Vasoactive intestinal peptide prevents PKCε-induced intestinal epithelial barrier disruption during EPEC infection

V. Morampudi,1,2* V. S. Conlin,1,2* U. Dalwadi,1,2 X. Wu,1,2 K. C. Marshall,1,2 C. Nguyen,1,2 B. A. Vallance,1,2 and K. Jacobson1,2,3

1Child and Family Research Institute, British Columbia Children’s Hospital, Vancouver, British Columbia, Canada; 2Division of Gastroenterology, British Columbia Children’s Hospital, Vancouver, British Columbia, Canada; and 3Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, British Columbia, Canada

Submitted 29 May 2014; accepted in final form 5 December 2014

The intestinal tract is lined with a single layer of intestinal epithelial cells bound together along their paracellular junctions by proteins of the apical junction complex, forming a physical barrier between luminal contents and the underlying mucosa. In the colon, a functionally intact epithelial barrier maintains a healthy homeostasis between water absorption and ion secretion while concurrently preventing escape of commensal bacteria from the lumen. Consequently, this barrier is a functional prerequisite for normal health, while rapid reopening of the barrier after injury is essential for prevention of exaggerated intestinal inflammation. Conversely, loss of epithelial barrier integrity is associated with increased passage of luminal antigens/metabolites across this barrier, resulting in aberrant activation of the underlying mucosal immune system and subsequent disease. There is increasing evidence that a dysfunctional intestinal epithelial barrier plays an important role in onset and pathogenesis of inflammatory bowel disease (IBD). The most apical junction of the apical junction complex is the zonula occludens [or tight junctions (TJs)]. These TJ proteins provide a barrier that regulates paracellular permeability, as well as cellular polarity, which helps maintain the health of the gut epithelium. Studies have identified defects in the structure and function of TJs in IBD patients and animal models of IBD. Moreover, altered epithelial barrier function has been observed in a subset (10–54%) of healthy first-degree relatives of IBD patients and in mouse models of colitis, supporting the hypothesis that epithelial barrier dysfunction represents an early event in disease pathogenesis.

Claudins constitute the backbone of TJs. It is clear that claudins play a key role in determining the permeability properties of the intestinal epithelium. Claudins are a multigene family of 27 tetraspan transmembrane proteins with different patterns of charged amino acids in the extracellular loops of individual isoforms. These proteins interact within epithelial cells to generate different-sized pores through which solute transfer occurs, conferring size and charge selectivity to the paracellular space. Decreased levels of claudins-1, -3, and -4 have been reported in IBD patients and mouse models of colitis. In contrast, claudin-2 was shown to be elevated in colonic tissues of IBD patients. Alteration in expression and cellular distribution of claudins during colitis likely impedes resealing of the epithelial barrier, resulting in ongoing passage of luminal antigens/metabolites across the barrier.

Claudin proteins are also regulated by serine/threonine and tyrosine kinases. While a range of protein kinase C (PKC) isoforms have been implicated in altered epithelial barrier permeability, their effects are often cell type-specific and dependent on conditions of activation. Overall, the effect of PKC isoforms on human intestinal epithelial TJs remains largely undefined. On the basis of the type of activation, PKC enzymes are classified as follows: 1) Ca2+- and diacylglycerol (DAG)-dependent conventional isoforms (α, βI, βII, and γ),
2) Ca\(^{2+}\)-independent, but DAG-dependent, novel isozymes (\(\delta\), \(\epsilon\), \(\theta\), \(\eta\), and \(\mu\)), and 3) neither Ca\(^{2+}\)-nor DAG-dependent atypical isozymes (\(\upsilon\) and \(\xi\)) (39).

While many factors control intestinal barrier function, the enteric nervous system plays a critical, yet incompletely understood, role in regulating this key aspect of intestinal health. The enteric nervous system regulates intestinal physiology and function, in part through secretion of neuropeptides, including VIP (31). VIPergic neurons are found throughout the intestinal lamina propria, forming a dense neural network that likely innervates intestinal epithelial cells and modulates intestinal epithelial cell function (51). Neunlist and colleagues (38) also showed that submucosal VIPergic neuronal pathways modulate intestinal epithelial barrier integrity and TJ protein expression.

