Bile acid deoxycholate induces differential subcellular localisation of the PKC isoenzymes $\beta_1, \epsilon$ and $\delta$ in colonic epithelial cells in a sodium butyrate insensitive manner

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Elevated levels of bile acids have been implicated in the abnormal morphogenesis of the colonic epithelium thus contributing to colorectal cancer (CRC). Alternatively sodium butyrate (NaB) produced by anaerobic fermentation of dietary fibre is regarded as being protective against colon cancer. Bile acids such as deoxycholic acid (DCA) are thought to mediate some of their actions by differentially activating protein kinase C (PKC). We examined the effects of DCA on the subcellular localisation of PKC-$\beta_1,-\epsilon$ and $-\delta$ and whether these responses could be modulated by NaB. HCT116 cells endogenously express PKC-$\epsilon$ and $-\delta$ but not PKC-$\beta$. DCA treatment results in endogenous PKC-$\epsilon$ translocation but not PKC-$\delta$ after 1 hr. To study the subcellular localisation of PKC isoforms in response to DCA in real time, PKC-$\beta_1$, PKC-$\epsilon$ and PKC-$\delta$ functionally interact green fluorescent protein (GFP) fusion constructs were used. Stimulation with 300 $\mu$M DCA induces rapid translocation of PKC-$\beta_1$-GFP and PKC-$\epsilon$-GFP but not PKC-$\delta$-GFP from the cytosol to the plasma membrane in 15 min. Interestingly, pretreatment with 4mM NaB does not modify the response of the PKC isoenzymes to DCA as PKC-$\beta_1$-GFP and PKC-$\epsilon$-GFP translocate to the plasma membrane in 15 min whereas PKC-$\delta$-GFP localisation remains unaltered. Immunofluorescence shows that PKC-$\beta_1$-GFP and PKC-$\epsilon$-GFP cells treated with DCA colocalise with the cytoskeletal elements actin and tubulin adjacent to the plasma membrane. Our findings demonstrate that the differential activation of the PKC isoenzymes by DCA may be of critical importance for the functional responses of colonic epithelial cells. Supplementary material for this article can be found on the International Journal of Cancer website at http://www.interscience.wiley.com/plugins/0020-7136/suppmat/index.html

Key words: bile acids; protein kinase C; translocation; cytoskeleton; colon cancer

Colorectal cancer is the third most common cause of cancer deaths in developed countries.1,2 Bile acids, in particular deoxycholic acid (DCA), have been associated consistently with colorectal cancer risk. Bile acids, normal constituents of the gastro-intestinal tract, are synthesised in the liver as primary bile acids and are converted in the colon by enteric bacteria to secondary bile acids, of which DCA is the most potent.3,4 DCA is thought to act as a tumour promoter by altering intracellular signalling and gene expression5,6 and has been shown to inhibit p53 activation by ionising $\gamma$-radiation.6 DCA induction of AP-1 and COX-2 is mediated by activation of protein kinase C (PKC),7,8 which is a family of serine/threonine kinases that play a fundamental role in a variety of cellular processes such as signal transduction leading to cell growth and differentiation.8,9 Given that DCA levels in the large intestine may be modulated by dietary factors, it is conceivable that high fat/low fibre diets associated with colorectal cancer might impact on PKC activation over a sustainable period of time. Other dietary factors such as NaB, generated in the intestine from anaerobic fermentation of dietary fibre, may also be important in this context. NaB has been shown to suppress proliferation10–12 and induce differentiation11 in many colonic cell lines. In vitro experimental studies showed that alterations in the PKC status in colonic cells can augment a range of butyrate dependent cellular processes.13

