Role of Protein Kinase C δ in the Age-Dependent Secretagogue Action of Bile Acids in Mammalian Colon

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Abstract

The role of specific PKC isoforms in the regulation of epithelial Cl⁻ secretion by Ca²⁺-dependent secretagogues remains controversial. In the developing rabbit distal colon, the bile acid taurodeoxycholate (TDC) acts via [Ca²⁺], to stimulate Cl⁻ transport in adult, but not in young, animals, whereas the PKC activator, phorbol dibutyrate (PDB), stimulates Cl⁻ transport at all ages. We tested the hypothesis that specific PKC isoforms account for the age-specific effects of TDC. The effects of conventional and novel PKC-specific inhibitors on TDC- and PDB-stimulated Cl⁻ transport in adult and weanling colonocytes were assessed using 6-methoxyquinolyl acetoethyl ester. In adult colonocytes, the cPKC inhibitor Gö6976 inhibited PDB, but not TDC action, whereas the cPKC and nPKC inhibitor Gö6850 blocked both TDC and PDB action. Additionally rottlerin and the PKCδ-specific inhibitor peptide (δV1-1) inhibited TDC- and PDB-stimulated Cl⁻ transport in adult colonocytes. Rottlerin also decreased TDC-stimulated short-circuit current in intact colonic epithelia. Only Gö6976, but neither rottlerin nor δV1-1 inhibited PDB-stimulated transport in weanling colonocytes. Colonic lysates express PKCα, -λ and -ι protein equally at all ages but not PKCγ or -θ at any age. Expression of PKCβ and PKCe protein was newborn>adult>weanling, whereas PKCδ was expressed in adult but not in weanling or newborn colonocytes. TDC (1.6-fold) and PDB (2.0-fold) stimulated PKCδ enzymatic activity in adult but failed to do so in weanling colonocytes. PKCδ mRNA expression showed age-dependence. Thus, PKCδ appears critical for the action of TDC in the adult colon, and its low expression in young animals may account for their inability to secrete in response to bile acids.

Keywords
epithelial chloride transport, taurodeoxycholate (TDC), signal transduction
Introduction

Neurohumoral agents, which act via intracellular calcium \([\text{Ca}^{2+}]_i\), are critical in fine-tuning epithelial fluid transport and are often linked to the protein kinase C (PKC) cascade (18, 38). The pathophysiological consequence of aberrations in this signaling is diarrhea. One class of \(\text{Ca}^{2+}\)-dependent secretagogues is bile acids, which are essential for fat digestion and absorption in the intestinal lumen. Under physiological conditions the mammalian intestine recycles the majority of the bile acids (>95%) back to the liver. While the colon can effectively handle the normal levels of bile acids that are excreted, excess bile acids in the colon cause fluid loss and prolonged exposure to bile acids promotes colon cancer (3, 17).

Conjugated or unconjugated dihydroxy bile acids, such as chenodeoxycholic acid or deoxycholic acid, stimulate \(\text{Cl}^\-) secretion in a variety of mammalian colonic preparations (20-22, 27, 44, 50, 69), in pancreatic ducts (46), in gallbladder-derived epithelium (12) and in cholangiocytes (60). Bile acids have a plethora of other actions ranging from carcinogenesis to mucin secretion and many of these functions involve PKC. There are at least 12 PKC isoforms, grouped into three major classes: conventional (cPKC: \(\alpha, \beta I, \beta II\) and \(\gamma\)); novel (nPKC: \(\delta, \epsilon, \eta, \theta; \mu, \nu\); viewed as a separate subclass or as PKD), and atypical (aPKC: \(\zeta, \lambda, \tau\)) (29, 45). A role for different PKC isoforms in bile acid action has been implicated in cholangiocarcinoma growth (1), mucin secretion in gallbladder (12), colon carcinogenesis models (33), and in the feedback inhibition of CYP 7A in hepatocytes (64). However, it is unclear if bile acids utilize the PKC pathway to stimulate \(\text{Cl}^\-) secretion. Interestingly the pharmacological activators of PKC, phorbol esters, have been demonstrated to regulate \(\text{Cl}^\-) secretion either by activating it (5, 38, 47, 57) or by inhibiting it (5, 8, 14, 72). These regulatory differences appear to be species-, cell-type- and isoform-specific. Interpretation is further complicated by the fact that the times of exposure to
phorbol esters can have differing effects since prolonged exposure to phorbol esters down-regulates the action of some, but not all, PKC isoforms. Thus, in primary rabbit and human colonocytes (57) and in the human colon carcinoma cell-line, HT-29cl.19A (2), short-term exposure (5 min) to phorbol esters increases Cl⁻ secretion. In contrast, in the T-84 cell-line, phorbol esters do not affect basal Cl⁻ secretion but attenuate cAMP-stimulated Cl⁻ secretion, by a mechanism linked to the PKCε-dependent internalization of the basolateral Na⁺-K⁺-2Cl⁻ cotransporter (19). In rats, estradiol inhibits female, but not male, rat colonic Cl⁻ secretion via the action of PKCδ (14). In pancreatic ductal cells, short-term exposure increases the magnitude of cAMP-stimulated CFTR currents, whereas prolonged exposure (consistent with PKC down-regulation) slowed CFTR current run-down suggesting that PKC affects stability of CFTR (72). Thus, in examining bile acid-mediated Cl⁻ secretion, the contrasting effects of different PKC isoforms have to be considered.

We (69) and others (21, 22) have demonstrated that, in colonic epithelia, bile acids activate phospholipase C (PLC) to increase inositol trisphosphate (IP₃) and [Ca^{2+}]. We also demonstrated that the secretagogue action of bile acids is segment-specific, occurring in the rabbit distal, but not proximal colon (69). Moreover this effect is age-specific, with bile acids evoking a response only in colonocytes of adult, but not in those of weanling or newborn animals (20, 69). Interestingly the recycling of bile acids is also age-dependent, with the neonatal mammal showing little and only passive absorption in the ileum (67). Therefore the refractoriness of the young animal to the secretory action of bile acids may serve as a protective mechanism against potentially deleterious luminal levels of this secretagogue.

In elucidating the basis of this refractoriness, we identified that the age-dependency shown by bile acids extends to other Ca^{2+}-dependent neurohumoral agents but that the Ca^{2+}
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ionophore A23187 (69) and phorbol esters (20) stimulate Cl⁻ transport at all ages. The refractoriness in the young animal to all tested Ca²⁺-dependent secretagogues and bile acids was due to the inability to generate IP₃ and increase [Ca²⁺]ᵢ (69). Interestingly, phorbol esters, but not bile acids, activate cPKC enzyme activity in colonocytes in vitro (69). This led us to conclude that at least cPKCs may not be involved in the secretagogue action of bile acids (69). However there is increasing evidence that the nPKCs and aPKCs play substantial roles in a number of biological processes, including in the actions of bile acids in other tissues (24, 29, 64).

The present study investigated if bile acid-stimulated Cl⁻ secretion in the rabbit colon involves any of the PKC isoforms and if the regulation of this cascade is age-dependent. The findings, for the first time, indicate that PKCδ is involved in bile acid-stimulated colonic Cl⁻ secretion and that the expression of PKCδ protein is a critical step in the age-related refractoriness to bile acid signaling in the mammalian colon.

