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Bile acids stimulate PKCα autophosphorylation and activation: role in the attenuation of prostaglandin E_1-induced cAMP production in human dermal fibroblasts

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Le, Man, Lada Krilov, Jianping Meng, Kelli Chapin-Kennedy, Susan Ceryak and Bernard Bouscarel. Bile acids stimulate PKCα autophosphorylation and activation: role in the attenuation of prostaglandin E_1-induced cAMP production in human dermal fibroblasts. *Am J Physiol Gastrointest Liver Physiol* 291: G275–G287, 2006; doi:10.1152/ajpgi.00346.2005.—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 of chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) led to YFP-PKCα and YFP-PKCδ translocation in 30–60 min followed by a transient 24- to 48-h downregulation 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, whereas 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 with PKC downregulation or inhibition, as well as PKC isoform dominant-negative mutants. Under these same conditions, neither phosphatidylinositol 3-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, whereas greater CDCA concentrations reduced the PMA-induced PKCα activation. CDCA alone did not affect PKCα activity in vitro. In conclusion, although 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.

ursodeoxycholic acid; chenodeoxycholic acid; taurocholic acid

PREVIOUSLY, WE HAVE REPORTED that dihydroxy bile acids were the most potent bile acids to inhibit stimulated adenosine 3′,5′-cyclic monophosphate (cAMP) production not only in hepatocytes (10, 12) but also in cells of nonhepatic 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 human 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). Hedenborg et al. (25) have reported that the bile acid concentration could be greater in these tissues than that measured in the serum. 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 come 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 Ref. 11 for review). However, the specific mechanism of PKC isoform activation by bile acids remains to be addressed.

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Based on structural properties and cofactor requirements, the PKC family has been subclassified into the classic (c) (PKCα, PKCβ1, PKCβ2, and PKCγ), novel (n) (PKCδ, PKCε, PKCθ, and PKCη), and atypical (a) (PKCζ, PKCμ, and PKCι) PKCs (41, 45, 58). Acute activation of most of the cPKC and nPKC 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 downregulates 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 Ref. 18). In human dermal fibroblasts, prostaglandin E_1 (PGE_1) stimulates cAMP production through its binding to the GPCR EP2 and/or EP4 receptors (see Ref. 57 for review). The PGE_1 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 remains 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 downregulation in human dermal fibroblasts. Furthermore, the role of these PKC isoforms in the bile acid-mediated attenuation of PGE_1-induced cAMP production was investigated. Finally, studies were also initiated to determine whether bile acids can stimulate PKCα transphosphorylation 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 (Freiburg, Germany). Taurocholic acid (TCA) was purchased from Steraloids (Wilton, NH). HeLa cells and cAMP antibody were a gift from A. Kumar (George Washington Medical Center, Washington, DC) and T. Gettys (Pennington Biomedical Research Center, Baton Rouge, LA), respectively. Human recombinant PKC was purchased from PanVera (Madison, WI). Yellow fluorescent protein (YFP)-labeled PKCα and YFP-PKCδ plasmids were provided by R. Kubitz (Heinrich-Heine University, Dusseldorf, Germany; Ref. 31), whereas the dominant-negative vectors, DN PKCα, DN PKCδ, and DN PKCε, were provided by Jae-Won Soh (Inha University, Incheon, South Korea; Ref. 60). Affinity-purified polyclonal rabbit anti-PKCα antibody was from Gibco Life Technologies (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 horseradish peroxidase (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 Probes. Hyperfilm and an enhanced chemiluminescence (ECL) detection kit were from Amersham (Arlington Heights, IL). 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). TCA and Whatman G4 anion exchange filter paper were from Fisher Scientific (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 ($3–5 \times 10^4$ 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 (1 μM), CDCA (100 μM), UDCA (100 μM), and TCA (100 μM) for a predetermined period of time. PGE_1 was used at the concentration 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 (20 mM Tris·HCl, pH 7.5, containing 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, and 0.1 mg/ml soybean trypsin inhibitor). Furthermore, except as otherwise mentioned, the fibroblasts were preincubated for 4 h in a medium containing 0.1% FBS before the cellular cAMP level was determined by radioimmunoassay after stopping the reaction with 12% $HClO_4$. It is worthwhile to mention that all the results were expressed as either a ratio to β-actin or GAPDH or even per milligram 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 35-mm glass-bottom dishes (MatTek, Ashland, MA) 24 h after transfection with YFP-PKCα or YFP-PKCδ 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 transfect fibroblasts with the dominant-negative PKCα, PKCδ, PKCε, and PKCζ mutants. 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-s to 2-min intervals using an Olympus IX-81 fluorescence microscope (×60 objective). For tyrosine phosphorylated protein residues and Golgi apparatus detection and/or colocalization, the cells were seeded onto poly-L-lysine-coated coverslips in 35-mm dishes and starved for 1 h before treatment with PMA or bile acid for 30–40 min at 37°C. After treatment, the cells were washed with PBS, fixed in 3.7% paraformaldehyde for 10 min, and incubated in 2% BSA/PBS (blocking buffer) for 1 h. 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-min 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 was analyzed as described above. Both fluorescence 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 (1:3,000) or anti-PKCα, anti-PKCβ$_2$, anti-PKCδ, anti-PKCε, and anti-PKCζ (1:1,000)]. The nitrocellulose membranes were next incubated for 1 h 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 preincubated 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 ECL. After exposure to the nitrocellulose membrane, the Hyperfilm was analyzed by densitometric scanning using photoimaging (Molecular Dynamics, Sunnyvale, CA). Although 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 imunoreactive 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 six-well plates using RNA Bee (Tel-Test, Friendswood, TX). Oligo-dT and SuperScript III were used for transcription containing 2–4 μg of RNA. Reverse transcription was conducted at 50°C for 60 min following RNase H treatment for 30 min. DNA (5–10 ng) 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 s, 55°C for 30 s, and 72°C for 40–60 s 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 (no. X52479: forward, 5′-CTT CAG ACA AAG ACC GAC GAC-3′, +725/+745; reverse, 5′-CAT GAC GAA GTA CAG CCG ATC-3′, +1,278/+1,258 for 554 bp), hPKCδ (no. L07860: forward, 5′-GTC ATC CAG ATT GTG CTA ATG CG-3′, +260/+282; reverse, 5′-TCT TGT GGA TGG CAG CGT TCA-3′, +653/+635 for 394 bp), and hGAPDH (no. M33197: forward, 5′-CCA TGA CAA CTT TGG TAT CGT GG-3′, +555/+577; reverse, 5′-CAG GTC CAC CAC TGA CAC GTT-3′, +798/+778 for 244 bp).