PKC is a major target for deoxyglycerol (DAG), which is produced after agonist stimulated phospholipid metabolism14,15 and serves as a receptor to the tumour promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA). At least 12 isoforms of PKC have been identified to date and are classified into 3 categories based on their structure and their requirements for Ca$^{2+}$ and DAG for activation. PKC isoforms have been implicated in the homeostasis of the colonic epithelium including $\beta$-catenin stability16 as well as in malignant transformation through alterations in isoform activity.17,18 Decreased protein expression or mRNA abundance of various PKC isoforms, in particular PKC-$\beta$, has been reported widely in human colorectal tumours.17,18 Furthermore decreased PKC levels may be seen after prolonged activation with PMA.19,20 Reduced PKC-$\beta_1$ expression seems to be an early event in the pathogenesis of colon cancer as it is seen in human colorectal adenomas.17 PKC-$\beta_1$ seems to be preferentially lost in comparison to the novel isoforms such as PKC-$\epsilon$ and PKC-$\delta$, which were used for comparison purposes. Another classical PKC isoform, PKC-$\epsilon$, seems to play an important role in colon cancer progression and has been linked with integrin activation, tumour cell migration and loss of E-cadherin expression.21,22 Studies have demonstrated that colonic cells resistant to DCA-induced apoptosis, had reduced PKC-$\beta_1$ expression23 and therefore suggests a functional consequence for reduced PKC expression.

Given that the colonic epithelium is physiologically exposed to luminal contents such as DCA and NaB and that PKC signalling elements regulate many of their biological effects, we explored the hypothesis that DCA may induce PKC-$\beta_1$ translocation, an isoenzyme that is preferentially lost at the adenocarcinoma stage. A hallmark of PKC activation has been the translocation of its isoforms from the cytosol to the plasma membrane24 with persistent activation resulting in down regulation and loss of expression.19,20,25 We examine the effects of DCA on PKC isoform sublocalisation and whether NaB can modulate those responses. For the first time, the effects of the physiologically important bile acid DCA on the translocation of PKC-$\beta_1$, have been verified by GFP constructs in real time. We examined PKC-$\epsilon$ and PKC-$\delta$ as examples of functionally distinctive, endogenously expressed PKC isoforms. This experimental approach provides an insight into the involvement of luminal contents in altering PKC expression in a colonic environment.

Material and method

Cell culture and reagents

HCT116 were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were used only between passages 5–20. Cells were maintained in McCoy’s 5A medium at 37°C in a 5% CO2 atmosphere.

Abbreviations: CRC, colorectal cancer; DAG, diacylglycerol; DCA, deoxycholic acid; GFP, green fluorescent protein; NaB, sodium butyrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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Received 22 April 2004; Accepted after revision 8 October 2004

DOI 10.1002/ijc.20803

Published online 11 January 2005 in Wiley InterScience (www.interscience.wiley.com).

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humidified atmosphere containing 5% CO\textsubscript{2}. The media was supplemented with 10% FBS, 4 mM l-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO BRL, Grand Island, NY). PMA and DCA were obtained from Sigma Chemical Co. (St. Louis, MO). NaB was from Calbiochem (La Jolla, CA).

Subcellular fractionation and Western blot analysis

HCT116 cells were lysed in 20 mM Tris-HCL (pH 7.5), 1% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5 mM PMSF and 10 μg/ml Leupeptin (all reagents from Sigma) and sonicated for 30 sec. The cells were then boiled for 5 min and centrifuged at 12,000 rpm for 10 min to remove unlysed cells and nuclei. The supernatant was designated the total cell lysate. For subcellular fractionation HCT116 cells were lysed in ice-cold hypotonic buffer (1 mM NaCO\textsubscript{3}, 5 mM MgCl\textsubscript{2}, 1 mM PMSF, 10 μg/ml leupeptin) and sonicated for 1 min on ice and centrifuged at 600 g for 5 min at 4°C. Supernatant was centrifuged at 100,000g for 10 min and the supernatant was designated the cytosolic fraction. The pellet was resuspended in Buffer B (20 mM Tris-HCL [pH 7.5], 1% NP-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF and 10 μg/ml Leupeptin) and centrifuged at 100,000g for 30 min at 4°C and the supernatant was the membrane fraction. Equal amounts of proteins were separated on a 10% SDS-polyacrylamide gel and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), probed with mAb to PKC isoforms ε, δ (Transduction Laboratories, Lexington, KY) and β (Seikagaku America, Rockville, MD). The PKC isoforms were visualised using photo horseradish peroxidase detection system (New England Biolabs, Hertfordshire, UK).