Materials and Methods

Materials

Tissue culture media, Ham’s F-12 nutrient mixture, fetal bovine serum, TRIZOL reagents, SuperScript™ II RNase H Reverse Transcriptase, Oligo(dT)₁₂₋₁₈ Primer, RNaseOUT™, and Recombinant Ribonuclease Inhibitor were obtained from Invitrogen, Carlsbad, CA; sterile lactated Ringer's was from Baxter International Inc., Deerfield, IL; iQ SYBR Green supermix and gel electrophoresis supplies were from Bio-Rad Laboratories, Hercules, CA; RNA-later from Ambion, Austin, TX; 6-methoxy-quinolyl acetoethyl ester (MQAE) was from Molecular Probes Inc., Eugene, OR; diphenylamine-2 carboxylate (DPC) was from Aldrich, Milwaukee, WI; and taurodeoxycholate, Gö6983, Gö6850, Gö6976 were from Calbiochem, San Diego, CA. Furosemide, phorbol dibutyrate, carbachol, chelerythrine, rottlerin,
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RedTaq Polymerase, oligonucleotide primers, 10 mM dNTP Mix and DNase kits were obtained from Sigma-Aldrich Corp., St. Louis, MO. Mouse monoclonal anti-PKCα, -β, -γ, -δ, -ε, -θ, -η, and -λ were purchased from Transduction Lab, Lexington, KY; goat anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. SuperSignal West Pico Chemiluminescent Substrate kit was from Pierce Biotechnology, Inc., Rockford, IL. PKC activity assay kit was purchased from Upstate Cell Signaling Solutions, Waltham, MA. PKCδ substrate was from Calbiochem, San Diego, CA. [γ-32P]ATP was obtained from MP Biomedicals, Inc. Irvine, CA. PKCδ-specific peptide inhibitor (δV1-1) and non-specific (scrambled sequence) peptide were a kind gift of Dr. Karen Ridge, Northwestern University, Chicago, Illinois. These peptides, which were linked (via an N-terminal Cys-Cys bond) to the Drosophila Antennapedia homeodomain-derived carrier peptide, were synthesized and purified at the Stanford Protein and Nucleic Acid Facility. All other reagents were obtained from either Sigma-Aldrich Corp., or Fisher Scientific, Hanover Park, IL, and were of analytical grade.

Tissue Procurement and Cell Isolation

New Zealand White adult (≥ 6 months), weanlings (25–28 days old) and newborn (7–9 days old) rabbits were purchased from New Franken Research Rabbits (New Franken, WI). Animals were housed in the UIC Biological Resources Laboratory (BRL), an accredited AAALAC facility. The care and handling of animals and tissue processing for our experiments were approved by the Institutional Animal Care Committee. Distal colon, from splenic flexure to rectum, was excised, and mucosal sheets separated from underlying muscle by blunt dissection. Colonocyte isolation and culture (18-24 hours) from adult and weanling animals were carried out as previously described (6, 53, 57, 69). Briefly, tissues were collected and isolated in oxygenated
lactated Ringer’s (LR) solution with 5% dextrose (LRG) and antibiotics (ABX: 25 μg/ml ampicillin, 125 μg/ml penicillin, 270 μg/ml streptomycin, and 1.25 μg/ml amphotericin B). For the isolation, colonic mucosa was incubated at 37°C in LRG+ABX, containing 0.03% collagenase, 0.1% pronase and 0.07% dithiothreitol (DTT) for adult samples (90 min), and 0.015% collagenase 0.05% pronase and 0.023% DTT for weanling samples (60 min) (6, 53). The resulting cell suspension was enriched for crypt colonocytes by sequential centrifugation twice at 4000 x g for 5 min and twice at 400 x g for 15 min. The final pellet was resuspended in 25 ml Ham’s F-12 media containing 20% fetal bovine serum, 0.5 units/ml insulin, 4 mM L-glutamine, 1 mM hydrocortisone, 500 μM selenium, 1 mM sodium butyrate, and ABX antibiotics, transferred to a T75 flask, and incubated 18-24 hrs at 37°C.

**Cl⁻ Transport**

The membrane permeable halide-sensitive fluorescent probe MQAE was used to assess Cl⁻ transport in isolated colonocytes as previously described (20, 57, 69). Isolated colonocytes were loaded with 10 mM MQAE (5 mins at RT and 90 mins on ice), in Buffer A containing, in mM: NaCl, 110, MgCl₂, 1, CaCl₂, 1, dextrose, 5, mannitol, 50, K₂SO₄, 1, and HEPES, 5, pH 7.4). Cells were then Cl⁻-depleted for 30 mins in Buffer B containing, in mM: Na⁺ isethionate, 110, MgSO₄, 1, dextrose, 5, mannitol, 50, K₂SO₄, 1, CaSO₄, 1, and HEPES, 5, pH 7.4. A PTI Alphascan spectrofluorometer (Princeton, NJ) was used to measure fluorescence at Ex₃: 350 nm and Em₃: 460 nm. Each test condition was run in triplicate with ~ 10⁴ cells/assay cuvette. Cl⁻ influx was measured, in triplicate, under basal conditions ± secretagogues ± Cl⁻ transport inhibitors (50 μM DPC ± 10 μM furosemide) ± various PKC inhibitors. We had previously shown that DPC, a Cl⁻ channel inhibitor, and furosemide, an NKCC cotransporter inhibitor, can be used in this system to determine the combined contributions of CFTR and NKCC to the Cl⁻...
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flux in the presence and absence of secretagogues (20, 57). The concentrations of PKC inhibitors were based on their known efficacy in T84 (62) and Caco2 cell systems (58, 59). The following PKC inhibitors were used: Chelerythrine (2 µM), a general PKC inhibitor; Gö6983 (100 nM), an inhibitor of most PKC isoforms with the exception of PKCδ; Gö6850 (5 µM), an inhibitor of cPKCs and nPKCs; Gö6976 (1 µM), an inhibitor of cPKCs; rottlerin (10 µM), commonly used as an inhibitor of PKCδ; and peptide inhibitor δV1-1 (0.5 µM, cross-linked via an N-terminal Cys-Cys bond to carrier peptide), a specific inhibitor of PKCδ. As a negative control, a non-specific control peptide (scrambled sequence cross linked to carrier peptide) was included in the δV1-1 studies.

Colonocytes were pre-incubated with δV1-1 or control peptide for 30 minutes and with the other PKC inhibitors for 5 minutes each prior to the start of the transport assay. As described in detail earlier, transport was calculated as 

\[ J_{Cl} = \frac{F_o}{(K_{Cl} \times F^2)} \times \frac{dF}{dt} \]

(57), where \( F_o \) and \( F^2 \) are initial and final fluorescence intensities, and \( K_{Cl} \) is the Stern-Volmer constant. The rate of transport was expressed as transport inhibitor (DPC + furosemide)-sensitive Cl⁻ influx in mM/sec. The secretagogue doses (TDC: 50 µM, PDB:1 µM) had previously been shown to elicit maximal Cl⁻ transport in rabbit distal colonocytes (20, 69).