Determination of PKC phosphorylation and kinase activity. Phosphorylation was determined by incubating human recombinant PKCα (0.1–1 ng) in HEPES buffer (pH 7.4) containing 0.3% Triton X-100, phosphatidylserine (PS), and diolein and sonicated 30 s at 4°C, as well as 100 μM [γ-^{32}P]ATP. The PKCα was incubated at 30°C for 30 min in the presence of the indicated agents, 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 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 [γ-^{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 X-100 (0.02%), and $CaCl_2$ (400 μM) or EGTA (5 mM). 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 was determined in an LS 3801 beta-counter (Beckman, Palo Alto, CA).

cAMP determination. Cells were incubated alone with the respective bile acid and/or with PGE_1 for the designated period of time. Cellular cAMP production was measured in $HClO_4$ extracts by radioimmunoassay as previously described (8, 10), using the method of Gettys et al. (20). The results were expressed as percent of the maximum obtained by incubating the cells with PGE_1 alone.

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

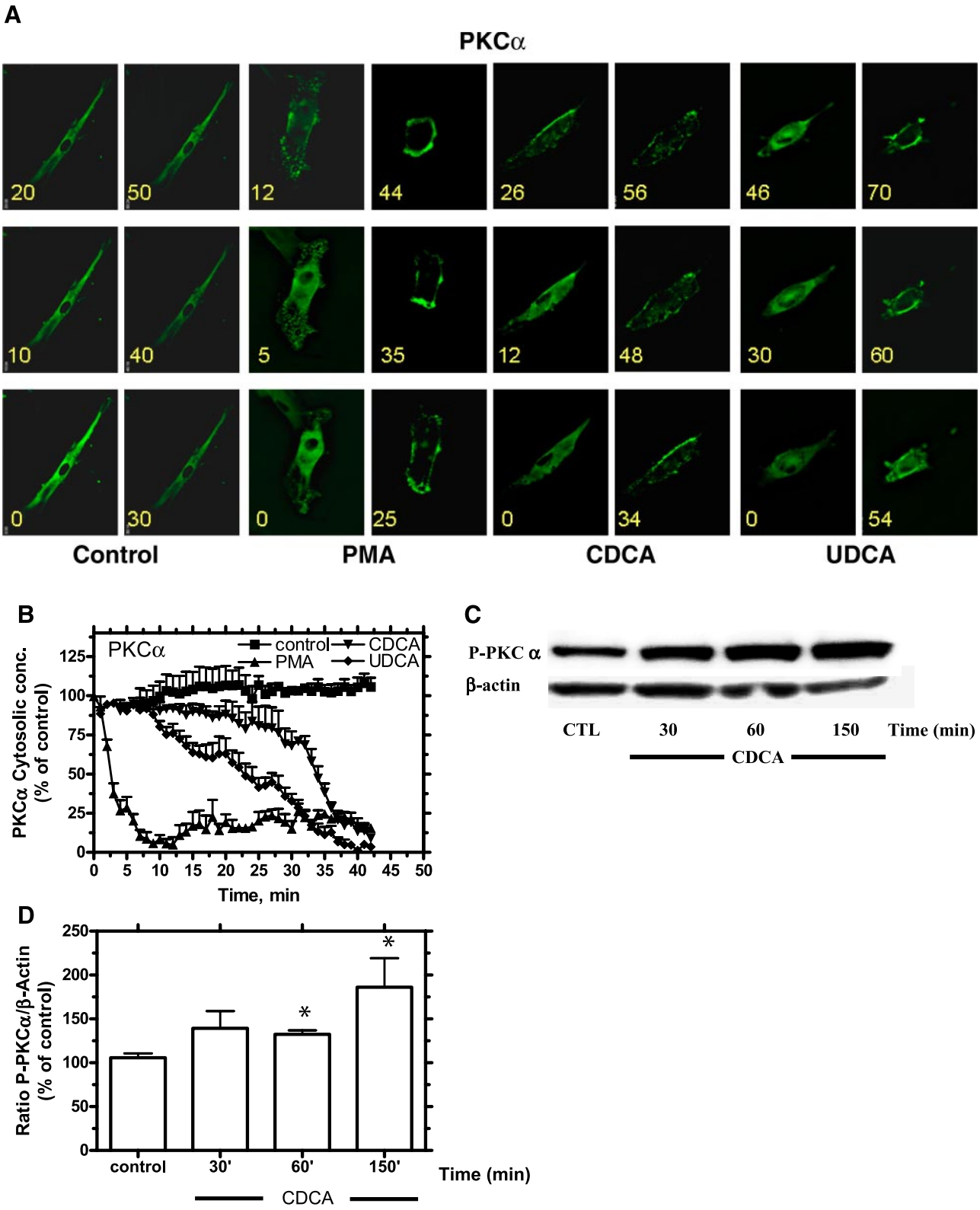
RESULTS

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. 1*A*). This was confirmed by measuring the disappearance of PKCα from the cytosol (Fig. 1*B*). 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. 1*A*). The disappearance of PKCα was quicker upon UDCA vs. CDCA stimulation (Fig. 1*B*). However, ~40 min incubation with either bile acid is required to induce a maximum PKCα translocation to the plasma membrane and disappearance from the cytosol (Fig. 1, *A* and *B*).