Transfection of the PKC-GFP fusion constructs into cultured HCT 116 cells

The plasmids PKC-ε-GFP, PKC-δ-GFP and pEGFP-N1 were kindly provided by Dr. N. Saito. The PKC-δ-GFP plasmid was purchased from Clontech (Palo Alto, CA). HCT116 cells were cultured on 8-well Permanox glass chamber slides (Nunc, Naperville, IL) for 24 hr to 50–70% confluence. Transient transfection was conducted using Gene Porter reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer’s standard protocol. All experiments were carried out 24 hr after transfection with transfection efficiencies of >75%.

Visualisation of PKC-GFP translocation by digital fluorescent microscopy

HCT116 cells expressing PKC-β\textsubscript{1}, ε- and δ-GFP were washed with CO\textsubscript{2}-independent medium (GIBCO BRL) pre-warmed to 37°C. Translocation of each PKC isoform was observed after the addition of either 1 μM PMA or 300 μM DCA. To analyse the effects of NaB on HCT116 cells transfected with either EGFP (Empty Vector), PKC-β\textsubscript{1}-GFP, PKC-ε-GFP or PKC-δ-GFP were pre-treated with 4 mM NaB for 50 min. After stimulation the cells were washed once and then stimulated with 300 μM DCA in the presence of 4 mM NaB. All experiments were carried out at 37°C in CO\textsubscript{2}-independent media. For the real-time imaging of the PKC translocation, a Nikon Diaphot TE300 (Nikon Europe, Badhoevedorp, The Netherlands) inverted microscope equipped with a cooled CCD camera (Photometrics, Tucson, AZ) and fluorescence attachment with fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) optimised filter cubes was used initially. Sequential images were obtained using the Qwin-standard acquisition program. Eight-hundred millisecond exposure intervals were routinely used in all experiments. Dynamic PKC intracellular localisation was subsequently verified using the Live Cell Imager confocal microscopy workstation (Perkin-Elmer, Cambridge, UK).

Immunofluorescence of HCT116 cells expressing PKC-GFP

HCT116 transfected cells were treated with 300 μM DCA or 1 μM PMA for appropriate periods of time as determined by real time translocation experiments. Cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were washed twice with PBS and stained for 20 min at room temperature with a TRITC conjugate of phalloidin (Sigma) to observe the filamentous actin (F-Actin). After washing with PBS, the cells were permeabilised with 0.1% Triton X-100 at 40°C for 24 hr and stained with a mAb to α-tubulin (Sigma) and a 7-amino-4-methyl coumarin-3-acetic acid (AMCA) labeled secondary affinity purified Ab (Dako, Bucks, UK). Analysis of cell immunofluorescence on the slides and microphotography were carried out on using a 100× oil immersion lens and equipped with Leica DC-100 colour digital camera.

Results

Analysis of endogenous PKC expression and translocation in colon cancer cells

To determine whether PKC-β\textsubscript{1}, ε- and δ- are endogenously expressed in HCT116 cells, Western blot analysis was carried out on total cell lysates from untransfected cells and cells transfected with either PKC-β\textsubscript{1}-GFP, PKC-ε-GFP or PKC-δ-GFP. As a control, HCT116 cells were also transfected with the pEGFP plasmid, which encodes only the green fluorescent protein. A monoclonal anti-PKC-β\textsubscript{1} antibody recognises PKC-β\textsubscript{1}-GFP as a band at 105kDa but did not detect any endogenous expression of PKC-β\textsubscript{1} in the HCT116 cells as no band of 78kDa is observed (Fig. 1a). Monoclonal antibodies against PKC-ε and PKC-δ show that these isoforms are expressed endogenously in HCT116 cells as bands of the appropriate molecular weights are observed, 90 kDa and 78 kDa, respectively. In addition, HCT116 cells transfected with either PKC-ε-GFP or PKC-δ-GFP expressed endogenous and fluorescently tagged proteins that are revealed on Western blots as bands of the predicted higher molecular weight, 117 kDa and 105 kDa respectively (Fig. 1a). The appropriate expression of GFP and PKC-GFP is verified using an anti-GFP antibody that only detects a 27 kDa band corresponding to GFP in cells transfected with the pEGFP plasmid alone (data not shown). Likewise no catalytic fragments of native PKC or PKC-GFP are observed by Western blotting using antibodies specific to each isoform and GFP.