Electrophysiological Measurements

The distal colon of adult rabbits was excised, opened along the mesenteric line, and placed in cold oxygenated LR solution with 10 mM glucose. The mucosal layer was stripped of underlying muscle and mounted in modified Ussing chambers (area: 0.33 cm²; Physiologic Instruments, Inc., San Diego, CA) and bathed with oxygenated (95% O₂: 5% CO₂) buffer C (5ml/reservoir) of the following composition in mM: \( \text{Na}^+ 141.8; \text{Cl}^- 5.4; \text{PO}_4^{3-} 3.0; \text{Ca}^{2+} 1.2; \text{Mg}^{2+} 1.2; \text{HCO}_3^- 21.0 \) (pH 7.4); \( \text{K}^+ 5.4; \) and D-glucose 10 at 37°C. Tissues from the same
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intestinal segment and animal were paired based on comparable tissue resistance ($R_t; \Omega \cdot \text{cm}^2, \pm 25\%$). Tissue conductance ($G_t; \text{mS/cm}^2$) and $R_t$ were monitored throughout the experiment. Transmural short-circuit currents ($I_{sc}; \mu\text{A/cm}^2$) were measured using the automatic voltage clamp apparatus (VCC-MC6; Physiologic Instruments) as described earlier (71). Tissues were allowed to stabilize for $\approx 30$ minutes until the $I_{sc}$ and $R_t$ reached plateau levels. Four tissues were mounted from each animal, two control (DMSO) and two treated with rottlerin (10 $\mu$M). Reagents were added to both the mucosal and serosal bathing solutions 30 min prior to the bilateral addition of TDC (100 $\mu$M). For dose response experiments, increasing concentrations of TDC were added sequentially (1-500 $\mu$M). Maximal increases in $I_{sc}$ were observed at 50-100 $\mu$M TDC (data not shown). At the end of each experimental run, carbachol (100 $\mu$M) was added to assess tissue responsiveness. This was confirmed in three experiments by measuring the $I_{sc}$ response to forskolin (10 $\mu$M) added at the end of the experimental run.

Protein Preparation and Assay

Using previously published methods (71), colonic mucosal epithelia were homogenized and sonicated in buffer D containing, in mM: EDTA 1; MgCl$_2$ 2; $\beta$-mercaptoethanol 5; DTT 1; Tris-HCl 25, pH 7.4 and 1 $\mu$g/ml each of leupeptin, pepstatin, and aprotinin. The homogenate was centrifuged at 3,000 x g for 10 min to obtain a post nuclear supernatant (total lysate) which was stored at -80$^\circ$C until use. For positive controls, rabbit and rat brain homogenates were similarly prepared. Total protein was measured via the Bradford method (Bio-Rad, Hercules, CA).

SDS-PAGE & Western Blotting

As described previously (69), the method of H. Towbin was adapted for SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and Western blotting. Briefly,
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samples (100 μg protein/lane for rabbit colonic epithelial samples, 30 μg/lane each for rabbit brain and Jurkat cell lysates and 10 μg/lane for rat brain) were separated on 7.5% polyacrylamide gels and transferred to an Immobilon PVDF transfer membrane (Millipore Corporation, Bedford, MA) at 250 mAmps for 2.5 hrs in transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS. All subsequent procedures were carried out in Tris-buffered saline (TBS-T) containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween-20. The membranes were blocked with Blotto (5% Carnation non-fat dry milk) for 1 hr at RT and then incubated with the primary antibody in the blocking agent overnight at 4°C. The antibodies used were as follows: mouse anti-PKCα (1:1000), mouse anti-PKCβ (1:250), mouse anti-PKCγ (1:1000), mouse anti-PKCδ (1:500), mouse anti-PKCε (1:1000), mouse anti-PKCθ (1:250), mouse anti-PKCτ (1:250), and mouse anti-PKCλ (1:250). After incubation the blots were washed 3 x 15 min in TBS-T and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) at RT for 1.5 hr. After the secondary antibody, the blots were washed 3 x 15 min in TBS-T at room temperature and the specific antibody-antigen interaction visualized using a SuperSignal West Pico Chemiluminescent Substrate kit. As reported earlier (51), to ensure equal loading and transfer, the blots were stained with Ponceau-S (10%) and the gels were stained with Coomassie-blue after electrotransfer. The gels post-transfer revealed no proteins and the blots showed uniform staining with Ponceau-S. In addition, for each set of protein samples, the blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and these blots did not exhibit age-specific differences in expression, suggesting that the loading was uniform and differences in PKC isoform expression were specific.

PKCδ Activity Assay
Specific PKCδ enzymatic activity was measured by custom designing a modification of the Upstate Cell Signaling Solution PKC activity assay kit. The original kit is composed of a PKC lipid activator, PKA/CaMK inhibitors, and a generic PKC substrate. We substituted the generic PKC substrate with a specific PKCδ peptide substrate, which has been shown to be phosphorylated by PKCδ, but not by PKCε and -η, and only slightly phosphorylated by PKCα, -β and -γ (37). We therefore also added Gö6976 to inhibit any cPKC activities. Adult and weanling distal colonocytes were isolated and incubated with 100 µM TDC or 10 µM PDB for 5 min. After the incubation, the buffer was immediately replaced with buffer D, and the cells were snap frozen in liquid nitrogen. The samples were thawed on ice, sonicated, and centrifuged at 2,000 x g for 5 min to obtain a postnuclear supernatant. Membrane and cytosolic fractions were obtained from this supernatant by ultracentrifugation at 100,000 x g for 30 min at 4°C as described earlier (69). Equal amounts of protein from control and treated samples (10-50 µg) were incubated with PKC lipid activator, PKA/CaMK inhibitors, Gö6976, PKCδ substrate and [γ-32P]ATP (10 µCi) for 10 min at 30°C. Samples (25 ul) were then spotted on P81 phosphocellulose paper and washed thrice with 0.75% phosphoric acid and once with acetone. 32P incorporated into the PKCδ substrate was determined by scintillation spectrometry using a Tri-Carb liquid scintillation analyzer (Perkin Elmer, Boston, MA) and Opti-Fluor scintillation cocktail (Perkin Elmer, Shelton, CT).

**RNA Isolation and Real-Time RT-PCR**

Total RNA was isolated from rabbit colonic mucosa using TRIZOL reagent according to manufacturer's instructions. Total RNA (1 µg) was treated with DNase and reverse-transcribed using Oligo(dT)12-18 Primer and Superscript II reverse transcriptase. For each real-time PCR reaction (25 µl), cDNA synthesized from 50 ng of RNA was mixed with 2x iQ SYBR Green
supermix containing 600 nM specific primers. The oligonucleotide primer sequences are listed in Table 1.

The PCR reactions were carried out in duplicate or triplicate, and the samples were analyzed on a BIO-RAD MyiQ Single Color Real-Time PCR Detection System. The PCR products were sequenced at the Marine DNA Sequencing Center (Mount Desert Island Biological Laboratory, Salisbury Cove, ME) to verify amplification of the desired target gene. The target genes examined were PKCδ, CFTR, NKCC and in preliminary studies PKCζ. GAPDH was used as the internal reference. Melting curve analyses were done for target genes and GAPDH to confirm the product specificity. The \(2^{-\Delta C_T}\) method, according to Pfaffl (48), was used to compare the relative mRNA level of target genes, where \(-\Delta C_T = -[^{\Delta C_T, \text{sample}} - \Delta C_T, \text{calibrator}]\), and \(C_T = C_T, \text{target gene} - C_T, \text{GAPDH}\). For each real-time PCR experiment, a newborn sample was selected as the calibrator sample (also called reference sample), and all other samples were normalized to this when performing relative quantification. The mRNA expression in this newborn sample was set as 1 (\(2^0\)), while the expression in the other newborn, weanling and adult samples in the same PCR run were calculated as \(n\)-fold of this newborn sample. Real time PCR amplification efficiencies for target genes and GAPDH were calculated to make sure that the amplification efficiencies were approximately equal.

**Statistics**

Data are expressed as mean ± SEM. For CI⁻ transport and PKCδ assay experiments, an \(n=1\) represents samples from either 1 adult rabbit or 2 pooled weanlings; for Western blotting, an \(n=1\) represents samples from 1 adult rabbit, 1 weanling or 2 pooled newborns; for the real-time PCR experiments, each “\(n\)” value depicts a separate animal. Student's t-test was used to determine statistical significance between means within an experiment and analysis of variance.
(ANOVA) followed by Tukey’s Test was used to determine significance for experiments with > 2 means. In all cases, a $p$-value of $\leq 0.05$ was considered significant.