Our previous research revealed a novel protective action of the neuropeptide VIP on barrier integrity during Citrobacter rodentium and enteropathogenic Escherichia coli (EPEC) bacteria infection. We showed that exogenous administration of VIP protects the colonic epithelial barrier by minimizing bacteria-induced redistribution of TJ protein, in part through actions of myosin light chain (MLC) kinase (MLCK) and phosphorylation of MLC (12). Here, we expand our findings by exploring further mechanisms of action by which VIP protects the intestinal epithelial barrier. Using Caco-2 monolayers, we show that degradation of PKC via long-term treatment with TPA protects epithelial barrier function during EPEC infection in a dose-dependent manner. Conversely, activation of PKC in the absence of EPEC by short-term TPA treatment resulted in disruption of the epithelial barrier, which was offset by pretreatment with VIP. Moreover, using PKCe and PKC\(\theta\) isozyme inhibitors, we show, for the first time, that VIP protects the epithelial barrier by inhibiting PKCe activation and, thereby, controlling claudin-4 degradation and attenuating EPEC-induced barrier disruption. Similarly, inhibition of PKCe activation in vivo in C. rodentium-induced colitis was found to significantly attenuate epithelial barrier disruption and the intestinal inflammatory response.

**METHODS**

**Epithelial Monolayer Cell Culture**

Caco-2 and HT-29 epithelial cells (\(1 \times 10^6\)), human colorectal adenocarcinoma cells isolated from a 72-yr-old male Caucasian and a 42-yr-old female Caucasian, respectively (American Type Culture Collection), were seeded in high-glucose DMEM growth medium onto 0.33-cm\(^2\) permeable polyester Transwell filters with 0.4-\(\mu\)m pore size (Costar/Corning). Cells were plated onto the upper surface of the filter, and transepithelial electrical resistance (TEER) of each well was measured every 48 h over a 21-day period until the monolayers reached 100% confluence, yielding resistances ranging from 1,200 to 1,000 \(\Omega\)·cm\(^2\). All resistance experiments were performed with 19- to 21-day-old monolayers.

**TEER Measurements**

TEER measures passive ion flow and is often used to describe paracellular permeability (33). TEER of Caco-2 epithelial monolayers growing on 0.3-\(\mu\)m, 12-mm-diameter polyester Transwell filters was measured using a voltohmmeter (EVOM, World Precision Instruments). Caco-2 cells (passages 40–50) were grown to full confluence on Transwell filters prior to addition of 1 \(\mu\)M VIP and/or the pharmacological inhibitor SQ-22536 (100–400 \(\mu\)M; Tocris Bioscience), forskolin (1 \(\mu\)M; Calbiochem), BAY 11-7082 (20 \(\mu\)M; EMD Biosciences), MG132 (30 \(\mu\)M; EMD Millipore), Y-27632 (10 \(\mu\)M; Calbiochem), TPA (100–400 nM; Sigma-Aldrich), PKC\(\theta\) pseudosubstrate inhibitor (50 nM; Calbiochem), PKCe translocation inhibitor peptide (20 \(\mu\)M; Calbiochem), or quercetin (200 \(\mu\)M; Sigma). Prior to selection of final dosages, preliminary assays were performed following the manufacturer’s recommendation or previously published results. TEER was measured at 0, 1, 2, 3, and 4 h, unless otherwise stated. After subtraction of TEER of the insert and bathing solution (120 \(\Omega\)·cm\(^2\)), measured TEER values for the effective growth area of the insert (0.33 cm\(^2\)) were normalized to 1,000 \(\Omega\)·cm\(^2\). Filters were then fixed and processed for immunocytochemical analysis or lysed for protein analysis.

**PKCe Transfection Mix**

Since PKCe inhibitor is a lipopholic peptide, 50 \(\mu\)l of transfection mix comprising 20 \(\mu\)M PKCe inhibitor, 3.5 \(\mu\)l of the protein transfection reagent ProteoJuice (EMD Millipore), and 7.5 \(\mu\)g of bovine serum albumin (BSA; Sigma-Aldrich) were used to transfet each Transwell insert according to the manufacturer’s recommendation. After the above-described mix was incubated for 20 min at room temperature, serum-free DMEM (200 \(\mu\)l) was added and distributed on the upper chamber, while 2% DMEM (1.5 ml) was added to the bottom chamber, of the Transwell insert. After the mix was incubated at 37°C in 5% CO\(_2\) for 2 h, cells were washed twice with serum-free medium to remove excess protein and used for TPA treatments or EPEC infections. Transwell inserts containing only ProteoJuice and BSA were used as controls.