The potential of the bile acid DCA to induce endogenous PKC translocation in HCT116 cells was investigated. As a control for PKC activation, these cells are stimulated with 1μM PMA, which has previously been shown to induce translocation of PKC-ε and δ in other cell types.\textsuperscript{26–28} Subcellular fractionation clearly illustrates that treatment with PMA, for 1 hr, induces translocation of endogenous PKC-ε and δ from the cytosolic fraction to the membrane fraction (Fig. 1b). Treatment of HCT116 cells with 300 μM DCA induces endogenous PKC-ε but not PKC-δ membrane translocation demonstrating that the PKC isoenzymes are differentially activated by DCA (Fig. 1b).

Analysis of PKC-GFP isoform translocation after DCA and PMA stimulation

Because PKC-β\textsubscript{1} expression is lost early in the adenoma–carcinoma sequence, PKC-β\textsubscript{1} was exogenously expressed in HCT116 cells to elucidate the effects of DCA on PKC-β\textsubscript{1} expression in addition to PKC-ε and δ. PKC-β\textsubscript{1}-GFP fluorescence is observed predominantly in the cytosol. To determine the optimal concentration for our study, a DCA dose response curve was conducted in PKC-β\textsubscript{1}-GFP transfecteds. HCT116 cells transfected with PKC-β\textsubscript{1}-GFP cells were treated with varying concentrations of DCA ranging from 0–500 μM. Low concentrations of DCA such as 0–200 μM do not induce PKC-β\textsubscript{1}-GFP transfection (Fig. 2a, upper panel). DCA, at concentrations at and above 300 μM, induces PKC-β\textsubscript{1}-GFP transfected plasma membrane protein translocation after 15 min of stimulation (Fig. 2a, lower panel). High concentrations of DCA, in particular 500 μM after 3 hr of stimulation, are associated with dramatic morphological changes indicative of apoptosis such as membrane blebbing and are deemed unsuitable for...
GFP and PKC- these studies (Fig. 2). From this point on, 300 µM DCA was used for all subsequent studies unless otherwise stated.

Treatment with 1µM PMA induces rapid translocation of PKC-β1-GFP and PKC-ɛ-GFP from the cytosol to the plasma membrane in 15 min (Table I; also see supplementary web movies PMA-beta, PMA-Epsilon on the International Journal of Cancer website at http://www.interscience.wiley.com/ipages/0020-7136/suppmat/index.html). PMA induces translocation of PKC-β-GFP to the plasma membrane and perinuclear region (Table I; also see supplementary web movie PMA-delta). Treatment with 300µM DCA induces plasma membrane directed translocation of PKC-β1-GFP and PKC-ɛ-GFP but not PKC-δ-GFP in 15 min (Table I; also see supplementary web movies DCA-beta, DCA-Epsilon). PKC-β1-GFP and PKC-ɛ-GFP remained at the membrane even 2 hr after treatment with DCA (data not shown). Control HCT116 cells, transfected with pEGFP were treated with 1 µM PMA and 300 µM DCA and cytosolic (c) and membrane (m) fractions were obtained as described in materials and methods. HCT116 were treated for 1 hr and fractionated. Immunoblot analysis was carried out using antibodies specific to PKC-ε and PKC-δ.

NaB did not modulate PKC translocation events in response to DCA

To determine whether NaB could modulate the responses of the PKC isoforms to DCA activation, cells were pretreated for 50 min NaB. As NaB is known to occur at millimolar concentrations in the gastrointestinal tract and to modulate the colonic cell proliferation and differentiation at millimolar concentrations, therefore NaB was used at a concentration of 4 mM. NaB alone does not induce activation or translocation of the PKC isoforms to the plasma membrane as observed with DCA treatment (Table II). DCA, in the presence of NaB, induces PKC-β1-GFP, PKC-ɛ-GFP but not PKC-δ-GFP plasma membrane directed translocation in 15 min (Table II, middle row). PKC-δ-GFP fluorescence remains unaltered even 1 hr after treatment with DCA in the presence of NaB. In cells transfected with EGFP, the pattern of GFP localisation remains unaltered and is expressed homogeneously throughout the cell (Table II).

Association of the PKC isoforms with the cytoskeletal elements actin and tubulin

Upon stimulation, rounding of cells is observed. This has been reported in other cell types. In A7v5 vascular smooth muscle cells, PMA induces PKC activation resulting in actin reorganisation. In HCT116 cells in the resting state, actin is distributed at the plasma membrane and in the stress fibres bundles. The microtubules are observed throughout the whole cell as well as the perinuclear region (Figs 3–6, upper panels). DCA treatment induces some reorganisation of the cytoskeletal element, F-Actin, in cells transfected with the empty vector, EGFP. PMA induces F-Actin and microtubule reorganisation (Fig. 3). HCT116 cells transfected with PKC-β1-GFP or PKC-ɛ-GFP are treated with 1 µM PMA (as a control) and 300 µM DCA. Upon treatment with DCA, remodeling of the actin cytoskeleton is observed in HCT116 cells transfected with either PKC-β1-GFP or PKC-ɛ-GFP. DCA-induced translocation of PKC-β1-GFP may be associated with localisation of the actin ring (Fig. 4), which is also observed with PMA (Fig. 4). In PKC-ɛ-GFP transfectants, stimulation with DCA or PMA results in attenuation of the actin stress...
fibres bundles, which are condensed around the periphery of the cell (Fig. 5). In resting PKC-δ-GFP transfected cells, some colocalisation of PKC-δ-GFP with actin is observed at the cell periphery. Nevertheless, PMA-induced PKC-δ-GFP translocation results in a specific and consistent pattern of association of the PKC isoform with the actin cytoskeleton, as colocalisation is evident (Fig. 6, merged image). DCA does not induce marked morphological changes such as disassembly of the actin stress fibres in PKC-δ-GFP transfected cells as observed with PMA treatment (Fig. 6). Partial colocalisation of PKC-δ-GFP with actin microfilaments adjacent to the plasma membrane is observed after DCA treatment, which is similar to that in the resting PKC-δ-GFP cells. Stimulation of PKC-δ-GFP with DCA does not induce a similar pattern of cytoskeletal rearrangement to that induced by the activation of PKC-

TABLE I – DCA INDUCES PLASMA MEMBRANE LOCALISATION OF PKC-β1-GFP AND PKC-ε-GFP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PKC-β1-GFP</th>
<th>PKC-ε-GFP</th>
<th>PKC-δ-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>Cytosol and nucleus</td>
<td>Cytosol</td>
<td>Cytosol</td>
</tr>
<tr>
<td>PMA Treated</td>
<td>Cytosol and nucleus</td>
<td>Plasma membrane</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>DCA Treated</td>
<td>Cytosol and nucleus</td>
<td>Plasma membrane</td>
<td>Plasma membrane</td>
</tr>
</tbody>
</table>

HCT116 cells were transfected with EGFP, PKC-β1-GFP, PKC-ε-GFP or PKC-δ-GFP as indicated in the top row. The left column represents the treatment conditions. Transfected HCT116 cells were either unstimulated or stimulated with 1 μM PMA or 300 μM DCA for 15 min and their subcellular localisation was subsequently analysed. The localization of the PKC isoforms after 15 min is indicated. The results shown here are representative of more than 5 independent experiments, all of which gave consistent results.

TABLE II – NaB DOES NOT ALTER DCA-INDUCED PLASMA MEMBRANE TRANSLLOCATION OF PKC-β1-GFP AND PKC-ε-GFP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PKC-β1-GFP</th>
<th>PKC-ε-GFP</th>
<th>PKC-δ-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DCA</td>
<td>Cytosol and nucleus</td>
<td>Cytosol</td>
<td>Cytosol</td>
</tr>
<tr>
<td>DCA-treated (15 min)</td>
<td>Cytosol and nucleus</td>
<td>Plasma membrane</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>DCA-treated (1 hr)</td>
<td>Cytosol and nucleus</td>
<td>Plasma membrane</td>
<td>Plasma membrane</td>
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</table>

HCT116 cells were transfected with EGFP, PKC-β1-GFP, PKC-ε-GFP or PKC-δ-GFP as indicated in the top row. The left column represents the treatment conditions. Transfected HCT116 cells were pretreated for 50 min with 4 mM NaB and then stimulated with 300 μM DCA or left unstimulated. Their subcellular localisation was subsequently analysed at 15 min and 1 hr after the addition of DCA. The subcellular localization of each PKC isoform at these time points is indicated. Results are representative of more than 5 experiments.
1-GFP and PKC-β1-GFP by DCA (Fig. 6). Alterations to the cytoskeletal elements are also associated with the rounding of cells after stimulation with DCA, suggesting that the remodeling in F-Actin may be a major contributing factor to the altered morphology of the DCA treated PKC-β1-GFP and PKC-ε-GFP transfectants.

Translocation of PKC isoenzymes involves extensive reorganisation of the actin cytoskeletal elements, however similar alterations in the microtubule network are also observed after treatment with DCA. In the resting PKC-β1-GFP and PKC-ε-GFP transfectants, the microtubules appear as an intricate network concentrated around the perinuclear region. After DCA-induced PKC-β1-GFP and PKC-ε-GFP translocation, the microtubules undergo a dramatic redistribution to the periphery of the cell such that they seem to associate with cytoskeletal elements adjacent to the plasma membrane (Figs. 4,5). Identical structural alterations in the microtubule network in both PKC-β1-GFP and PKC-ε-GFP cells are observed upon treatment with PMA (Figs. 4,5). In HCT116 PKC-δ-GFP transfectants, the microtubule network is substantially remodeled from a highly organised network to a more diffuse distribution after PMA treatment (Fig. 6). Upon DCA stimulation, negligible rearrangement of the microtubule networks is observed in cells transfected with PKC-δ-GFP. The pattern of microtubule rearrangement in the PKC-δ-GFP transfectants differs from that induced in PKC-β1-GFP and PKC-ε-GFP transfectants.

**Discussion**

Extensive research has focused on the ability of bile acids to affect multiple signalling pathways. Our study investigated the potential of the bile acid DCA to modulate the expression of PKC isoforms, PKC-β1,-ε and -δ in colon cancer cells. To date, the involvement of the PKC isoforms in bile acid mediated events has been implicated by the use of inhibitors specific for each PKC isoform. The involvement of the PKC isoforms was investigated in a human colonic adenocarcinoma cell line, HCT116. In vivo, during malignancy, a decrease in total protein kinase C levels of expression and activity as compared to adjacent normal mucosa has been noted.17,36 In particular, a decrease in the levels of PKC-β1 has been reported in human adenomas.17 Western blot analysis of the HCT116 cells exhibited no endogenous expression of PKC-β1. This is in agreement with results published by other investigators who could not detect PKC-β1 expression in HCT116 cells.37 We demonstrate that transfection of these cells with PKC-β1-GFP construct restores expression of the PKC-β1 isoenzyme (Fig. 1a). Endogenous expression of PKC-ε and -δ as well as their GFP counterparts is observed in this cell line (Fig. 1a). No catalytic products of native or transfected PKCs are observed during Western blotting indicating the appropriate expression of both the native and the fluorescent PKC isoforms and preservation of their functional properties (Fig. 1a). To test our hypothesis that DCA could modulate PKC expression and translocation, we incubated parental cells with DCA and preformed subcellular fractionation to examine the intracellular localisation of PKC-ε and -δ. DCA induces translocation of endogenous PKC-ε but not PKC-δ from the cytosol to the membrane (Fig. 1b). Consequently DCA has the ability to selectively modulate the intracellular localisation of diverse PKC isoenzymes.
Fluorescently tagged PKCs are utilised in our study to monitor the dynamics of translocation in response to treatment with PMA and DCA in real time. Unique translocation patterns of PKC-β/H9280-GFP and PKC-γ/H9253-GFP have been demonstrated in response to distinct fatty acids in CHO-K1 cells. In the same cell type, translocation and activation of PKC-γ/H9254-GFP has been reported in response to PMA, ATP and Hydrogen peroxide (H₂O₂) stimulation. Similarly, the PKC-GFP fusion proteins exhibit a remarkably different pattern of distribution in comparison to the distribution of GFP alone (Table I). The GFP localisation pattern is unaltered after treatment with either PMA or DCA, remaining uniformly distributed throughout the cytoplasm and the nucleus. PMA stimulates rapid translocation of PKC-β/GFP and PKC-ε/GFP to the plasma membrane in 10 min (Tables I,II) and remains at a membrane location for at least 2 hr (data not shown). PMA induces translocation of cytoplasmic PKC-δ-GFP to the perinuclear region as well as the plasma membrane (Table I). In contrast, an equal concentration of PMA induces PKC-δ-GFP translocation to the same target sites after 5 min in CHO cells. These data correlate strongly with previous studies showing that the kinetics of PKC translocation to a given activator is both isoform- and cell type-specific.

PKC-β and -ε respond to a physiologically relevant concentration of DCA, 300 μM. DCA, similar to the PMA, induces rapid translocation of PKC-β1-GFP and PKC-ε-GFP from the cytosol to the plasma membrane in 15 min (Table I). In contrast, DCA does not induce translocation of PKC-δ-GFP (Table I) but the isoform resides at the plasma membrane after 18 hr (data not shown). Apoptotic changes are also observed at 18 hr and therefore the PKC-δ-GFP membrane association may not be a DCA specific effect. Worthy of note is that the endogenously expressed isoenzymes, PKC-ε and -δ, elicit similar responses to PMA and DCA as their GFP fusion counterparts (Fig. 1, Table I), which demonstrates the specificity of the PKC-GFP fusion proteins responses.

Our findings clearly demonstrate that DCA can differentially regulate subcellular localisation of diverse PKC isoforms. Although the delayed response of PKC-δ-GFP to DCA might have more profound indirect effects in the long term such as apoptosis, the early activation of PKC-β1 and PKC-ε emphasises the importance of these 2 isoforms in early DCA mediated intracellular events. The rapid activation of PKC isoforms by DCA, the levels of which are elevated in colon cancer patients, may result in their subsequent down regulation and depletion from the colonic epithelium although the effects may be differential for each isoform. Downregulation of the PKC isoforms due to persistent activation by the tumour promoter PMA and other stimuli has been reported in several cell types. Interestingly, observations from a cDNA microarray study showed that PKC-β1 mRNA is downregulated in HCT116 cells resistant to apoptosis. In Ha-ras transformed colonic epithelial cells, PKC-ε expression is increased 5-fold. Overexpression of PKC-ε is associated with morphology changes such as anchorage independent colony formation and increased saturation densities. In other malignancies, prolonged activation of PKC-ε is associated with downregulation of PKC-ε and increased cell survival and clonal expansion.
In light of the protective role NaB is thought to have in colon tumourgenesis, it was of crucial importance to investigate if NaB could modulate the responses of the PKC isoforms to DCA. McBain et al.12 speculated that activation of PKC by PMA may play a central role in determining the sensitivity of the colon cancer cells to NaB-induced apoptosis. The synergistic effect of PKC activation and NaB has been reported for other biological effects of NaB, such as enhanced differentiation, in other colonic cancer cell lines. 13,48 McMillan et al.49 demonstrated that, in AA/C1 colon cancer cells, NaB-induced apoptosis is dependent upon PKC-H9254 activation and translocation to the membrane. In our study, NaB alone does not induce translocation of the PKC isoforms to any particular subcellular site (Table II). In the presence of NaB, DCA is uninhibited and continues to induce translocation of PKC-H9252-GFP and PKC-H9280-GFP but not PKC-H9254-GFP in 15 min (Table II). Furthermore, no apoptotic changes were observed at these time points and even 1 hr after treatment. Stimulation with NaB does not modulate the response of the PKC isoenzymes to DCA, suggesting that DCA may be able to circumvent the action of NaB and thus highlighting the critical importance of DCA in the pathogenesis of colorectal cancer.