**Results**

*Effect of PKC Inhibitors on TDC Action in Adult Rabbit Distal Colon*

We had previously shown that, in rabbit colonocytes, phorbol dibutyrate, but not TDC, increases cPKC activity *in vitro* (69) and that the effects of PDB are not age-specific (20). However, neither of these studies examined if perturbation of the PKC signaling pathway would interfere with TDC action. We first examined if inhibition of PKC activity by the broad-specificity PKC inhibitors, chelerythrine or Gö6983 affected TDC (50 μM)-stimulated Cl$^-$ transport in adult distal colonocytes. In all the inhibitor studies, PDB (1 μM) was used as a positive control. Chelerythrine (IC$_{50}$ ≈ 0.66 μM) (32) inhibits all PKC isoforms, while Gö6983 (IC$_{50}$ ≈ 7-60 nM) (30) inhibits c-, n- and aPKCs but not PKCμ (IC$_{50}$ >20 μM). As shown in Figure 1, when isolated adult colonocytes are pre-incubated (5 min) with either chelerythrine (2 μM; Fig. 1A) or with Gö6983 (100 nM; Fig. 1B), both TDC- and PDB-stimulated Cl$^-$ transport are inhibited to basal levels. These compounds do not affect basal transport confirming they specifically affect furosemide- and DPC-sensitive transport stimulated by TDC or PDB. The above data indicate that activation of PKC(s), but not PKCμ is involved in the Cl$^-$ secretory response to TDC and, not surprisingly, in that of phorbol esters.

To dissect the roles of c-, n- and aPKCs, isoform-specific inhibitors were used. The compound Gö6850 inhibits c- and nPKCs but not aPKCs (68). As seen in figure 2A, Gö6850 (5μM) inhibited both TDC- and PDB-stimulated Cl$^-$ transport to basal levels. In marked contrast Gö6976 (1 μM), an inhibitor that specifically affects cPKCs (42), has no effect on TDC action but inhibited PDB-stimulated Cl$^-$ transport to basal levels (Figure 2B). Again, basal transport
was not affected by either of these inhibitors. These data imply that, while PDB action involves both c- and nPKCs, the action of TDC may involve nPKCs but not cPKCs.

Of the four nPKC isoforms, -δ, -ε, -θ and -η, PKCδ has been implicated in a variety of functions. Rottlerin, which inhibits at the ATP binding site (31), has been extensively utilized as a fairly specific inhibitor of PKCδ action (59). As shown in Figure 3, rottlerin (10 μM) had no effect on basal levels of Cl⁻ transport. However rottlerin inhibited both TDC- and PDB-stimulated Cl⁻ transport, suggesting a role for PKCδ in TDC action. Although our cumulative evidence suggests that rottlerin specifically inhibits PKCδ in rabbit colonocytes (see discussion), rottlerin has been implicated to have non-PKCδ effects in a few cell types. Therefore, we examined the effects of a specific PKCδ inhibitor, δV1-1. The specifically designed δV1-1 peptide blocks PKCδ translocation and activation in a variety of cells including alveolar epithelial cells (55). As shown in Figure 3, δV1-1 had no effect on basal levels of Cl⁻ transport, but inhibited both TDC- and PDB-stimulated Cl⁻ transport. A control scrambled peptide had no effect on basal, TDC- or PDB-stimulated Cl⁻ transport. These studies confirmed a role for PKCδ in TDC and PDB action. The observation that PDB action was completely inhibited by δV1-1 and rottlerin is intriguing and its possible implications are explored in the discussion.

To determine if the role of PKCδ seen in isolated colonocytes can be observed in intact epithelia, adult colonic mucosa stripped of underlying muscle were mounted in Ussing chambers, and the effects of TDC ± rottlerin on short-circuit current (Isc) assessed. Shown in Figure 4 are the effects of 100μM TDC on tissues (n=5-6 animals, each representing the mean of duplicates) pre-treated with 10μM rottlerin (30 min) or DMSO (vehicle). This concentration of TDC caused a 3-fold increase in Isc (p<0.05). In data not shown, this concentration yielded the maximal Isc in a dose-response study. While rottlerin alone had no effect on basal Isc (p>0.05), it caused a
significant inhibition of TDC-stimulated $I_{sc}$ (p<0.05 as compared to TDC alone); equally important there was no difference in $I_{sc}$ of rottlerin-treated tissues ± TDC (p>0.05). To assess tissue responsiveness, carbachol or forskolin were added at the end of the experiment. The $I_{sc}$ responses to these secretagogues in TDC- and TDC + rottlerin- treated tissues were similar. These results, in the intact epithelium, further support the notion that TDC appears to act via PKCδ to stimulate Cl⁻ transport.

**Effects of PKC Inhibitors on the Weanling Colon**

We next examined if the differences in PKC signaling employed by TDC (nPKC) and PDB (cPKC and nPKC) could account for the fact that TDC, but not PDB, exhibits age-specific effects. Therefore, we compared the effects of the c/n PKC inhibitor Gö6850, with those of the cPKCs inhibitor Gö6976 and the PKCδ inhibitors rottlerin and δV1-1 in PDB action in weanling colonocytes. As shown in Figure 5 A and B, both Gö6850 and Gö6976 inhibited PDB action. In marked contrast, although rottlerin and δV1-1 inhibited PDB action in the adult colonocytes (Fig. 3), they failed to inhibit PDB action in the weanling colonocytes (Figure 5C). None of the inhibitors affected basal transport. These results were provocative and suggested that the observed refractoriness to TDC in the weanling animal was perhaps due to reduced PKCδ activity in the weanling animal.

**Expression of PKCδ Transcript**

Therefore, we examined the expression of PKCδ in distal colonic mucosa of adult, weanling and newborn rabbits. Only some of the PKC isoforms have been cloned from the rabbit. GeneBank only lists PKCα, -β and -ζ and there is an accession number (M19338) listed as PKCδ. However this is misleading as a comparison of this sequence with that of PKC isoforms cloned from other species reveals that the GeneBank M19338 is more than 90%
identical to human PKC\(\gamma\) (GeneBank Accession Number BC047876) and shares low identity with human PKC\(\delta\) (GeneBank Accession Number NM_006254.3) or rat PKC\(\delta\) (GeneBank Accession Number BC076505.1). We designed degenerate oligonucleotide primers based on the conserved regions of published PKC\(\delta\) mRNA sequences of human, mouse and rat, and obtained a partial (1423bp) sequence of rabbit PKC\(\delta\). A GeneBank Blast search revealed that the predicted partial amino acid sequence of rabbit PKC\(\delta\) has 90% identity to human PKC\(\delta\) and 89% identity to rat PKC\(\delta\). We have registered this rabbit PKC\(\delta\) sequence in GeneBank (Accession number: AY847776). It is interesting that in contrast to the rat brain, where there is a robust expression of PKC\(\delta\), it is very low in adult rabbit brain (\(\approx 1.5\%\) of that in adult distal colon).

As shown in Table 2, by real-time PCR, we observe a significant age-dependent expression of PKC\(\delta\) with the adult having 1.6-fold greater transcript level than the weanling and 2.3-fold (\(p<0.05; n\geq 3\)) greater than the newborn. In contrast to PKC\(\delta\), the expression of the transport proteins CFTR and NKCC1 did not vary with developmental age (Table 2). In preliminary experiments, we examined PKC\(\zeta\), the only other non-conventional rabbit PKC that has been cloned. Expression of PKC\(\zeta\) mRNA was adult \(\geq\) weanling \(>>\) newborn. Since inhibitor studies imply that atypical PKCs may not be involved, an examination of these isoforms was not further pursued in this study. These data demonstrate that, at the level of the mRNA transcript, PKC\(\delta\) specifically shows an age-related expression.

*Age-Dependent Expression of PKC Protein*

Since PCR is a highly sensitive method of detection and demonstration of a transcript does not necessarily imply protein expression, we examined if PKC protein expression was different in adults and weanlings. There have been few thorough analyses of PKC isoform expression in
primary preparations of colon as opposed to cell-lines (13) and there is one report on expression of PKC isoforms in adult rabbit ileum (36). Using an array of commercially available antibodies, we screened rabbit colonic lysates for the expression of various PKC isoforms (Figure 6 and Table 3), using the rat and rabbit brain and Jurkat cells as positive controls. As described in the methods, samples were checked for uniform loading and transfer. Of the cPKCs, the rabbit colon expresses PKCα and -β but not -γ (Figure 6A). Furthermore PKCβ shows a biphasic age-dependent expression with high expression in the adult and newborn (2.1±0.5-fold of adult) and low expression in the weanling (0.5±0.5-fold of adult). However it is unlikely that expression of PKCβ is responsible for the age-dependent effects of TDC since TDC does not affect cPKC activity (Figure 2B) (69). Of the nPKC isoforms, the rabbit colon only expresses PKCε and -δ but not PKCθ (Figure 6B). Expression of both PKCε and PKCδ was age-dependent. Like PKCβ, PKCε shows a biphasic expression, with low expression in the weanling and high expression in the newborn (0.5 and 2.5 fold of the adult respectively, Table 3). PKCε is an unlikely candidate as a mediator of TDC action; it is not sensitive to rottlerin or δV1-1, and its biphasic age-dependent expression does not parallel the transport effects of TDC (see discussion). In marked contrast, there is a dramatic age-dependent increase in the expression of PKCδ, with highest expression in the adult and barely detectable expression in the weanling and newborn. Confirming our PCR findings, there is no detectable expression of PKCδ protein in the rabbit brain, whereas, as shown by others (52), we find high expression of PKCδ in rat brain (Figure 6). As shown in Figure 6C, the rabbit colon expresses atypical PKCτ and -λ and there was no dramatic change in expression with age.

PKCδ Activity
To relate protein expression to activity, we next examined PKCδ enzymatic activity in response to TDC and PDB. We first attempted to measure activity by immunoprecipitating the protein. However, although the antibodies were good for immunodetection by Western blotting, neither the mouse monoclonal nor the goat polyclonal commercial antibodies yielded consistent immunoprecipitation (data not shown). Therefore by using a PKCδ-specific substrate and a series of selective inhibitors, we modified the commercially available PKC Activity Assay Kit to specifically measure PKCδ activity (see methods). PKCδ activity was measurable in both cytosol and membrane fractions, and both TDC (1.6-fold) and PDB (2.0-fold) caused significant increase over basal in membrane-associated PKCδ activity (Figure 7A). There was a concomitant, statistically significant, 11% decrease in PKCδ activity in the cytosolic fraction of PDB-treated cells, and a similar (9%), but statistically insignificant, decrease in that of TDC-treated cells. Our data thus far suggested that, in the weanling, PDB stimulation of Cl⁻ transport was PKCδ-independent (i.e., rottlerin and δV1-1-insensitive, Figure 5C), and this may be due to low expression of PKCδ protein (Figure 6B). To confirm this further, we examined PKCδ activity in weanling colonocytes treated with PDB or TDC. As shown in Figure 7B, neither agent caused a stimulation of PKCδ activity. These results strongly suggest that TDC acts via PKCδ and that the age-specific effects of TDC are in part due to the lack of PKCδ in the young animal.
Discussion

The PKC cascade is often linked to the action of neurohumoral modulators acting via [Ca$^{2+}$]. We had earlier demonstrated that Ca$^{2+}$-dependent segretagogues, including bile acids, exhibited age-refractoriness in the rabbit colon, and that TDC does not alter the activity of conventional PKCs (69). The present study is the first to demonstrate a role for PKC$\delta$ in the secretagogue action of bile acids and that a lack of PKC$\delta$ protein and activity most likely account for the refractoriness of the colon of young animals to bile acid-stimulated Cl$^-$ secretion. There are multiple PKC isoforms and the availability of isoform-specific inhibitors allows the delineation of specific signaling pathways. Another useful tool for studying the PKC signaling cascade is the phorbol esters, general activators of PKC. While there are some inherent limitations in the use of such pharmacological manipulations, collectively, they help understand the mechanisms underlying hormonal signaling pathways. Our earlier results (69) had indicated that, in contrast to neurohumoral agents, neither PDB nor A23187, showed age-refractoriness, and in contrast to TDC, PDB stimulated conventional PKC enzymatic activity. The implications of these results were that the age-dependent effects of TDC did not involve cPKCs, were different from the actions of PDB, and occurred at a proximal step in signaling. This was confirmed by the finding that in the weanling, secretagogues such as TDC and neurotensin failed to increase colonic IP$_3$ production, [Ca$^{2+}$], or to stimulate Cl$^-$ transport. However these studies did not examine the roles of the nPKCs and aPKCs in TDC action. Cognizant of the potential differences in TDC and PDB signaling, we compared the effect of PKC isoform-specific inhibitors on the actions of TDC and PDB in the developing rabbit colon to elucidate the basis of age-refractoriness in TDC signaling.
PKCδ in the age-dependent action of TDC

The pan-specific PKC inhibitors, chelerythrine and Gö6983, completely inhibited the stimulatory action of both TDC and PDB. Chelerythrine acts on the phosphate acceptor site of the catalytic domain of PKC and selectively inhibits PKC as compared to PKA, CAMK and tyrosine kinases (TK) (32). Gö6983 also acts on the catalytic site to inhibit several PKC isoforms but does not inhibit PKCµ (30). Therefore, TDC-stimulated Cl⁻ transport involved PKCs but perhaps not PKCµ. Gö6850 is a potent and selective inhibitor of cPKCs and nPKCs, but not of PKA and TKs (68). The IC₅₀ of Gö6850 for aPKCs (>5.8 µM) was much higher than those for cPKCs or nPKCs (2-210 nM) (42); therefore, at the concentration used in this study, 5 µM, Gö6850 specifically inhibited cPKCs and nPKCs, but not aPKCs. In contrast, Gö6976, a compound which specifically inhibits cPKCs and PKCµ with a similar potency (30), blocked PDB but not TDC action. Collectively, these data imply that TDC action did not depend on aPKCs, PKCµ or cPKCs and is consistent with our previous report that only PDB, but not TDC, activated cPKC enzymatic activity (69).

The studies with Gö6850 and Gö6976 imply that TDC action may involve only nPKCs. Of the nPKCs, rottlerin inhibits PKCδ (31, 62) (IC₅₀ of 3-6 µM) and PKCθ (70) with greater selectivity. Rottlerin has significantly reduced inhibitory activity on PKCε (IC₅₀ = 80-100 µM). The latter concentrations are 8 times greater than used in the present study and as PKCθ was not expressed in the rabbit distal colon (Figure 6B), it is likely that any action of rottlerin reflects involvement of PKCδ. While some reports have disputed the selectivity of rottlerin in inhibiting PKCδ (16), our results cumulatively demonstrate that rottlerin’s effects are specific for PKCδ in the rabbit colon. The most compelling evidence is that the effects of rottlerin and those of the specific peptide inhibitor, δV1-1 are similar. In contrast, a scrambled sequence control peptide
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had no effect on TDC-stimulated transport. In addition rottlerin shows age-specificity affecting only the adult and not weanling colonocytes. If it had non-specific effects, this would be unlikely. Thus, in other systems, rottlerin has been reported to uncouple mitochondrial oxidative phosphorylation and to inhibit the ubiquitous elongation factor 2-specific CAMK III (31). If rottlerin were acting by either of these mechanisms in the rabbit colon, then it should have inhibited Cl− transport in both adult and weanling and this is not the case (Figure 3 and Figure 5C).