The association between PKCα membrane translocation and activation was further evidenced by measuring phosphorylation of PKCα by CDCA over time (Fig. 1, *C* and *D*). The results indicated a 1.2- to 1.9-fold increase in phospho-Ser657 PKCα induced by CDCA over the 150-min test period, 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. 1, *A* and *B* vs. *C* and *D*). 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. 2*A*). 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 at ~30 min (Fig. 2, *A* and *B*). Upon stimulation with either CDCA or UDCA, PKCδ progressively accumulated in the perinuclear region to a maximum of approximately

twofold in 25–30 min (Fig. 2, *A* and *B*). However, none of the bile acids tested induced an initial loss in perinuclear PKCδ (Fig. 2*B*), and although the stimulatory effect of CDCA was rapid and required <5 min to reach a noticeable increase, that of UDCA was delayed by 15–20 min (Fig. 2*B*). Furthermore, since PKCδ can be phosphorylated at Thr505 and Tyr311 and since 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 colocalization



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. 2*D*) accumulated almost exclusively in the nucleus with little or no colocalization with the Golgi (data not shown). Nuclear PKCδ Tyr311 accumulation is increased by ~2-fold by PMA and by ~1.6- to 1.8-fold by CDCA and UDCA, respectively (Fig. 2*E*). The accumulation of at least phospho-PKCδ Thr505 in the nucleus upon stimulation has also been recently reported by Wiedlocha et al. (66) to occur in 3T3 fibroblasts. 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 downregulation following chronic exposure of human dermal fibroblasts to either bile acids or PMA. The major PKC isoforms detected in fibroblasts were PKCα, PKCδ, PKCε, and PKCζ (Fig. 3*A*). 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 24 h resulted in the downregulation of the total expression of PKCα by 99 ± 1%, 37 ± 4%, and 45 ± 4% of control, respectively ($n = 4$; $P < 0.05$; Fig. 3, *A* and *B*). Similar results were observed when UDCA and CDCA were used at a concentration of 50 μM (data not shown). As far as the novel PKC subfamily was concerned, PKCδ was identified as a doublet of 85/80 kDa, which was validated using a PKCδ peptide against which the antibody was raised. PMA, UDCA, and CDCA downregulated the total expression of PKCδ by 90 ± 5%, 48 ± 8%, and 34 ± 7%, respectively ($n = 3$; $P < 0.05$; Fig. 3, *A* and *B*). PKCε was the other novel PKC isoform detected in fibroblasts. This isoform has a molecular mass of 92 kDa, and its total expression was decreased by 95 ± 8%, 30 ± 12%, and 28 ± 7% following incubation of the cells with PMA, UDCA and CDCA, respectively, for 24 h ($n = 3$; $P < 0.05$; Fig. 3, *A* and *B*). Furthermore, the atypical PKCζ was detected as an ~82-kDa protein and was not significantly downregulated after a 24-h incubation of the cells with either PMA, UDCA, or CDCA ($n = 3$; Fig. 3, *A* and *B*).

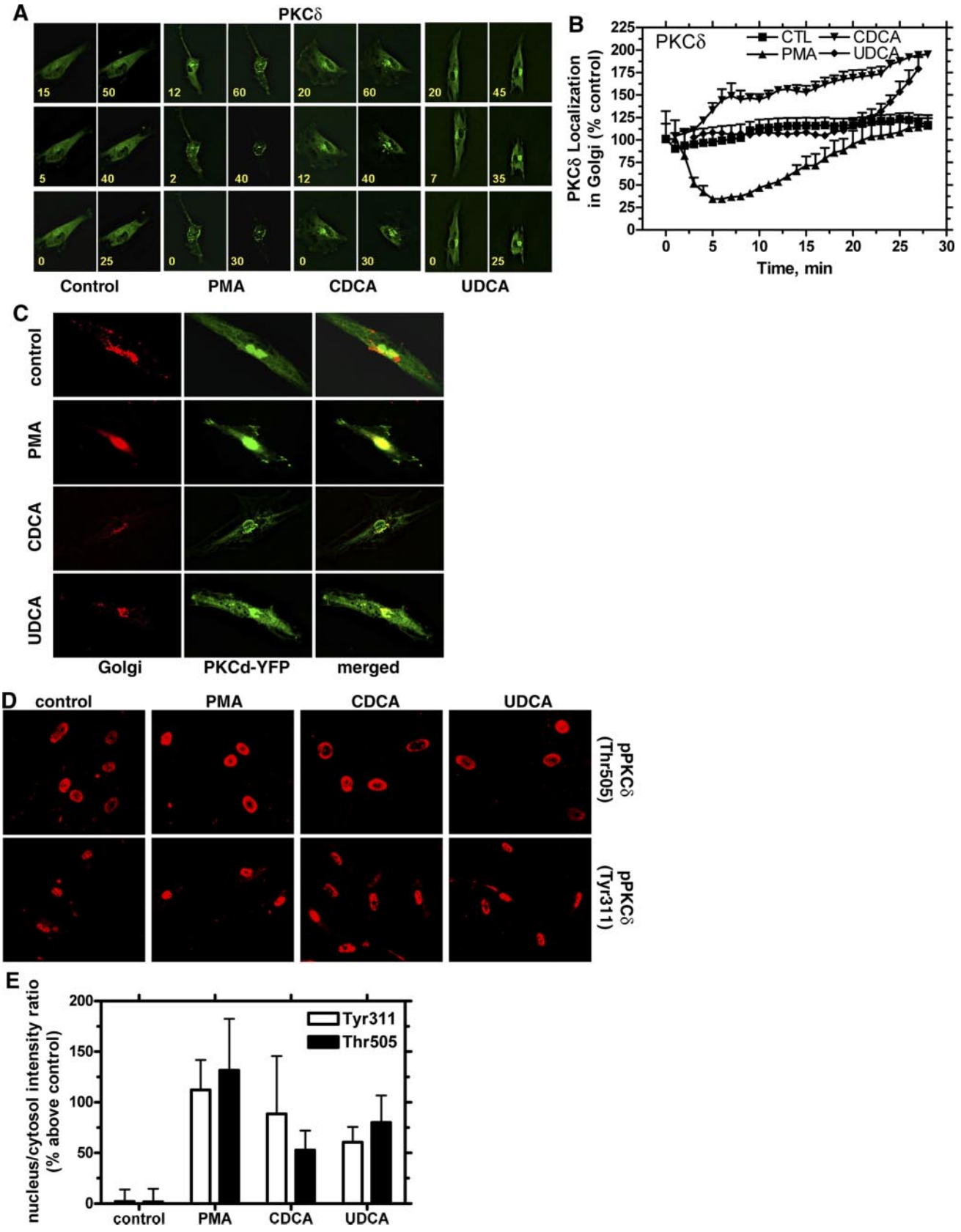
Since under pathological conditions, tissues could be exposed to bile acids for a prolonged period of time, we compared the effect of 1 μM PMA and 100 μM UDCA or CDCA on PKCα and PKCδ total expression following incubation of the cells with these respective agents for 24 and 48 h (Fig. 3*C* and Fig. 3*D*). Although the effect of both PMA and UDCA on PKCα protein expression remained significant, that of CDCA was transient and completely disappeared after 48 h of incubation (Fig. 3*C*). Under these conditions, the PKCδ downregulation by the different agents tested was transient and was not significantly different from control after 48 h of incubation with UDCA and CDCA (Fig. 3*D*).

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

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 assessement of the downregulation of PKC expression level by either bile acids or PMA. The phosphorylation of histone III-S in cell homogenate in the presence of DAG and PS 24 h after incubation of the fibroblasts with the different bile acids or PMA was investigated. As reported in Fig. 5, 24 h of 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^{-1}·min^{-1} with PMA and to 31 ± 2 and 27 ± 8 pmol·mg protein^{-1}·min^{-1} after UDCA and CDCA exposure, respectively. Compared with the control, the reduction in the total kinase activity following 24 h of incubation of the cells with PMA and bile acids were ~99% and 32% ($n = 3$; $P < 0.05$; Fig. 5), respectively.

Role of PKC downregulation 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 24 h to study the effect of PKC downregulation in CDCA-induced inhibition of stimulated cAMP production. Downregulation of the PKCs by PMA did not significantly affect the basal cellular cAMP level (Fig. 6*A*). Furthermore,

Fig. 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 yellow fluorescent protein (YFP)-labeled PKCα (YFP-PKCα) using the transfection kit for primary cells. Twenty-four to forty-eight hours after transfection, the cells were serum-starved for 1 h, placed on a heated stage, and treated with 100 nM PMA, 50 μM chenodeoxycholic acid (CDCA), or 50 μM ursodeoxycholic acid (UDCA). Images were collected every 0.5–2 min using a fluorescence microscope (×60 objective). Values in each image represent time in minutes. Data shown is representative of three independent experiments. *B*: 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 (10–20 mg/ml) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with specific antibodies against phospho-Ser657-PKCα and β-actin followed by horseradish peroxidase (HRP)-labeled secondary antibody. *D*: results were quantified by densitometric scanning using photoimaging and normalized against the respective β-actin optical density signals. Values are expressed as percent of control and are means ± SE of three different experiments. *Significantly different from respective control, $P < 0.05$.



CDCA did not significantly alter the basal cAMP level, as previously reported (8). However, the respective significant 20% and 45% inhibitory effect observed after 2 h of incubation with 10 and 25 μM CDCA on 0.7 μM PGE_1-induced cAMP production was completely abolished after 24 h of PMA treatment (Fig. 6*A*). 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. 6*B*). 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 only a partial UV-induced activation of this inhibitor. Therefore, the results may respresent 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 mutants, and the regulation of PGE_1-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 the PKCα dominant-negative mutant (Fig. 7). The inhibitory effect of CDCA was enhanced to the same extent and by around threefold when the cells were transfected with PKCδ and PKCζ dominant-negative mutants. Transfection of the cells with the PKCε dominant-negative 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 phosphatidylinositol 3-kinase (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 (42) reported that several PKCs, including PKCα, presented 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. Furthermore, results described in Fig. 1, *A* and *B*, suggested that bile acids stimulate PKCα phosphorylation at Ser657. Therefore, experiments were designed to study PKCα crossphosphorylation 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 PS, and phosphorylation was determined using [γ-^{32}P]ATP. The PKC protein level was determined by Western blotting once the radioactivity had significantly decayed. The results reported in Fig. 8, *A* and *B*, 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. 8, *A* and *B*). The bile acid effect was significant at a concentration as low as 10 μM, whereas 250 μM CDCA stimulated PKCα phosphorylation by up to threefold and to the same or greater extent as that induced by 1 μM PMA (Fig. 8, *A* and *B*). Finally, preincubation of PKC with 1 μM Gö6976 resulted in the abrogation of both the PMA- and bile acid-induced PKCα phosphorylation (Fig. 8*C*), thus supporting the specificity of the effect. These results suggest that CDCA can stimulate the crossphosphorylation 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 III-S as a substrate (Fig. 9). Indeed, CDCA concentrations up to 250 μM did not significantly increase the phosphorylation of histone III-S 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- to 7-fold increase in activity observed with 1 μM. Furthermore, CDCA potentiated 10 nM PMA-induced histone III-S phosphorylation in a dose-dependent manner, with a maximum increased activity of ~60% observed at concentrations ranging from 5–25 μM (Fig. 9). However, greater CDCA

Fig. 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 YFP-PKCδ and processed as described in the legend of Fig. 1. Data shown is representative of three independent experiments. *B*: PKCδ localization to the Golgi was measured over time. *C*: fibroblasts transfected with PKCδ-YFP were seeded onto poly-L-lysine-coated coverslips. Twenty-four to forty-eight hours after transfection, the cells were serum-starved for 1 h and treated with 100 nM PMA, 50 μM CDCA, or 50 μM UDCA for 30 min at 37°C. 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 phospho-PKCδ was studied using either anti-phospho-PKCδ Thr505 or anti-phospho-PKCδ Tyr311. *E*: results were analyzed and quantified using the Slidebook digital microscopy software.

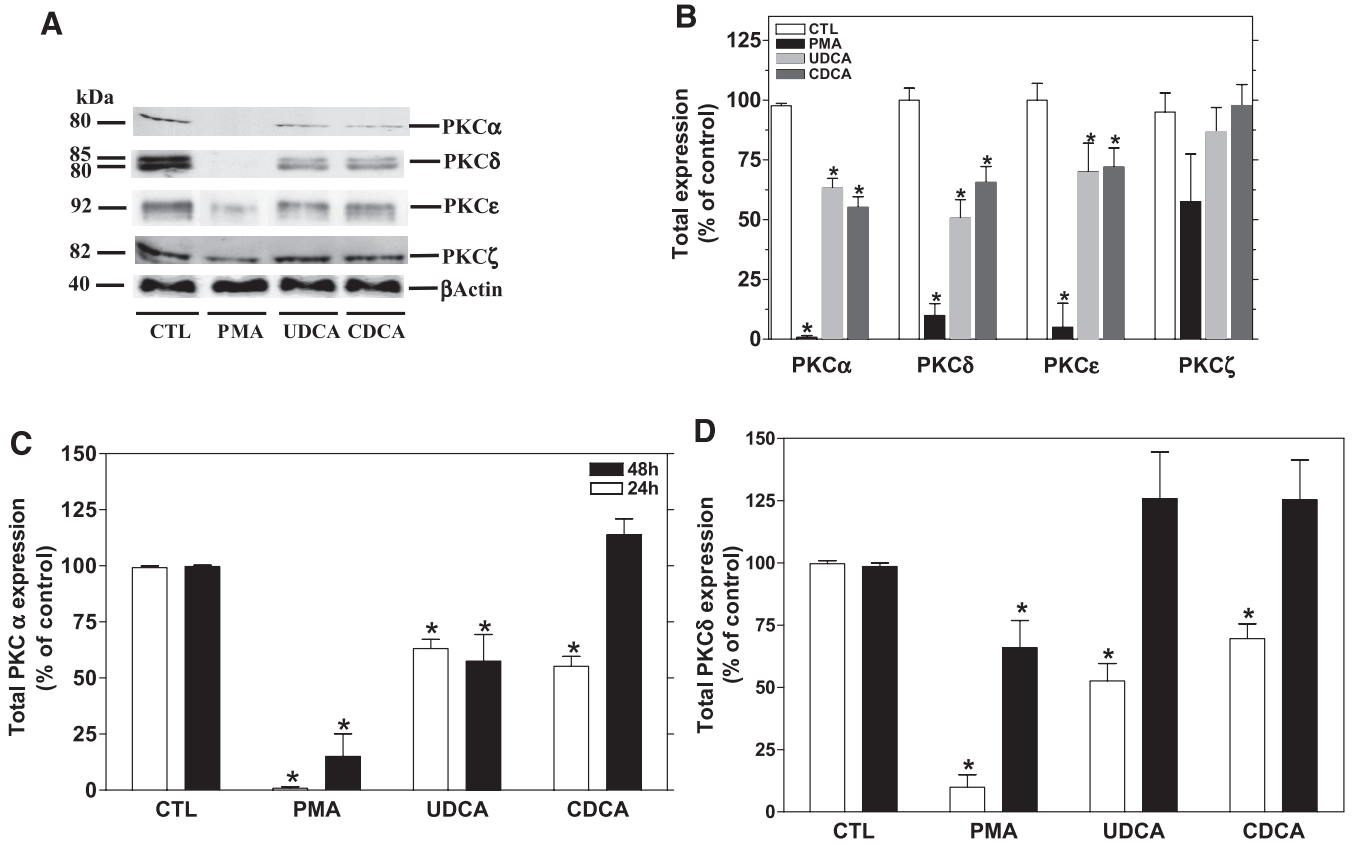


Fig. 3. Total PKC expression following 24- and 48-h exposure to either bile acids or PMA. *A*: cultured human dermal fibroblasts were incubated for 24 h in the absence (CTL) and presence of 1 μM PMA, 50 μM UDCA, or 50 μM CDCA. Total cellular proteins (10–20 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with specific polyclonal antibodies against PKCα, PKCδ, PKCε, and PKCζ, as well as β-actin, followed by HRP-labeled secondary antibody. *B*: results were quantified by densitometric scanning using photoimaging and normalized against the respective β-actin optical density signals. Values are expressed as percent of control and are means ± SE of three different experiments performed in duplicate. *C*: cultured human dermal fibroblasts were incubated for 24 and 48 h in the presence of 1 μM PMA, 100 μM UDCA, or 100 μM CDCA. The total cellular proteins were analyzed as described above for PKCα (*C*) and PKCδ (*D*). Values are means ± SE of at least three experiments performed in duplicate. *Significantly different from respective control, $P < 0.05$.

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, PI3 kinase, or MAP kinase, 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), and proliferation and differentiation (26; see Ref. 18 for review). One additional action of PKC is to modulate the glucagon-associated responses of 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 24 h pretreatment of the cells with PMA at least partially abolished the inhibitory effect of both PMA and CDCA on cAMP production induced by PGE_1. 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, although 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- to 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 nonhepatic origin. In dermal fibroblasts, CDCA and UDCA significantly stimulated PKCα and PKCδ translocation and phosphorylation, whereas long-term bile acid stimulation resulted in partial downregulation of these PKCs, as well as of PKCε total protein expression. Two different hypotheses can be proposed to explain the increased accumulation of phospho-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 stimulating the phosphorylation of the PKCδ present in the nucleus. However, since PKCδ can be activated in the absence of the phosphoryation, the increased phosphorylation on Thr505 and Tyr311 could be im-

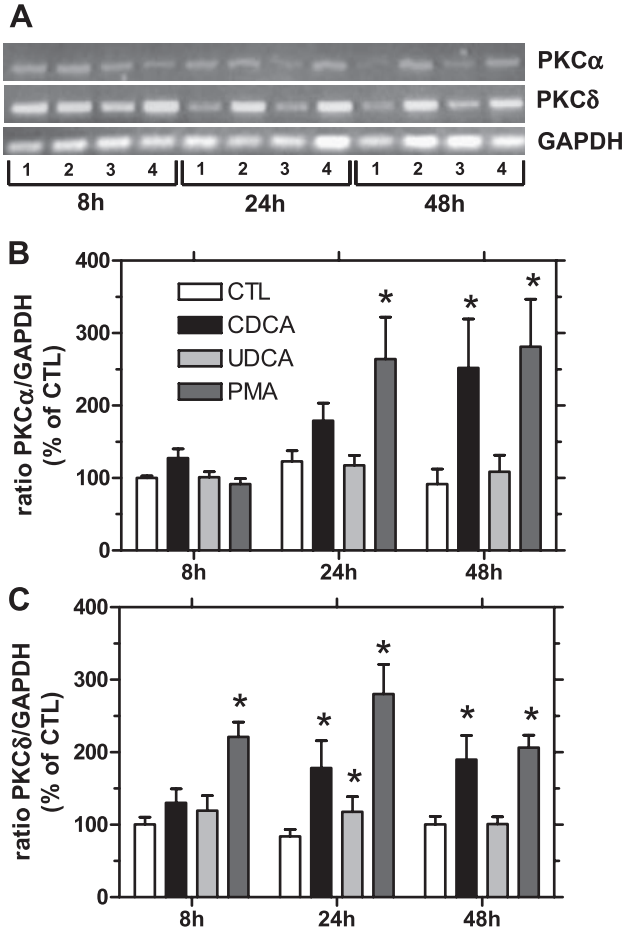


Fig. 4. Total PKCα and PKCδ mRNA in human dermal fibroblasts exposed to either bile acids or PMA. *A*: cells were incubated for up to 48 h in the presence of 1 μM PMA, 100 μM UDCA, or 100 μM CDCA. PKCα and PKCδ mRNA levels were determined by RT-PCR according to the method described in MATERIALS AND METHODS at 8, 24, and 48 h. The results for PKCα (*B*) and PKCδ (*C*) were analyzed as described above and normalized to GAPDH mRNA expression and expressed as a percent of control. Values are means ± SE of five experiments. *Significantly different from respective control, $P < 0.05$.

portant for the stability of the PKC isoform or in the localization, i.e., nucleus vs. Golgi (see Ref. 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 downregulation, 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. (7) have, nevertheless, reported that 12-*O*-tetradecanoylphorbol 13-acetate activated and subsequently downregulated 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 downregulation is indirect and involves mechanisms that 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 downregulation. 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 48 h. Interestingly, the downregulation of PKCδ by the different agents tested, including PMA, UDCA, and CDCA was short lived, since it was not significantly different from control after 48 h of incubation and with little correlation between protein and mRNA expression levels. The activation of PKCs, and of PKCα in particular, by TCA remains controversial. Although 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 24 h of incubation with TCA. Although 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 cPKC isoforms (α and β_2) 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 (Ref. 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

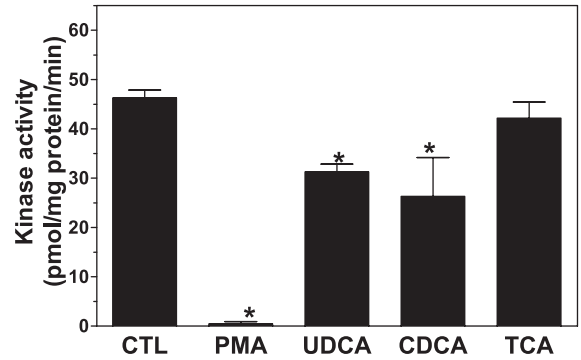


Fig. 5. Total kinase activity in cultured human dermal fibroblasts following a 24-h exposure of the cells to either bile acids or PMA. The cells were incubated as described in the legend of Fig. 2 in the presence of either 1 μM PMA, 100 μM UDCA, or 100 μM CDCA for 24 h, and the cells were kept frozen at −80°C until the activity was determined. The cells were lysed on ice using a Sonicator W380 (Heat Systems Ultrasonics, 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), L-α-phosphatidyl-L-serine (PS) (50 ng/μl), Triton X-100 (0.02%), [γ-^{32}P]ATP (~50,000 dpm/tube), and 400 μM $CaCl_2$. The incubations were terminated by precipitating histone III-S with a solution of trichloroacetic acid (TCA) (0.25%) onto a Whatman filter paper, and the radioactivity was determined by liquid scintillation spectrometry in an LS 3801 beta-counter. Values are expressed as percent of control and are means ± SE of three different experiments performed in duplicate. *Significantly different from respective control, $P < 0.05$.

