycholic acid (CDCA) before the cellular cAMP production was determined 8 min after the addition of 0.7 μM PGE1. The total cellular cAMP level was determined by radioimmunoassay. Results are expressed as % of the control cAMP level (approx. 2200 pmol/mg protein), corrected for the basal level (~28 pmol/mg protein), determined in the presence of PGE1, and in the absence of bile acids and PKC inhibitor. Results are the means +
SE of 3 determinations. **B** The cells were preincubated at 37°C for 1h with 100 nM Calphostin C followed by the addition of increasing concentrations (25 and 50 µM) of chenodeoxycholic acid (CDCA) for 2h. The total cellular cAMP level was determined by radioimmunoassay. Results are expressed as % of the control cAMP level determined in the presence of PGE1, in the absence of bile acid and PKC inhibitor, and corrected for the basal level. Results are the means + SE of 3 determinations.

*, Significantly different vs PBS alone, P<0.05, **, Significantly different vs respective control, P<0.05, ***, Significantly different vs PBS condition, P<0.01

**Figure 7: Effect of PKC isoform dominant negative mutants on the cAMP production by CDCA:** Fibroblasts (60-80% confluency) were transfected with 1-2 µg of the respective PKC (α, δ, ε, ζ) DN mutant or GFP as described in Legend of Fig. 1. After 18-24h, the cells were incubated in serum-free medium and with and without 1 µM PGE1 and in the presence and absence of 50 µM CDCA. The total cellular cAMP level was determined by radioimmunoassay. Results are expressed as % of the respective basal value determined in the absence of any agent. *, Significantly different vs respective control determined in the sole presence of PGE1.

**Figure 8: Comparative dose-dependent effect of chenodeoxycholic acid on PKCα phosphorylation:** **A** PKCα (0.1-1 ng) was incubated *In vitro* with 100 µM [γ32P]-ATP and without (control, CTL), 1 µM PMA or with increasing concentrations (2.5-250 µM) of chenodeoxycholic acid (CDC) in the absence and presence of 5 µg PS. The preparation was incubated at 30°C for 30 min, the reaction stopped by boiling in SDS, the proteins separated by SDS-PAGE, transferred to nitrocellulose membranes and exposed to a phosphor-screen. **B** The level of phosphorylation (32P) was determined using a STORM phosphor-imager (Molecular Dynamics) and was normalized to total PKCα protein concentration (PKCα) and analyzed by densitometric scanning (ImageQuant software, Molecular Dynamics). The results are the mean±SE of 3-4 determinations and were expressed as % of respective control. **C** PKCα (0.1-1 ng) was preincubated for 5-10 min with 1µM Gö6976 and further incubated without (control, CTL) or with either 1 µM PMA or 250 µM chenodeoxycholic acid (CDCA) and in the presence of PS. The preparation was incubated at 30°C for 30 min, the reaction stopped by boiling in SDS, the
proteins separated by SDS-PAGE, transferred to nitrocellulose membranes and exposed to a phosphorscreen. The level of phosphorylation was determined and analyzed as described above. The results are the mean±SE of at least 3 determinations.

Abbreviations: PS, L-α-phosphatidyl-L-serine.

*, Significantly different from CDC 2.5 µM, p<0.05; **, Significantly different from CTL, p<0.05.

Figure 9: Dose-dependent effect of chenodeoxycholic acid plus or minus PMA on PKCα activation: PKCα (0.1-1 ng) was incubated for 10 min at 30°C with [γ³²P]-ATP, Histone III-S and in the presence and absence of 10 nM PMA and increasing concentrations (1-250 µM) chenodeoxycholic acid (CDCA) as described in the legend of Fig. 4 and 6. The reaction was stopped and processed as described in the legend of Fig. 4. The results are the mean + SE of 3 experiments performed in duplicate.

*, Significantly different from CTL, p<0.05.
**Figure 1A:**

PKCα

Control  PMA  CDCA  UDCA
Figure 1B:
Figure 1C:
Figure 1D:
Figure 2A:
Figure 2B:
Figure 2C:
Figure 2D:
Figure 2E:
Figure 3A
Figure 3B:
Figure 3C
**Figure 3D**

A bar chart showing the total PKCδ expression (% of control) for different treatments: CTL, PMA, UDCA, and CDCA. The chart indicates a significant increase in expression for UDCA and CDCA compared to control and PMA.
Figure 4A
Figure 4B & 4C
Figure 5:
Figure 6A:
Figure 6B:
Figure 7:

![Bar graph showing cAMP production (% of basal)]

- GFP
- PKCαDN
- PKCδDN
- PKCεDN
- PKCζDN

Conditions:
- Basal
- CDCA 50 μM
- PGE1 1 μM
- PGE1 + CDCA 50 μM

Bars marked with asterisks (*) indicate significant differences.
Figure 8A:
Figure 8B:
Figure 8C:
Figure 9: