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Ursodeoxycholic acid inhibits translocation of protein kinase C in human colonic cancer cell lines

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Abstract

Deoxycholic acid (DCA) has been implicated in colonic carcinogenesis through effects mediated by protein kinase C (PKC) activation. By contrast, ursodeoxycholic acid (UDCA) is reported to reduce colon cancer incidence in ulcerative colitis. The aim of this study was to investigate whether UDCA modulated DCA-induced PKC isoenzyme translocation to its site of activity. HCT116 cells were treated with DCA, UDCA alone or pre-treated with UDCA followed by DCA. Analysis of translocation of endogenous and enhanced green fluorescent protein (EGFP) constructs of PKC isoenzymes was performed. Both DCA and phorbol myristate acetate (PMA) but not UDCA caused translocation of endogenous PKC α, ε and δ and transfected PKC β1-, ε- and δ-EGFP from cytosol to plasma membrane, reflecting isoenzyme activation. Furthermore, UDCA inhibited DCA-induced translocation of PKC isoenzymes. Inhibition of DCA-induced PKC translocation may be a mechanism for UDCA-mediated chemoprevention of colon carcinogenesis.

Keywords: Ursodeoxycholic acid; Deoxycholic acid; Protein Kinase C; Colorectal cancer

1. Introduction

The protein kinase C (PKC) family of enzymes has previously been implicated in the pathogenesis of colorectal carcinoma [1–3]. PKC isoenzymes are a pleiotrophic family of molecules with diverse roles in the regulation of cell signalling for proliferation and for stimulus-secretion coupling [4]. PKC was first identified as a receptor for the tumour promoter phorbol myristate acetate (PMA) and indeed PKC activation through PMA is involved in the promotion of tumour growth in many animal models of malignancy [5]. While PKC was initially thought to be a calcium and phospholipid-dependent enzyme activated through phorbol esters or endogenous ligands such as 1,2-sn-diacylglycerol (DAG), it is now recognised that both novel (calcium-independent) and atypical (diacylglycerol-independent) members of this family play an important role in cellular functions [6].

PKC plays an important role in colorectal carcinogenesis. In animal models, PKC activation has been shown to play a role in tumour promotion [1,2]. In human malignancy, the situation is more complex with evidence that PKC depletion occurs at an early stage in the malignant process, possibly before the development of the multiple mutations associated with CRC [7]. Of particular importance, PKC isoenzymes may be depleted *in vitro* by activation and it is possible that their depletion *in vivo* may reflect the effects of chronic activation. The mechanisms whereby PKC enzymes could be activated and or depleted in the colon are potentially

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complex. PKC enzymes are responsive to dietary fatty acids and it is possible that fatty acid composition of the diet could play a role in activation or depletion [8].

There is considerable suggestive evidence from epidemiological studies that a high fat content in Western diet predisposes to colorectal cancer [9]. One possible mechanism for the role of a high fat diet relates to stimulation and abundance of tumour promoting bile acids [10,11]. High serum and faecal levels of bile acids are found in patients with adenomas and colorectal carcinomas [12,13]. It has been suggested that bile acids act as tumour promoters by disturbing the balance between proliferation, differentiation and apoptosis in colonic epithelial cells [14]. Of particular note, it has been demonstrated by previous researchers that the secondary bile acid deoxycholic acid is a potent activator of PKC *in vitro* with effects on PKC activation resembling those induced by the tumour promoter PMA [15]. Carcinogen-treated rats, fed with a diet supplemented with DCA developed more colonic tumours than those treated with carcinogen alone [16,17]. Furthermore, surgically altering the bile acid flow in rat colon, hence changing the faecal bile acid excretion also altered the incidence of tumours [18]. There is a plethora of studies exploring the tumour promoting effects of DCA and the mechanisms behind these effects have gradually been unravelling. DCA is reported to activate NF-κB and IL-8 in human colon cancer cells [19]. It has also been reported that DCA induces cyclooxygenase 2 (COX-2), vascular endothelial growth factor (VEGF), *c-fos* and β-catenin signalling, which are all implicated in colon cancer growth and invasiveness [20,21].

There is significant data linking the PKC activator DCA with the pathogenesis of colorectal carcinoma. By contrast, recent data suggests that ursodeoxycholic acid, a bile acid used in the treatment of gall-stones and more recently in inflammatory liver disease, may act to reduce the occurrence of colorectal dysplasia and cancer in patients with inflammatory bowel disease in humans [22,23]. Some of the mechanisms of UDCA-mediated chemoprotection have recently been published. In hepatocytes, UDCA inhibited formation of reactive oxygen intermediates and blocked mitochondrial membrane permeability transitions, while preventing apoptosis induced by more hydrophobic bile acids [24,25]. UDCA is reported to inhibit the overexpression of cyclin D1 and COX-2 and progression of low-grade dysplasia in colorectal cancer [26,27], its effects on COX-2 illustrating a potential mechanism of antagonism of DCA activity. UDCA has also been shown to inhibit DCA-induced apoptosis through EGFR/Raf-1/ERK signalling in human colon cancer cells [28].

The aim of the current study was to determine if UDCA could mediate its chemoprotective effect by modulating PKC isoenzyme activity in colon cancer cells. We have demonstrated that UDCA did not cause translocation, but actually inhibited DCA-induced translocation of PKC isoenzymes, indicating a potential mechanism for UDCA-mediated chemoprevention of colon carcinogenesis.

2. Materials and methods

2.1. Cell culture and materials

Colorectal adenocarcinoma cell lines (HCT116 and SW480) and the human breast adenocarcinoma cell line (MCF7), obtained from American Type Culture Collection (ATTC, Rockville, MD), were cultured in McCoy's 5a medium, L-15 medium and RPMI medium respectively. All media were supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (GIBCO BRL, Grand Island, NY). According to the manufacturer, the FBS was free from conjugated bile acids. FBS was present in the growth medium in all experiments except at the stage of transfection, which was carried out in serum-free medium. The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. The PMA and bile acids, DCA and UDCA were obtained from Sigma Chemical Co. (St. Louis, MO) C2-Ceramide and dihydroceramide were purchased from Clontech (Palo Alto, Ca).

2.2. Immunofluorescent staining for endogenous PKC isoenzymes

HCT116 cells were cultured on 8 well Permanox chamber slides (Nunc, Naperville, IL) for 24 h to 60–70% confluence, treated with 150–300 μM DCA, 100 nM PMA, 150–300 μM UDCA and 50 μM C2-Ceramide alone for 2 h or pretreated with UDCA for 2 h and then exposed to DCA or PMA or C2-Ceramide. Cells were washed with warm PBS and fixed by gentle immersion in acetone at −20 °C for 10 min. Permeabilisation of fixed cells was carried out using 0.1% Triton X-100/PBS for 30 min. This was followed by blocking with 40 μl of normal goat serum. PKC α, β, ε, δ, ζ-rabbit polyclonal antibody kits (R&D CA) were used to determine their subcellular localisation. Cells were incubated with primary antibodies for 1 h at room temperature. The slides were washed and incubated with Alexa-Fluor 488 conjugated anti-rabbit secondary antibody and mounted beneath a clean coverslip with fluorescence-preserving mounting medium (DAKO) and photographed using Nikon TE 300 inverted microscope equipped with Leica DC 100 color digital camera.

2.3. Transfection of the HCT116 cells with PKC-EGFP fusion constructs

The plasmids PKC ε-EGFP and pEGFP were a gift from Dr. Naoaki Saito. PKC β1-EGFP, PKC δ-EGFP

and PKC ξ-EGFP plasmids were purchased from Clontech (Palo Alto, Ca). PKC inhibitors, Calphostin C, Indolocarbazole (Gö6976), Bisindolylmaleimide were from Calbiochem. Plasmids were prepared either utilizing GeneElute Endotoxin free plasmid midiprep kit (Sigma) or Plasmid DNA purification system (Promega). Plasmid quantification was done by agarose gel electrophoresis and ultraviolet photography and plasmids were stored at −20 °C. Cells were cultured on 8 well Permanox chamber slides as described above for transfection experiments. Transient transfections were conducted using Gene porter transfection reagent according to protocols (Gene Therapy Systems, San Diego, CA). Cells were transfected with either EGFP (Empty Vector) or PKC β1-EGFP, PKC ε-EGFP, PKC δ-EGFP, PKC ξ-EGFP, depending upon the experiment. All experiments were performed 24 h after transfection. Transfection efficiency of 80–90% was achieved with PKC β1-EGFP, PKC ε-EGFP, PKC ζ-EGFP, and 60–70% with PKC δ-EGFP. Minimum of three experiments were performed for each PKC isoenzyme.

2.4. Cell activation and analyses of EGFP-tagged PKC

Transfected HCT116 cells expressing PKC-β1-EGFP, PKC ε-EGFP or PKC δ-EGFP were treated with 150–300 μM DCA, 1 μM PMA, and 150–300 μM UDCA alone for 2 h or pretreated with UDCA for 2 h and then treated with DCA or PMA. HCT116 cells transfected with PKC ζ-EGFP were treated with C2-Ceramide and dihydroceramide (control) at doses of 25–200 μM. It was observed that 300 μM was the optimal concentration of the bile acids and 2 h the optimal time to observe changes in all the PKC isoenzymes. SW480 cells and MCF7 cells were transfected with PKC-β1-EGFP and similarly treated. Fast rate acquisition photo microscopy using Photometrics KAF-0400, cooled digital CCD camera was used to observe the effects of treatments. For each experiment, 'resting' cells imply those untreated with bile acid or any other stimulus. PMA was used as a positive control for PKC activation and translocation, except in the case of PKC ζ (which is unresponsive to PMA) where C2-Ceramide was used. In experiments involving UDCA pretreatment of cells, the UDCA was washed out before stimulation of the cells with DCA.

After establishing the consistent pattern of effects of treatment by real time photomicroscopy, cells were prepared for subsequent examination. Cells were washed with sterile filtered PBS twice and fixed with 4% parafarmaldehyde in PBS and stained with tetramethylerhodamine isothionate (TRITC) conjugate of phalloidin (Sigma), for 20 min at room temperature to visualize PKC translocation to F-actin. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Slides were then mounted and examined under Nikon TE 300 inverted microscope equipped with Leica DC 100 color digital camera. Photomicrographs were taken with 100 X oil immersion lens.

2.5. PKC inhibitors

HCT116 cells, transfected with PKC β1 were treated with Calphostin C, at doses of 100–600 nM; Gö6976, 1–6 μM; Bisindolylmaleimide, 1–6 μM for half an hour and then stimulated with either PMA or DCA to elucidate the mechanisms involved in PKC translocation and its relevance to cell shape change.

2.6. Western blot analysis

To confirm the effects observed by fluorescence microscopy, subcellular fractions of PKC β1 transfected and treated cells were prepared using the protocol of Chakravarthy BR [29]. Protein concentrations were determined using the Lowery method. Equal amounts of proteins were separated on a 10% SDS-polyacrymide gel, electrotransferred onto polyvinyldene difluoride membrane (PVDF, Millipore Corp. Bedford, MA). Nonspecific binding of antibody was blocked by incubating blots with 5% non-fat milk in PBS with Tween-20 at room temperature for 1 h. Membranes were processed for immunoblotting by incubation with PKC β1-specific antibody (Zymed) followed by incubation with secondary antimouse HRP-conjugated antibody. Protein bands were visualized by enhanced chemiluminescence's (ECL) method (New England Biolabs, Hertfordshire, UK), with exposure time adjusted to ensure linear responses.

3. Results

3.1. UDCA treatment inhibits DCA and PMA-induced membrane translocation of endogenous PKC α, δ, and ε in HCT116

Both PMA 100 nM and DCA 300 μM caused translocation of the endogenous PKC α (Fig. 1A), PKC ε to the cell membrane and PKC δ to the nucleus (Fig. 1B and C). In all cases, this was inhibited by pre-treatment of the cells by 300 μM UDCA (UDCA-mediated inhibition of PMA-stimulated PKC δ and ε translocation not shown). UDCA was used in this concentration range as it has been reported that UDCA protects against membrane-damaging effects of more hydrophobic bile acids at millimolar concentrations [30–32]. In a recent study, 500 μM UDCA was used for ERK signalling in HCT116 cell lines [28]. C2-Ceramide (50 μM) caused translocation of the atypical PKC ζ isoenzyme to the nucleus (Fig. 1D); dihydroceramide, used as con-

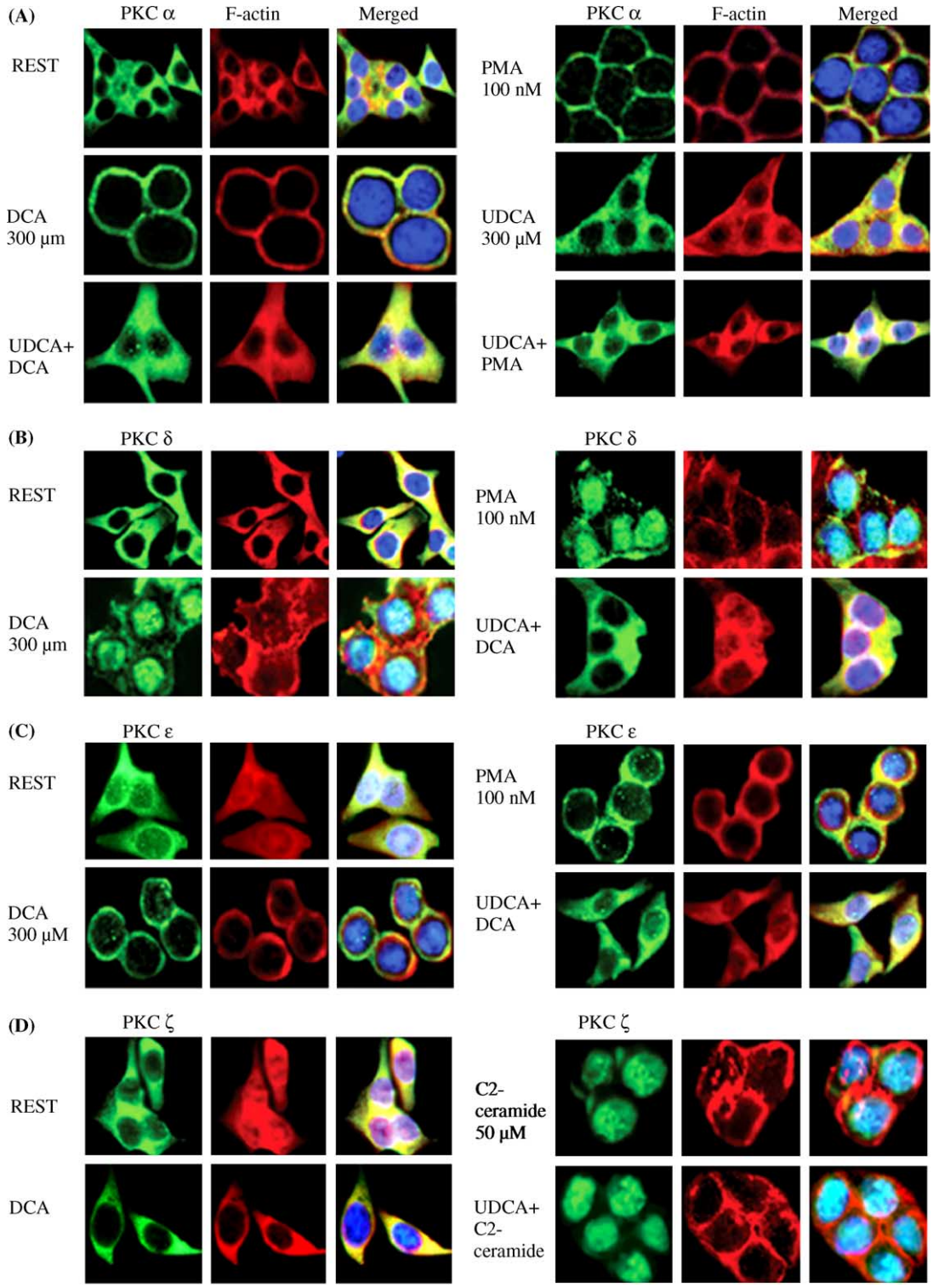

Fig. 1. Immunoflourescent staining of endogenous PKC isoenzymes in HCT116 cells. HCT116 cells were treated with ursodeoxycholic acid (U, UDCA), deoxycholic acid (DCA), phorbol myristate acetate (PMA) or C2-Ceramide for 2 h, or pre-treated with UDCA for 2 h followed by stimulation with DCA, PMA or C2-Ceramide. Immunofluorescent microscopy showed PMA and DCA caused translocation of PKC α and PKC ε to the membrane, and PKC δ to the nucleus (A–C). UDCA did not cause translocation of PKC α, ε or δ, but inhibited both PMA and DCA-induced translocation of PKC α, ε and δ. C2-Ceramide caused translocation of PKC ζ to the nucleus and DCA and UDCA had no effect on PKC ζ translocation (D). Untreated cells are labelled Rest. F-actin is stained using TRITC conjugate of Phalloidin and nuclei with DAPI. Merged photos show co-localization of PKC isoenzymes to the F-actin, seen as yellow.

trol did not cause PKC ζ translocation (data not shown). Neither UDCA nor DCA induced PKC ζ translocation while the C2-Ceramide-stimulated translocation was not inhibited by UDCA. Endogenous PKC β1 was not seen in these cells. The effect of PMA- or DCA-mediated PKC activation on cell morphology is illustrated by staining of the actin cytoskeleton.

3.2. UDCA treatment inhibits DCA-induced membrane translocation of EGFP tagged PKC β1 in HCT116 and SW480 transfected cells

Both DCA and PMA treatment of colon cancer cell lines, HCT116 and SW480 cells, transfected with PKC β1-EGFP resulted in translocation of PKC β1 from cytosol to the cell membrane (Fig. 2A and B). However, a higher dose of PMA, 1 μM was required to induce translocation of the EGFP-tagged PKC isoenzymes compared with endogenous PKC, where 100 nM PMA was sufficient to cause translocation of PKC α, δ and ε. Previous studies have demonstrated a requirement for micromolar doses of PMA to induce translocation of GFP-tagged PKC isoenzymes in cell model systems [33]. UDCA did not cause PKC β1 translocation and pre-treatment of both cell lines with UDCA inhibited DCA-induced membrane translocation of PKC β1. Pre-treatment with UDCA did not prevent PMA-induced membrane translocation of the PKC β1-EGFP in either cell lines (data not shown). There was no change in fluorescence pattern in cells transfected with the empty GFP vector whether treated with PMA or DCA. Localisation of PKC β1 to the membrane showed co-distribution with F-actin distribution pattern in cells treated with DCA or PMA. Similar observations were made in both colon cancer cells lines. These results demonstrate that DCA-induced PKC β1 activation was inhibited by UDCA pre-treatment.

To further elucidate the specificity of results obtained in colonic cancer cells, a breast cancer cell line, MCF7 were transfected with PKC β1-EGFP and then treated with UDCA, DCA or PMA described above. The MCF7 cell line was chosen for use in these experiments as control based on the hypothesis that MCF7 cells may not have bile acid receptors. Although PMA treatment caused membrane translocation of the PKC β1-EGFP in these cell lines, UDCA and DCA treatment had no effect on PKC β1 distribution (Fig. 2C).

To confirm these data, Western blot analyses (Fig. 3) were performed on subcellular fractionations of HCT116 cells transfected with PKC β1-EGFP and treated as above. In untreated cells and cells treated with UDCA, PKC β1-EGFP was found predominantly in the cytosolic fraction of the HCT116 cells. After DCA and PMA stimulation, PKC β1-EGFP was found predominantly in membrane fraction of the cells consistent with observation made by immunofluorescence. Pre-treatment of cells with UDCA inhibited the membrane translocation of PKC β1-EGFP caused by DCA.

3.3. UDCA treatment inhibits DCA-induced membrane translocation of novel PKC isoenzymes delta and epsilon-EGFP in HCT116 cells

Treatment of PKC ε-EGFP transfected cells with DCA or PMA resulted in translocation of PKC ε-EGFP from cytosol to the membrane (Fig. 4A). Pre-treatment of HCT116 cells with UDCA inhibited DCA-induced membrane translocation of PKC ε-EGFP reflecting inhibition of PKC ε-EGFP activation. Prior treatment with UDCA did not prevent PMA-induced membrane translocation of the PKC ε-EGFP (data not shown). The distribution of PKC ε also appeared to correspond with the F-actin distribution pattern.

In PKC delta transfectants, PMA and DCA treatment resulted in translocation of PKC δ-EGFP to the cell membrane as well as the nucleus, though the effect of DCA was much slower, occurring in 1–2 h compared with PMA, which caused PKC δ-EGFP translocation in a few minutes. Pre-treatment of cells with UDCA inhibited the PKC δ-EGFP translocation caused by DCA (Fig. 4B) but not by 1 μM PMA (data not shown).

Cells transfected with the atypical isoenzyme PKC ζ-GFP were treated with C2-Ceramide, PMA, DCA or UDCA, with or without pre-treatment with UDCA (Fig. 4C). C2-Ceramide caused translocation of PKC ζ-EGFP to the cell membrane and also to the nucleus at a higher dose of 200 μM compared with endogenous PKC ζ. DCA, UDCA or PMA treatment, on the other hand had no effect on PKC ζ-EGFP at the doses described. Pre-treatment of cells with UDCA did not inhibit C2-Ceramide -induced PKC ζ-EGFP translocation.

3.4. Cell shape change in response to PKC activation and role of PKC inhibitors

In all experiments PKC activation either with DCA or PMA caused cell shape change and rounded phenotypes were observed in association with PKC translocation to the membrane and F-actin distribution. To further elucidate the specific role of PKC in this PMA- or DCA-induced change in cell morphology, PKC inhibitors, Calphostin C, Indolocarbazole (Gö6976), Bisindolylmaleimide were used. Calphostin C and bisindolylmaleimide are competitive inhibitors of the enzyme, bisindolylmaleimide acting as a competitive inhibitor of the ATP-binding site while calphostin C competes at the binding site of diacylglycerol and phorbol esters. Gö6976 selectively inhibits calcium-depen-

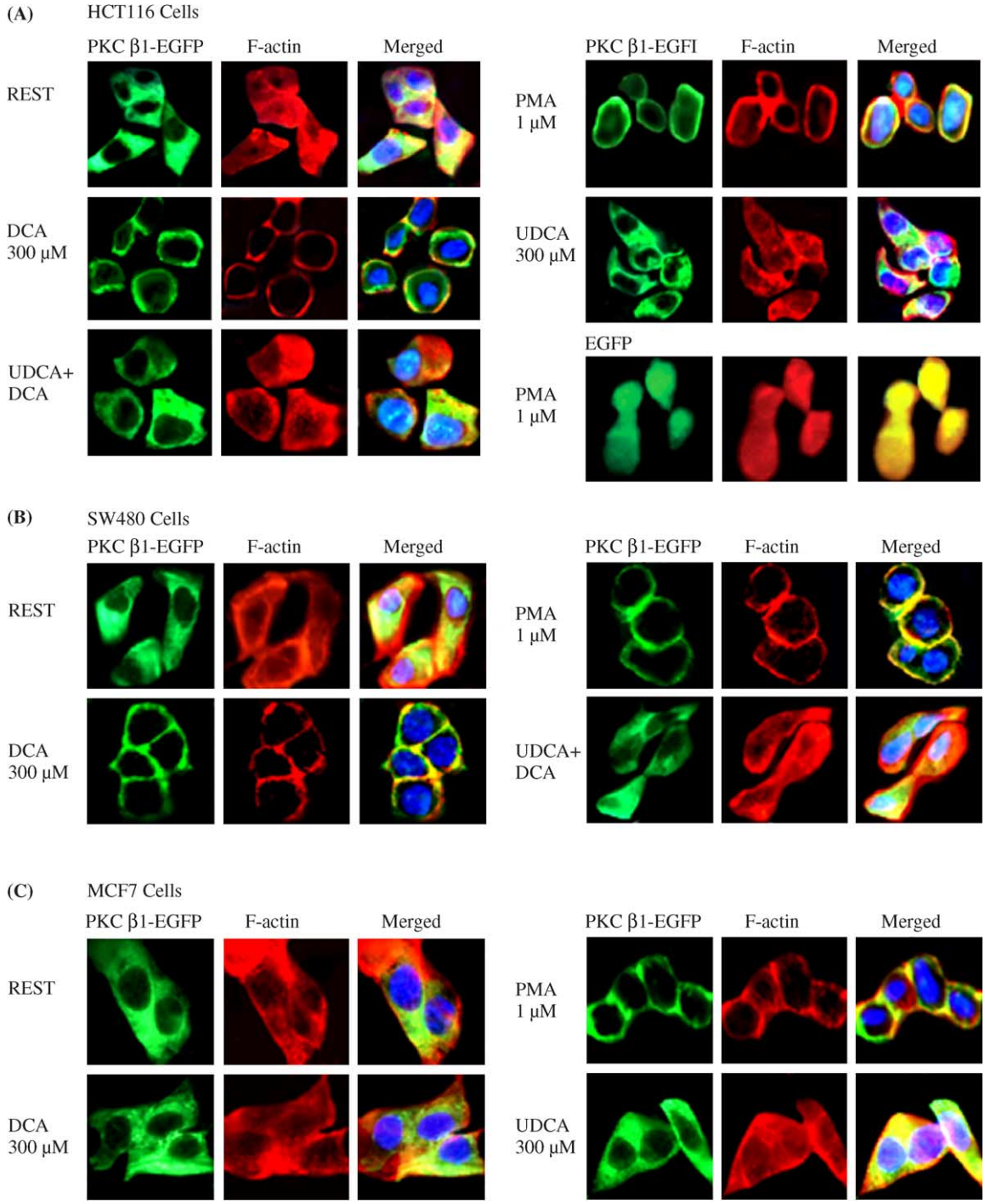

Fig. 2. Subcellular localization of transfected PKC β1-EGFP in HCT116, SW480 and MCF7 cells as determined by immunofluorescence. HCT116, SW480 and MCF7 cells transfected with enhanced green fluorescent protein (EGFP) or PKC β1-EGFP for 24 h, then either treated with UDCA, DCA or PMA for 2 h, or pre-treated with UDCA for 2 h followed by stimulation with either DCA or PMA. UDCA inhibited DCA-induced PKC β1-EGFP translocation (A, B). No effect of DCA or UDCA treatment was seen on PKC β1-EGFP in the MCF7 breast cancer cell line (C). F-actin is stained with TRITC conjugate of Phalloidin and nuclei with DAPI. Translocation of PKC β1-EGFP to membrane and F-actin is clearly seen in DCA and PMA activated cells. UDCA + DCA, denotes pre-treatment of the cells with 300 μM UDCA, followed by activation with DCA. Untreated cells are labelled as Rest. Empty Vector (EV), indicates cells transfected with EGFP alone. EGFP transfected cells were treated with either DCA or PMA and were used as controls in all experiments.

dent PKC isoenzymes α and β. The effect of these PKC inhibitors on cell shape and inhibition of cell rounding shown in Table 1.

High concentrations of PKC inhibitors were required to block PMA-induced PKC β1-EGFP translocation and block cell shape change. However, DCA-induced

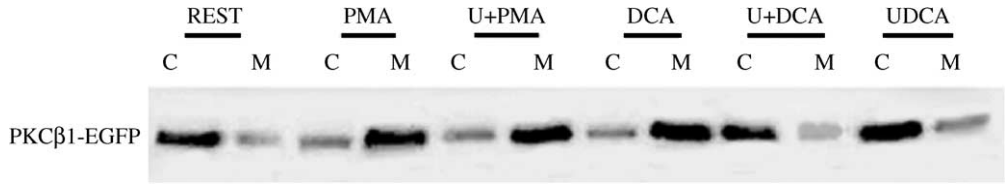

Fig. 3. Subcellular localization of PKC β1-EGFP in HCT116 cells as determined by Western blot analysis. Cells were transfected with PKC β1-EGFP and treated with UDCA, DCA or PMA as shown. Cellular proteins were then fractionated into cytosol and membrane fractions as described in materials and methods. Equal amounts of proteins were separated on a 10% SDS-polyacrymide gel, electrotransferred onto PVDF membrane. Membranes were incubated with PKC β1-specific antibody followed by incubation with secondary anti-mouse HRP-conjugated antibody. Protein bands were visualized by enhanced chemiluminescence (ECL). U, UDCA (ursodeoxycholic acid), DCA (deoxycholic acid), PMA (phorbol myristate acetate).

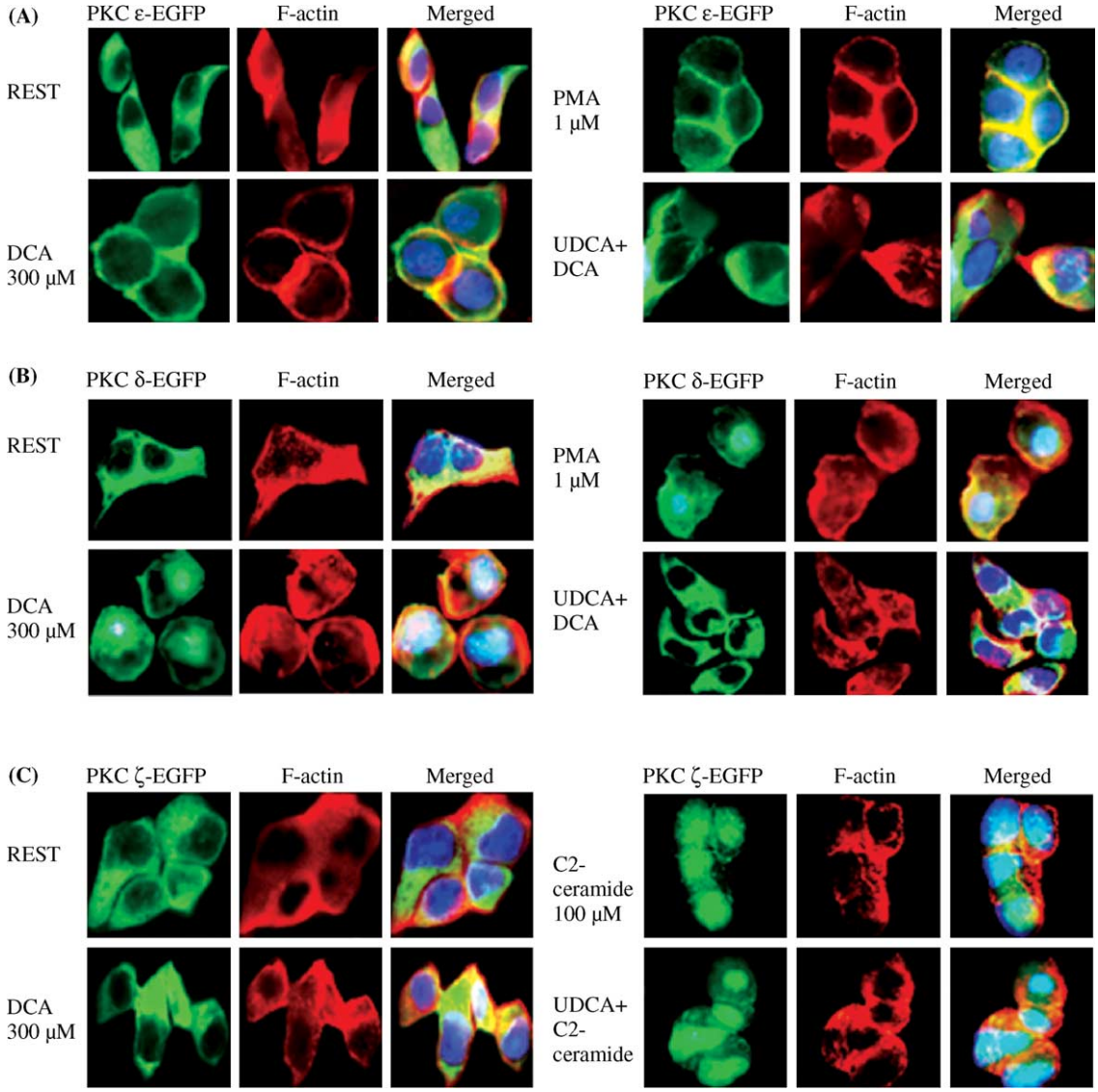

Fig. 4. Subcellular localization of transfected PKC ε-EGFP, PKC δ-EGFP and PKC ζ-EGFP in HCT116 cells as determined by immunofluorescence. HCT116 cells transfected with EGFP or PKC ε-EGFP, PKC δ-EGFP or PKC ζ-EGFP. Twenty-four hours post-transfection, cells were either pre-treated with UDCA for 2 h followed by stimulation with DCA, PMA or C2-Ceramide or they were treated with DCA, PMA, C2-Ceramide or UDCA alone. Untreated cells are labelled as Rest. UDCA inhibited DCA but not PMA-induced PKC ε-EGFP and PKC δ-EGFP translocation (A, B). UDCA and DCA had no effect on PKC ζ-EGFP translocation (C).

Table 1
Effect of PKC Inhibitors on PKC β1 translocation and its relationship to cell shape change

	PMA-induced PKC β1 translocation	Change in cell shape	DCA-induced PKC β1 translocation	Change in cell shape
Calphostin C				
100 nM	−	−	+	+
200 nM	−	−	+	+
400 nM	+	+	+	+
600 nM	+	+	+	+
Gö6976				
1 μM	−	−	−	−
2 μM	−	−	−	−
4 μM	+	+	+	+
6 μM	+	+	+	+
Bisindolylmaleimide				
1 μM	−	−	−	−
2 μM	−	−	+	+
4 μM	+	+	+	+
6 μM	+	+	+	+

Symbols: −, translocation of PKC β1 and change in cell shape not inhibited; +, translocation of PKC β1 and change in cell shape inhibited. Inhibition of PKC β1 translocation by PKC inhibitors corresponds with inhibition of cell shape change.

shape change was inhibited by concentrations of PKC inhibitors predicted to inhibit PKC activation. These data may reflect the higher dose used for PKC β1-EGFP and potency of PMA in mediating PKC activation.

4. Discussion

UDCA is used therapeutically in clinical practice in order to modify the biochemical parameters of cholestasis in various cholestatic disorders including primary biliary cirrhosis and primary sclerosing cholangitis [34]. UDCA is a hydrophilic non-toxic bile acid, and it becomes the predominant bile acid in serum and bile by competitively inhibiting the ileal absorption of natural, toxic endogenous bile salts after oral administration [35,36]. Compared with chenodeoxycholic acid and deoxycholic acid, UDCA is relatively hydrophilic, a chemical property suggested to contribute to its cytoprotection against these more hydrophobic membrane-damaging bile acids [37].

In this setting there is some evidence that UDCA may be protective in reducing the risk of human colorectal cancer associated with inflammatory bowel disease (IBD). In animal models, UDCA has also been shown to inhibit the development of malignant disease [38,39]. UDCA treatment reduces hepatic carcinogenesis in mice fed a diet supplemented with the carcinogen diethylnitrosamine (DEN) [40]. Rats treated with azoxymethane (AOM) and intrarectal *N*-methylnitrosourea and fed a diet supplemented with UDCA also had a significant reduction in aberrant crypt formation (ACF) and a lower incidence of colon tumours [38,41]. In another animal model of colonic cancer, UDCA significantly reduced faecal and colonic concentration of deoxycholic acid (DCA) thus inhibiting its ability to mediate potentially toxic effects in pathogenesis of colonic polyps and tumours [42]. More recently, it has been formally demonstrated that UDCA has an additive chemopreventive effect with Sulindac on colonic adenomas in a murine model of adenomatous polyposis [43] and of chemical induced carcinogenesis in the rat [44]. UDCA treatment is associated with a reduction in the rate of malignant progression in patients with ulcerative colitis and primary sclerosing cholangitis, a clear pre-malignant combination [45], in which elevated particulate PKC activity has previously been demonstrated [46]. In addition, there are anecdotal reports that UDCA may limit the growth of adenomas in Familial Adenomatous Polyposis in humans [47]. Hence there is now compelling data that suggests that UDCA is chemopreventive for colorectal cancer in both animal and human models. However, the mechanism of such chemoprevention is currently poorly understood.

PKC isoforms are the major receptor for the tumour-promoting phorbol esters and are therefore thought to play an important role in signal transduction and carcinogenesis [5]. Diacyglycerol (DAG), produced by the receptor-coupled hydrolysis of membrane phospholipids, activates PKC [48]. Phorbol esters bind to the DAG binding site of the regulatory domains of all but the atypical PKC isoenzymes and activate their catalytic activity [49]. DCA has previously been reported to induce PKC activation [15] and evidence for tumour promoting effects of DCA comes from both human and animal studies. Hence, we investigated the hypothesis that the chemopreventive effects of UDCA could derive from inhibition of DCA-induced PKC activation. In the current study, we have examined PKC translocation in response to DCA both using immunofluorescence for endogenous PKC and analyses of GFP tagged PKC isoenzymes. In untreated cells, DCA induced translocation of multiple PKC isoenzymes to subcellular sites. UDCA on the other hand did not cause PKC translocation; rather it inhibited the translocation of the endogenous PKC α and PKC ε and PKC δ caused by DCA and PMA at a dose of 300 μM and 100 nM, respectively. In case of transfected PKC, pre-treatment with UDCA inhibited DCA-induced translocation of PKC β1, ε and δ but did not inhibit PMA-induced translocation of these PKC isoenzymes, as a higher dose of PMA, 1 μM, was required for the optimal translocation of EGFP tagged PKC isoenzymes. Similarly a higher dose of C2-Ceramide was required for transfected PKC ζ translocation compared with endogenous PKC ζ. The reason for this effect is not clear. The concentration of bile acid in the colon varies with the fat content of the

diet and it has been reported that subjects on high fat diet can have as high as 800 μM DCA concentration [50].

A second observation was the finding that cells treated with PMA or DCA showed significant shape change with appearance of rounded phenotypes of the cells. The PKC inhibitors inhibited this change in cell shape. Cytoskeletal structures including F-actin play an important role in cell shape and migration as well as their adherence to matrix through connections with surface molecules. Thus conformational changes and remodelling of F-actin induced by the activation of PKC might have important implications in cell polarity, cell spreading, tight junction function and ultimately cancer cell growth and migration [51]. Specifically the maintenance of cell polarity has been associated with cell differentiation in other systems and the alteration of normal patterns of intracellular contact may play a significant role in the development of malignant change in cultured cells. In these experiments indirect data from PKC β1 transfectants suggest that the effects of DCA on shape change may be mediated through PKC. Firstly, DCA induced translocation of PKC isoenzymes to locations adjacent to the F-actin ring. Secondly, protein kinase C inhibitors blocked the development of shape change in the cell lines examined. Though other PKC isoforms may also be responsible for these conformational changes in F-actin and phenotype modifications, the PKC β1 isoform was used in this study because of the high transfection efficiency of this PKC isoenzyme in our cell lines.

UDCA has been shown to inhibit the development of malignancy in both animal and human studies. The results of a phase I multiple-dose clinical study of UDCA indicate that there are no serious adverse effects associated with UDCA treatment [52]. Our data suggest the possibility that UDCA might mediate its anticancer properties by direct prevention of PKC activation and the consequent downstream effects. It has been reported that secondary bile acids may increase PKC activity by facilitating its association with phospholipids or alternatively may increase DAG levels or intracellular calcium [53,54]. Chronic exposure could also lead to depletion of isoforms such as PKC-β. In this regard exposure to DCA on a chronic basis could be predicted to have similar effects in terms of tumour promotion to conventional PKC agonists such as PMA. Data from this study clearly demonstrate that this potentially tumorigenic pathway is not activated if DCA is co-administered with UDCA and suggest a possible mechanism for chemopreventive effects of UDCA in both human and animal studies.

Conflict of interest statement

None declared.

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