Rottlerin and the specific peptide inhibitor δV1-1 inhibited TDC-stimulated Cl− transport in isolated adult colonocytes (Figure 3). Equally important, in intact colonic epithelia, rottlerin inhibited TDC-stimulated, but not basal I_{sc}, a measure of Cl− secretion (Figure 4). It is intriguing that rottlerin and δV1-1 completely inhibited PDB-stimulated transport in the adult colonocytes. If PDB activated different PKC isoforms, which then converged on a common target (e.g. a transporter), then one might have predicted a partial inhibition. However these data imply that multiple PKC isoforms may act at different steps of the signaling cascade resulting in Cl− secretion (see below).

In contrast to the adult, neither rottlerin nor δV1-1 had an effect on PDB-stimulated Cl− transport in weanling colonocytes. More important, the absence of function attributable to PKCδ in the weanling colon was associated with reduced PKCδ transcript (Table 2), low PKCδ protein expression (Figure 6) and undetectable PKCδ activity (Figure 7). These activity assays were conducted under conditions where all second messenger-regulated kinases, including cPKCs, PKA and CAMK were inhibited. Furthermore, a PKCδ specific peptide was used as the substrate. We are intrigued by the marked differences in PKCδ protein expression at the different age groups and the modest 2-fold difference in PKCδ mRNA expression. While a more detailed
PKCδ in the age-dependent action of TDC

comparison of the transcript vs. proteins of the various isoforms is warranted, at this juncture, we can only speculate that there is perhaps greater PKCδ protein stability in the adult. Taken together, the inhibitor (rottlerin and δV1-1) studies, the age-dependence and the activity measurements demonstrate that PKCδ is critical for stimulation of Cl⁻ transport by TDC in the rabbit distal colon.

What then are the targets for PDB and TDC in the signal transduction cascade leading to Cl⁻ secretion? The simplest canonical sequence is as follows: Ca²⁺ dependent secretagogues → receptor → PLC activation → IP₃ and DAG production → [Ca²⁺]ᵢ release and PKC activation → activation of a combination of transporters including Cl⁻ channels, NKCC cotransporter, K⁺ channels and Na⁺/K⁺ ATPase. This scenario is compounded by the ability of key signaling molecules to affect multiple steps in the cascade (cross-talk) resulting in a complex signaling network. It is clear from the age-dependence studies that while PDB and TDC may both activate PKCδ, PDB affects other PKCs and therefore perhaps other targets. Furthermore, PKCδ may be affecting more than one step. The age-dependency of PKCδ expression and activity together with our earlier data (69) that TDC cannot increase IP₃ and [Ca²⁺]ᵢ in the weanling, suggests that PKCδ may be affecting at least one early step in signal transduction. One possible site is the activation of PLCγ rather than its expression since our preliminary data indicate that PLC protein expression is not age-dependent (Prasad, R., et. al. unpublished observation). Such a role for PKC was previously reported in the regulation of bradykinin-stimulated phosphoinositide breakdown in astrocytes (10).

Based on the lack of age-specificity of PDB action, we speculate that it is unlikely that PDB activates these initial steps. In addition the evidence for PDB to stimulate [Ca²⁺]ᵢ in colonocytes is spotty at best. Thus in T-84 cells it does not increase [Ca²⁺]ᵢ, but attenuates the
ability of agents such as carbachol to increase it (54). However, in oligodendrocytes PDB increases \([\text{Ca}^{2+}]_i\), (73). Our evidence that rottlerin and the \(\delta V1-1\) peptide completely rather than partially inhibit PDB action leads us to the intriguing speculation that PKC\(\delta\) may be acting at another step in the cascade and this step supercedes the activation of the cPKCs by PDB. Albeit highly speculative, we provide one example of many cross-talk signaling mechanisms we envisaged. It is conceivable that associated with the increased expression of PKC\(\delta\) during development from weanling to adult, two steps are introduced: first, PKC\(\delta\) plays a role in an early step in signal transduction (as discussed above) and second, PKC\(\delta\) may be involved in increasing transporter recruitment to the membrane. In contrast, cPKCs cause direct activation of the transporter in the weanling and adult, but in the latter due to the emerging role of PKC\(\delta\), the action of cPKC could be superceded by that of PKC\(\delta\). Thus, if there are insufficient transporters in the membrane, activation will be attenuated. This might explain our data wherein PKC\(\delta\) inhibition results in a complete attenuation of TDC and PDB stimulated Cl\(^-\) transport in the adult.

What then might be the physiological role of the TDC-PKC\(\delta\) refractoriness? In contrast to PKC\(\delta\), there was no age-dependency in the expression of the Cl\(^-\) transporters NKCC1 and CFTR in the rabbit distal colon. This supports the observation that the colon of young animals is able to transport Cl\(^-\) in response to some secretagogues, including cAMP and PDB. Many neurohumoral agents utilize Ca\(^{2+}\)-signaling, and the lack of a fully operative cascade may be a protection against excess loss of fluid with baseline fluid secretion occurring in response to cAMP.

Our studies have also to be examined in the wider context of what is known about the functions of PKC\(\delta\) and specifically the role of PKCs in Cl\(^-\) secretion. Although roles ranging
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from a proapoptotic signal (39), to a tumor suppressor and to a regulator in stress, immune and inflammatory responses have been ascribed to PKCδ (29, 63), the PKCδ-knock-out mice generally develop and grow normally. The knock-out mice show a clear phenotype only in certain cell functions (63); examples include reduced migration of neutrophil to the injured site and loss of preconditioning-induced cardio-protection (63). Intestinal ion transport has not been examined in these animals. While a role for PKCs has been implicated in a variety of electrolyte transport processes, specific isoforms have been identified only in some systems, and it is clear that there are considerable cell-type and species differences. For example, PKCδ mediates the stimulation of \( \text{Na}^+/\text{H}^+ \) exchange by phorbol esters in \( \text{C}_6 \) glioma cells (11), and PKC\( \alpha \) mediates the inhibition of \( \text{Na}^+/\text{H}^+ \) exchange by serotonin in Caco-2 cells (28). In contrast, inhibition of \( \text{Cl}^-/\text{OH}^- \) exchange in Caco-2 cells by serotonin involves PKCδ (59) and by phorbol esters involves PKC\( \varepsilon \) (58). Activation of PKCδ, but not PKC\( \varepsilon \) or PKC\( \alpha \), decreases IEC-18 epithelial integrity (66). In heterologous expression systems, the transporters involved in \( \text{Cl}^- \) secretion are differentially regulated by PKC. For example, in BHK cells, direct phosphorylation by PKC of CFTR, presumably on its PKC consensus site at S686, results in partial activation of the channel (9). However, phorbol esters inhibit NKCC1 activity in \( \text{Xenopus} \) oocytes overexpressing NKCC1 (25, 49). In HEK-293 cells with stably expressed Kir3.1/3.2 channels, PKCδ-mediated channel inhibition was caused by activation of muscarinic receptors (7). Thus, PKC isoform-specific modulation is dependent on cell type.