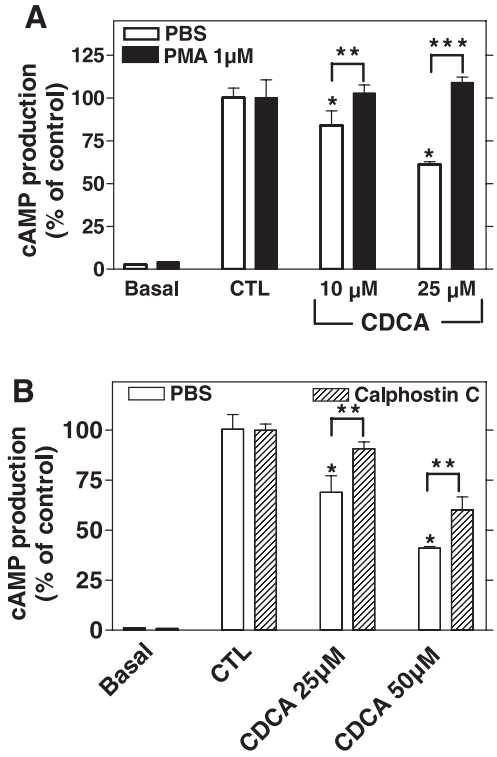


Fig. 6. Effect of PKC downregulation and inhibition on the bile acid-induced reduction of stimulated cAMP production. *A*: cultured human dermal fibroblasts were incubated for 24 h in the presence of either 1 μM PMA or PBS used to dissolve PMA. The cells were then incubated for 2 h either in the absence (CTL) or presence of increasing concentrations (10 and 25 μM) of CDCA before the cellular cAMP production was determined 8 min after the addition of 0.7 μM PGE_1. The total cellular cAMP level was determined by radioimmunoassay. Results are expressed as the percent of the control cAMP level (~2,200 pmol/mg protein), corrected for the basal level (~28 pmol/mg protein), determined in the presence of PGE_1 and in the absence of bile acids and PKC inhibitor. Values are means ± SE of three determinations. *B*: cells were preincubated at 37°C for 1 h with 100 nM calphostin C followed by the addition of increasing concentrations (25 and 50 μM) of CDCA for 2 h. The total cellular cAMP level was determined by radioimmunoassay. Results are expressed as the percent of the control cAMP level determined in the presence of PGE_1 and in the absence of bile acid and PKC inhibitor and were corrected for the basal level. Values are means ± SE of three determinations. *Significantly different vs. PBS alone, $P < 0.05$. **Significantly different vs. respective control, $P < 0.05$. ***Significantly different vs. PBS condition, $P < 0.01$.

by Martiny-Baron et al. (38) showed that Gö6976 displays greater selectivity for the cPKCα and cPKCβ1 over the nPKCδ and nPKCϵ. The IC_{50} for inhibition of PKCα was in the nanomolar range and was around threefold less than that necessary for PKCβ1 inhibition and required a several hundred-micromolar concentration to inhibit nPKCs (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 dominant-negative 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

Table 1. *Comparative effect of various protein kinase inhibitors*

	cAMP Production, % of control	
	PGE_1 (0.7 μM) + 25 μM CDCA	PGE_1 (0.7 μM) + 50 μM CDCA
Control	69±7*	44±6*
Gö6976, 10 μM	106±5†	83±10†
SB202190, 25 μM	60±7	45±4
LY294002, 20 μM	58±4	46±2
UO126, 20 μM	67±5	41±4
PD98059, 25 μM	60±5	42±8
H 89, 1 μM	ND	35±9

Cells were preincubated at 37°C with PBS (control), Gö6976 (PKC inhibitor), SB202190 (P38 MAP kinase inhibitor), LY294002 (phosphatidylinositol 3-kinase inhibitor), UO126 (MEK inhibitor), PD98059 (P42/44 MAP kinase inhibitor), and H89 (cAMP-dependent protein kinase inhibitor). After 30 min to 1 h, PBS (vehicle) or either 25 or 50 μM chenodeoxycholic acid (CDCA) was added for 2 h before the cellular cAMP level was determined in the presence of PGE_1. The results are expressed as the percent of control determined in the sole presence of PGE_1. The cAMP production induced by PGE_1 was not significantly affected by any of the agents tested in the period of up to 20 min tested. ND, not determined. Values are means ± SE. *$P < 0.05$ vs. PBS alone, †$P < 0.05$ vs. control.

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 PS and acting as a PKC cofactor (19, 27, 64). Recently, another study

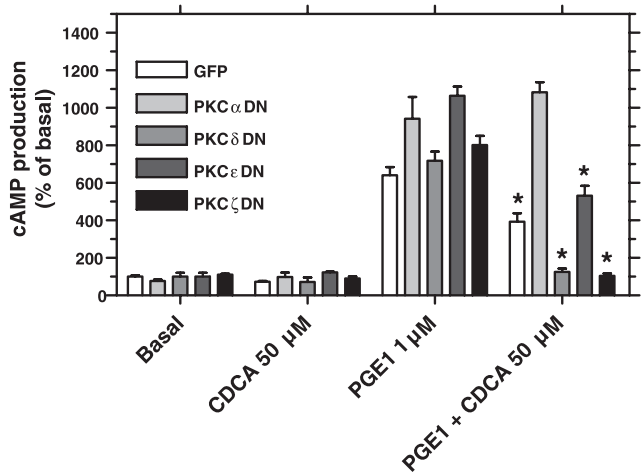


Fig. 7. Effect of PKC isoform dominant-negative (DN) mutants on the cAMP production by CDCA. Fibroblasts (60–80% confluency) were transfected with 1–2 μg of the respective PKC (α, δ, ϵ, and ζ) DN mutant or YFP as described in the legend of Fig. 1. After 18–24 h, the cells were incubated in serum-free medium, with or without 1 μM PGE_1, and in the presence or absence of 50 μM CDCA. The total cellular cAMP level was determined by radioimmunoassay. Results are expressed as the percent of the respective basal value determined in the absence of any agent. *$P < 0.05$ vs. respective control determined in the sole presence of PGE_1.

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 transphosphorylation or autophosphorylation, possibly increasing sensitization of PKC for activation and deactivation.

The requirement for posttranslational phosphorylation of PKC as a necessary step to produce an activatable kinase is generally accepted (see Refs. 43 and 61 for review). Phosphorylation of the activation loop at least at Thr497 but also at Thr495 is necessary for activation of PKCα, whereas 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. (62) 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. This latter observation could explain, at least in part, the results in the present study that the increased phosphorylation of PKCα by CDCA, although not leading to a direct activation of this kinase, increases the affinity and, in turn, the activation of PKCα by PMA. Sando et al. (54) have observed a correlation between autophosphorylation and activation of PKC with a lipid-dependent, normally distributed activation pattern. 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).