Resting HCT116 cells transfected with PKC-β1-GFP, PKC-ε-GFP or PKC-δ-GFP show no significant morphological differences to resting untransfected or cells transfected with empty vector. Upon stimulation, some of the transfectants undergo dramatic morphological changes such as rounding, which is associated with reorganisation of cytoskeletal microfilaments. In NIH 3T3 cells overexpressing PKC-γ, PMA treatment causes PKC-γ overexpressers to rapidly round up. In the same cells, PMA stimulation of PKC-βH causes pronounced ruffling at the plasma membrane and the cells flattened out.50 Translocation of the PKC isoforms to the membrane and the coordinate redistribution of the actin and microtubule networks also to the membrane suggest possible functional associations. In T84 intestinal epithelial cells, treatment with PMA is associated with the translocation of PKC-ε from the cytosol to the plasma membrane as well as remodeling of the actin cytoskeleton. This synchronized association facilitates basolateral endocytosis.51 In the context of the present study, it is likely that remodeling of both cytoskeletal networks by DCA ensures the rapid trafficking of the PKC isoforms to the cell periphery and therefore in closer proximity to their intracellular targets. Previous work has demonstrated that PKC activation by PMA induces reorganisation of the actin cytoskeleton, which is associated with alterations in the Rho signaling pathway35 modulating multiple aspects of cell morphology and proliferation.52,53 Bile acid-stimulated invasion of colonic carcinoma cells is shown to be dependent upon the Rho pathway. Specifically treatment of HCT-8/E11 colon cells with 250 μM DCA for 10 min leads to an 18-fold increase in the amount of active Rho-A and Rac-1.54 It is possible that DCA associated cytoskeletal changes in HCT116 cells may involve alterations in the Rho GTPase signaling cascade.

Conversely DCA did not induce explicit reorganisation of either cytoskeletal element in PKC-δ-GFP transfectants as observed with PKC-β1-GFP and PKC-ε-GFP (Fig. 6). PKC-δ-GFP expressing cells, after PMA treatment, exhibited a more rounded morphology, which is potentially due to the reorganisation of the actin cytoskeleton. In BaF3 cells, a murine pre-B lymphoid cell line, activation of PKC-δ by PMA resulted in the dissolution of actin based membrane ruffles.54 Control HCT116 cells, containing EGFP
alone and expressing endogenous PKC-ε and PKC-δ, experienced limited changes in the cytoskeletal systems in response to stimuli, whereas cells overexpressing these isoforms, PKC-ε-GFP and PKC-δ-GFP transfectants, underwent substantial cytoskeletal reorganisation.

In summary, our study provides the first direct evidence that in living cells the bile acid DCA differentially activates the PKC isoforms. Alterations in the isoforms activity are likely to have an essential role in the development and maintenance of the abnormal morphogenesis of the colonic epithelium. The fact that a naturally occurring substance, DCA, may trigger PKC translocation in colon cancer cells is compelling evidence for the importance of DCA involvement in this malignant process. As PKC changes are early and DCA levels can be modulated by dietary factors, these mechanisms may be important in mediating the environmentally induced component of colorectal carcinogenesis.

Acknowledgements

We thank N. Saito for the generous gifts of the GFP plasmid constructs and technical advice regarding GFP technology. The Cancer Research Advancement Board, the Health Research Board and Enterprise Ireland supported this work. The equipment was supported by joint a Health Research Board/Wellcome Trust Grant.

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