The most compelling evidence for specific roles for PKC isoforms in \( \text{Cl}^- \) secretion comes from studies in airway epithelia by Liedtke and colleagues. Thus, while PKC\( \varepsilon \) is involved in CFTR activation, a complex of kinases, including PKCδ, and protein phosphatases (PP2A) plays a role in the adrenergic activation of NKCC1 and thereby \( \text{Cl}^- \) secretion (40, 41). Recent evidence
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suggests that PKCδ directly associates with actin (61) and that actin serves as a scaffold for PKCs, PP2A and STE-20 related proline alanine-rich kinase to optimally modulate NKCC activity (41). With respect to colonic Cl⁻ secretion, phorbol esters, and therefore PKCs, stimulate it in rabbit (50, 57) and rat distal colon (23), the human colonic cell line HT-29cl.19A (2) and in primary human colonocytes (57). In some studies, the effects of phorbol esters on epithelial cells has been demonstrated to occur by activation of secretagogues, such as prostaglandins from the lamina propria (8). However, we demonstrated that the action of TDC in isolated colonocytes (69) is not dependent on prostaglandin release. In contrast, PKC activation by phorbol ester is inhibitory in some cell types (5), see introduction); For example, it inhibits Ca²⁺-dependent Cl⁻ secretion and NKCC activity in T84 cells, which appear to involve PKCε but not PKCδ (5, 13, 19). The phorbol ester-induced internalization of NKCC1 in T84 cells occurs via PKCε (19). Our present study on the developmental expression of PKC isoforms provides some insights into isoform-specific regulation. Only PKCδ, -β and -ε showed age-specific protein expression (Figure 6, Table 3) with reduced expression in weanlings as compared to adult. While we have shown that PKCδ (Figure 5), but not PKCβ (Figure 2B), plays a role in the age-specific secretagogue action of TDC, the role, if any, of PKCε in TDC remains to be explored. Based on the T84 studies, it is tempting to make a couple of speculations. First, in the adult it is conceivable that PKCε may serve to temper the overall response to PDB or TDC by inhibiting NKCC. Second is the suggestion that the high expression of PKCε (and thereby reduced NKCC) in the newborn may account for the lack of TDC responsiveness in the newborn. While an attractive suggestion, this does not account for the weanling data (low PKCε and no TDC effect) and the fact that both PDB and cAMP stimulate Cl⁻ transport in the newborn (20). With the
caveat that there are tissue/cell-line-specific differences, short-term PKCδ activation appears to stimulate colonic Cl⁻ transport.

Age-dependent expression of PKCδ has been reported in other tissues, but to our knowledge ours is the first study to link developmental expression of PKCδ to function in a physiological model. For example, in developing rat brain PKCδ is involved in ventilatory regulation, and its expression of PKCδ, -μ and -β, but not -ι/λ, are low in 2-day and 10-day-old pups and high in adult cortex (4). However, PKCδ expression was greater in the fetal and neonatal rat heart and declined 2 weeks postnatally (56). In the rat colon, PKCδ mRNA expression was lower in the colonic mucosa of 10-day-old compared to that of 25-day-old rats and hypoxia increased PKCδ translocation to the membrane (15). In none of these studies were the mechanisms regulating PKCδ expression and signaling during development examined. Potential candidates include glucocorticoids and thyroxine that rise during weaning and which have been implicated in the developmental regulation of proteins involved in ileal bile acid transport (34, 35, 43). Analysis of the promoter region of PKCδ has revealed a number of potential regulatory elements including NFK-B, GATA, p53 and MYOD, but not any consensus sequence motifs for FXR, the putative bile acid receptor (65). However the PKCδ promoter contains a related motif, the androgen response element, and PKCδ transcription increases in an androgen dependent manner in prostate cells (26). It is conceivable that a combination of other hormones, diet and intestinal flora could dictate PKCδ expression.

In summary, we provide one of the first demonstrations wherein age-dependent expression of a signaling molecule like PKCδ, is correlated with functional changes. Our data strongly implies that the expression of PKCδ protein may be a critical step in the age-related
refractoriness to bile acid signaling in the rabbit colon. This signaling cascade may be utilized by a number of neurohumoral agents such as histamine, serotonin and neurotensin, and such Ca\textsuperscript{2+}-dependent secretagogues are responsible for the minute-by-minute regulation needed in intestinal fluid homeostasis. In the young animal, where excess fluid loss can have grave consequences, this refractoriness to Ca\textsuperscript{2+}-dependent secretagogues may represent an overall protective mechanism.
Acknowledgment

The authors are deeply indebted to Dr. Karen Ridge, Division of Pulmonary & Critical Care, the Feinberg School of Medicine, Northwestern University for her generous gifts of PKCδ-specific peptide inhibitor (ΔV1-1) and non-specific (scrambled sequence) peptide.

Grants

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Reference:


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Figure Legends

Figure 1: Effect of chelerythrine and Gö6983, general PKC inhibitors, on TDC-stimulated Cl\(^-\) transport in adult distal colonocytes. Colonocytes were pre-incubated with 2 µM chelerythrine (1A, n=4) or 100nM Gö6983 (1B, n=3) for 5 min prior to the addition of 50 µM TDC or 1 µM PDB (positive control). Cl\(^-\) transport in colonocytes was then measured at 5 min and is expressed as DPC (50 µM) and furosemide (10 µM)-sensitive Cl\(^-\) transport as described in the Methods. Open bars: control; Striped bars: +inhibitors. Data are mean ± SEM, *p<0.05 vs. basal. #p<0.05 vs. TDC or PDB alone.

Figure 2: Effect of Gö6850 (c- and nPKC inhibitor) and Gö6976 (cPKC inhibitor) on TDC-stimulated Cl\(^-\) transport in adult distal colonocytes. Colonocytes were pre-incubated with 5 µM Gö6850 (2A, n=4) or 1 µM Gö6976 (2B, n=4) for 5 min prior to the addition of 50 µM TDC or 1 µM PDB (positive control). Cl\(^-\) transport in colonocytes was measured as described in the Methods and legend of Figure 1. Open bars: control; Striped bars: +inhibitors. Data are mean of inhibitor-sensitive Cl\(^-\) transport ± SEM, *p<0.05 vs. basal. #p<0.05 vs. TDC or PDB alone.

Figure 3: Effect of rottlerin and δV1-1, PKCδ inhibitors, on TDC- and PDB-stimulated Cl\(^-\) transport in adult distal colonocytes. Colonocytes were pre-incubated with 10 µM rottlerin for 5 min or 0.5 µM δV1-1 for 30 min prior to the addition of 50 µM TDC or 1 µM PDB (positive control). Cl\(^-\) transport in colonocytes was measured as described in the Methods and legend of Figure 1. Open bars: control; Vertically striped bars: +Rottlerin; Diagonally striped bars: Control peptide; Black bars: +δV1-1. Data are mean of inhibitor-sensitive Cl\(^-\) transport ± SEM, n=7 for
control and rottlerin experiments, and n=3 for δV1-1 and control peptide experiments. *p<0.05 vs. basal. #p<0.05 vs. TDC or PDB alone.

Figure 4: Effect of rottlerin on the TDC-stimulated short-circuit current (I_{sc}) in adult rabbit distal colonic epithelia. Adult distal colonic epithelia stripped of underlying muscle were mounted in Ussing chambers. Tissues from the same animal were paired based on comparable tissue resistance, and I_{sc} in response to 100 μM TDC ± 10 μM rottlerin or DMSO (control) was measured as described in the Methods. The colonic epithelia were preincubated with rottlerin (10 μM) for 30 min before adding 100 μM TDC. Carbachol (100 μM) was added at the end of the experiment to assess tissue responsiveness. n=6 for rottlerin + TDC treated tissues, and n=5 for control + TDC treated tissues. *p<0.05 vs. control. #p<0.05 vs. TDC alone.