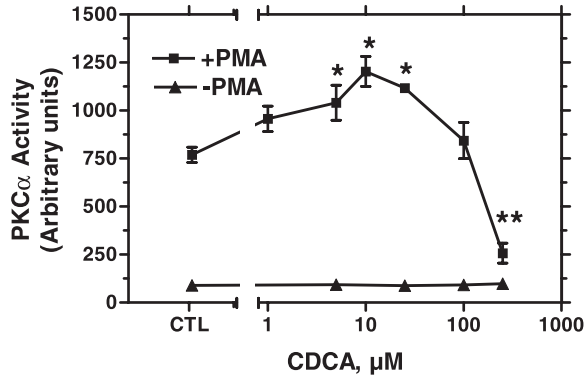


Fig. 9. Dose-dependent effect of CDCA ± PMA on PKCα activation. PKCα (0.1–1 ng) was incubated for 10 min at 30°C with [γ-^{32}P]ATP, histone III-S, in the presence or absence of 10 nM PMA, and increasing concentrations (1–250 μM) of CDCA as described in the legends of Figs. 4 and 6. The reaction was stopped and processed as described in the legend of Fig. 4. Values are means ± SE of three experiments performed in duplicate. *Significantly different from CTL, $P < 0.05$.

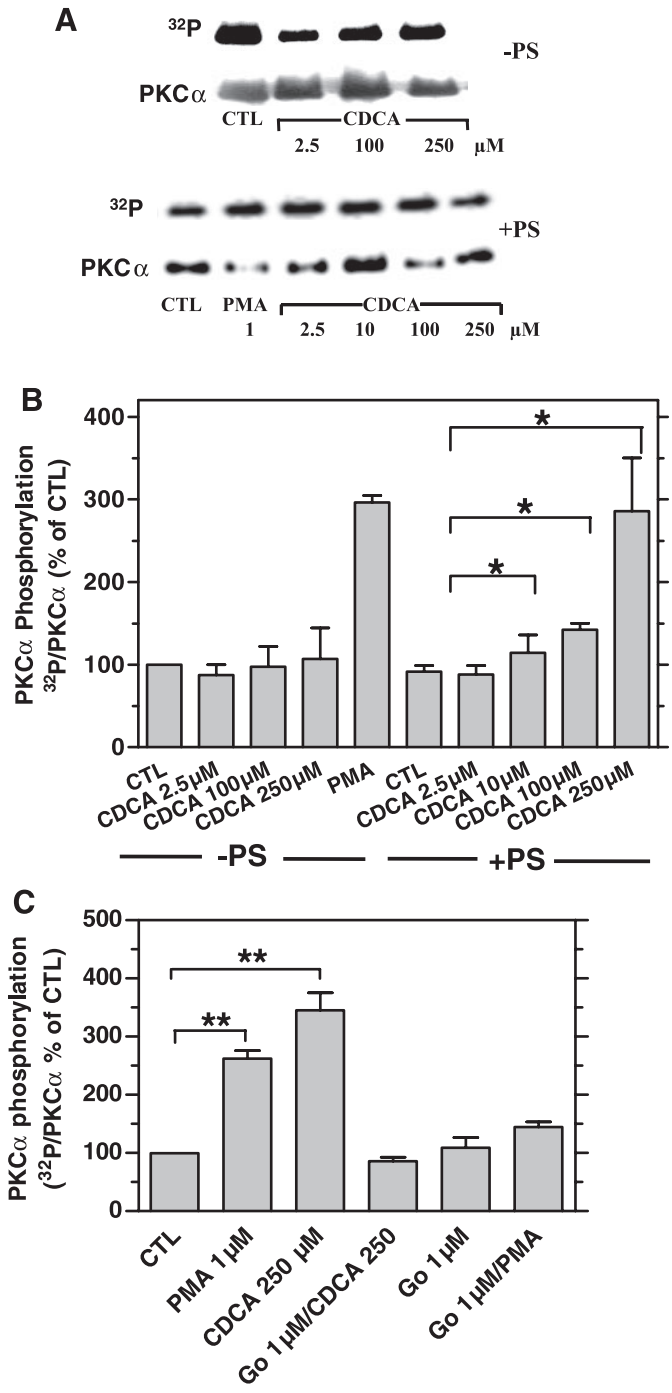


Fig. 8. Comparative dose-dependent effect of CDCA on PKCα phosphorylation. *A*: PKCα (0.1–1 ng) was incubated in vitro with 100 μM [γ-^{32}P]ATP and without (CTL), 1 μM PMA, or with increasing concentrations (2.5–250 μM) of CDCA in the absence or presence of 5 μg PS. The preparation was incubated at 30°C for 30 min, the reaction was stopped by boiling in SDS, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and exposed to a phosphor screen. *B*: levels of phosphorylation (^{32}P) were 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). Values are means ± SE of three to four determinations and were expressed as the percent of respective control. *C*: PKCα (0.1–1 ng) was preincubated for 5–10 min with 1 μM Gö6976 and further incubated without (control, CTL) or with either 1 μM PMA or 250 μM CDCA and in the presence of PS. The preparation was incubated at 30°C for 30 min, the reaction was stopped by boiling in SDS, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and exposed to a phosphor screen. The level of phosphorylation was determined and analyzed as described above. Values are means ± SE of at least three determinations. *Significantly different from CDCA 2.5 μM, $P < 0.05$. **Significantly different from CTL, $P < 0.05$.

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 human 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 two 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 PGE_1 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 epxression in a PKC-dependent manner (data not shown). Therefore, not only activation but also downregulation of specific PKC isoforms could mediate, at least in part, the toxic effect associated with chronic bile acid stimulation.

In conclusion, this study is the first to suggest that although 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 nonhepatic origin. The activation of this kinase by bile acids is accompanied by an increased transphosphorylation 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.

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