Figure 5: Effect of Gö6850 (c- and nPKC inhibitor), Gö6976 (cPKC inhibitor), rottlerin and δV1-1 (PKCδ inhibitors) on PDB-stimulated Cl⁻ transport in weanling distal colonocytes. Weanling colonocytes were pre-incubated with 5 μM Gö6850 (5A), 1 μM Gö6976 (5B), 10 μM rottlerin (5C) for 5 min, or 0.5 μM δV1-1 (5C) for 30 min prior to the addition of 1 μM PDB. Cl⁻ transport was measured as described in the Methods and legend of Figure 1. Open bars: control; Diagonally striped bars: + Gö6850; Gray bars: +Gö6976; , Vertically striped bars: +rottlerin; Dashed vertically striped bars: +control peptide; Black bars: +δV1-1. Data are mean of inhibitor-sensitive Cl⁻ transport ± SEM, n=4 for 5A and 5B, n=7 for 5C control and rottlerin experiments and n=3 for δV1-1 and control peptide experiments. *p<0.05 vs. basal. #p<0.05 vs. PDB alone.
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Figure 6: Protein expression of cPKC (6A), nPKC (6B) and aPKC (6C) in the developing rabbit distal colon. Rabbit brain lysates (Rb Br, 30 µg), and lysates of mucosal epithelia of adult distal colon (AD DC, 100 µg), weanling distal colon (WN DC, 100 µg), and newborn distal colon (NB DC, 100 µg) were subjected to western blot analyses as described in the Methods. Positive control (+ve control) represents rat brain (Rt Br, 10 µg) for PKCα, -β, -γ, -δ, -ε and -ι and Jurkat cell lysates (30 µg) for PKC0. Blots were stripped and re-probed with anti-GAPDH antibody. Representative blots for n=3 determinations are shown.

Figure 7: Effect of TDC and PDB on PKCδ activity in adult and weanling distal colonocytes. Adult (7A, n=3) and weanling (7B, n=4) colonocytes were incubated with 100 µM TDC or 10 µM PDB for 5 min, and cytosolic and membrane fraction were prepared. A protocol specifically designed to assess PKCδ was used to measure activity as described in Methods. Open bars: basal; Striped bars: +TDC; Dotted bars: +PDB. *p<0.05 vs. basal.
Table 1: Oligonucleotide primer sequences used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences: 5' to 3'</th>
<th>Amplicon(bp)</th>
</tr>
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<tbody>
<tr>
<td>PKCδ</td>
<td>sense CCATGTACCCAGAGTGGA</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>antisense ATTGTTCTTCTTGACG</td>
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</tr>
<tr>
<td>CFTR</td>
<td>sense GGGCTGCTCTGGGAACTACTAC</td>
<td>86</td>
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<td></td>
<td>antisense CCTAGCCCGGCTTGACAA</td>
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<tr>
<td>NKCC1</td>
<td>sense ATCCGAATTATTGGAGCC</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>antisense AACCTTTGGGTTTCTTGC</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>sense CCAGTATGATTCCACCCA</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>antisense TCCTGGAAGATGGTGATG</td>
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Table 2: Relative mRNA expression of PKCδ, CFTR and NKCC1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative mRNA Expression in Distal Colon (-Fold of 1 newborn sample)</th>
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<tbody>
<tr>
<td></td>
<td>Adult</td>
</tr>
<tr>
<td>PKCδ</td>
<td>1.88 ± 0.29*</td>
</tr>
<tr>
<td>CFTR</td>
<td>0.98 ± 0.20</td>
</tr>
<tr>
<td>NKCC1</td>
<td>1.24 ± 0.37</td>
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</table>

Total RNA was extracted from adult, weanling and newborn distal colon, and real-time PCR was performed as described in the Methods. Each n-value represents one animal. The $2^{\Delta\Delta C_T}$ method was used to compare the relative mRNA level of target genes. For each real-time PCR experiment, a newborn sample was selected as the calibrator sample, and all other samples were normalized to this when performing relative quantification. The mRNA expression in this newborn sample was set as 1 ($2^0$), while the expression in the other newborn, weanling and adult samples were calculated as n-fold of this newborn sample. (Also see methods). Data are mean ± SEM. *p<0.05 vs. newborn distal colon. n≥3.
PKCδ in the age-dependent action of TDC

Table 3: Relative density of PKC isoforms.

<table>
<thead>
<tr>
<th>Relative Density (-Fold of adult distal colon)</th>
<th>Adult</th>
<th>Weanling</th>
<th>Newborn</th>
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<tbody>
<tr>
<td>PKCα</td>
<td>1</td>
<td>1.8±1.0</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>PKCβ</td>
<td>1</td>
<td>0.5±0.5</td>
<td>2.1±0.5*</td>
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<tr>
<td>PKCγ</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>PKCδ</td>
<td>1</td>
<td>0.1±0.1**</td>
<td>0.2±0.2*</td>
</tr>
<tr>
<td>PKCe</td>
<td>1</td>
<td>0.5±0.5</td>
<td>2.5±1.3</td>
</tr>
<tr>
<td>PKCθ</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>PKCτ</td>
<td>1</td>
<td>1.2±0.1</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>PKCλ</td>
<td>1</td>
<td>1.2±0.0</td>
<td>0.7±0.4</td>
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Quantitation of proteins represented in Figure 6 was determined by densitometric scans of each blot. Blots were tested for uniform loading as described in methods. Data are mean ± SEM. *p<0.05 vs. adult distal colon; ** P<0.001 vs. adult distal colon; N.D.: Not Detectable. n=3.
Figure 1

A

- **Control**
- **Chelerythrine (2 µM)**

Inhibitor-sensitive Cl-Flux (mM/sec)

Basal | TDC (50 µM) | PDB (1 µM)

B

- **Control**
- **Gö6983 (100 nM)**

Inhibitor-sensitive Cl-Flux (mM/sec)

Basal | TDC (50 µM) | PDB (1 µM)
**Figure 2**

**A**

- **Control**
- Gö6850 (5 µM)

Inhibitor-sensitive Cl-Flux (mM/sec)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Inhibitor-sensitive Cl-Flux (mM/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.5</td>
</tr>
<tr>
<td>TDC (50 µM)</td>
<td>1.5</td>
</tr>
<tr>
<td>PDB (1 µM)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**B**

- **Control**
- Gö6976 (1 µM)

Inhibitor-sensitive Cl-Flux (mM/sec)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Inhibitor-sensitive Cl-Flux (mM/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.5</td>
</tr>
<tr>
<td>TDC (50 µM)</td>
<td>1.5</td>
</tr>
<tr>
<td>PDB (1 µM)</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Figure 3

Inhibitor-sensitive Cl- Flux (mM/sec)

- Control
- Rottlerin (10 µM)
- Control peptide (0.5 µM)
- δ V1-1 (0.5 µM)
Figure 4
Figure 6

A  cPKC

+ve  Rb  AD  WN  NB  control

PKCα

82 kDa

PKCβ

80 kDa

PKCγ

80 kDa

B  nPKC

PKCδ

78 kDa

PKCε

80 kDa

PKCθ*

80 kDa

C  aPKC

PKCλ

74 kDa

PKCα

74 kDa

GAPDH

36 kDa
Figure 7

PKCδ activity (Fold over the basal)

A

- Basal
- TDC (100 µM)
- PDB (10 µM)

B

- Basal
- TDC (100 µM)
- PDB (10 µM)