**Paracellular Flux Experiments**

EPEC cultures were grown overnight in Luria broth (LB, optical density \(= 1 \times 10^6\)) on 5 x 10^6 bacteria/ml and then pelleted by centrifugation at 5,000 rpm for 5 min. The pellet was washed three times in PBS and then resuspended in 2% DMEM (without antibiotics) containing 1 mg/ml FITC conjugated to dextran (4 kDa). Caco-2 monolayers confluent grown on the apical chamber of Transwell chambers were infected with the bacterial suspension at a concentration of 10^7 bacteria per well. At 4 h postinfection, 100 \(\mu\)l from the bottom well were collected for measurement of tracer fluxes of FITC-dextran by the Thermo Electron Electroskan EX plate reader at 488 nm. Background readings were obtained from wells containing 2% DMEM only and subtracted from each experimental well.

**Immunocytochemistry**

Transwell filters from resistance experiments were fixed at 4 h postinfection in 4% paraformaldehyde, and mouse colonic tissues were fixed in 10% formalin (Fisher) overnight at 4°C and then processed to generate 5-\(\mu\)m paraffin sections. Mouse tissue sections were deparaffinized by heating at 55–56°C for 10 min, cleared with xylene, and rehydrated through an ethanol gradient to water. Fixed Transwell filters and mouse tissue were then blocked in 1% BSA and labeled with phallolidin-A594 (Invitrogen) or one of the following rabbit or rat primary antibodies (all at 1:200 dilution): PKC\(\alpha\), PKC\(\beta\), PKC\(\gamma\), PKC\(\delta\), PKC\(\theta\), or PKC\(\varepsilon\) (Santa Cruz Biotechnology). The secondary goat anti-rabbit and anti-rat antibodies were conjugated to Alexa Fluor 488 and 568, respectively, and used at 1:200 dilution. Stained cells were mounted in VECTASHIELD (Vector Laboratories, Burlington, ON, Canada) on glass slides and screened using a Zeiss microscope.

**Gene Expression Analysis by Quantitative PCR**

Noninfected Caco-2 cells or Caco-2 cells treated with EPEC for 4 h were collected from tissue culture plates by incubation with trypsin-EDTA (0.25%), and total RNA was extracted using the Qiagen RNeasy kit following the manufacturer’s protocol. Extracted RNA was quantified using a spectrophotometer (model ND1000, Nano-
Drop), and 500 ng of RNA were used to generate cDNA using the Quagen Omniscript RT kit following the manufacturer’s protocol. cDNA generated from reverse-transcription PCR on RNA was diluted 1:5 in RNase/DNase-free water. For quantitative PCR, 5 μl of cDNA was added to 15 μl of PCR mix containing 10 μl of SYBR green dye (Bio-Rad) and 5 μl of RNase/DNase-free water and primers to a final concentration of 0.6 μM. All reactions were assembled in duplicates, and quantitative PCR was carried out using the Bio-Rad Opticon 2 system. Melting-point analysis confirmed the specificity of the PCR. Results were quantified using GeneEx Macro OM 3.0 software (Bio-Rad) following the comparative threshold (2^(-ΔΔCt)) method. Primer sequences and reaction schemes are given in Table 1.

**TJ Fractionation Analysis**

The ProteoExtract subcellular proteome extraction kit (EMD Biosciences) was used for extraction of lysates from infected/treated Caco-2 monolayers and collection in four different fractions: cytosol, membrane-bound, nucleus, and cytoskeleton. The collected fractions were processed for Western blot analysis using polyvinylidene difluoride membranes (Bio-Rad). Rabbit anti-claudin-4, PKCε, PKCe, and β-actin antibodies were used at 1:1,000 dilution, and secondary anti-rabbit horseradish peroxidase-labeled antibodies were used at 1:2,000 dilution. Blots were visualized by an enhanced chemiluminescence detection system (Perkin Elmer). Incubation with anti-β-actin was used as a loading control. Intensity of the bands was quantified using ImageJ software, and values were normalized against α-tubulin.

**Immunoprecipitation**

Anti-rabbit claudin-4 antibody was conjugated to Dynabeads (Invitrogen) or β-actin (Santa Cruz Biotechnology). Monolayers were lysed using 300 μl of lysis buffer [in mM: 150 NaCl, 20 Tris, pH 7.5, 1 EDTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 phenylmethylsulfonyl fluoride, 1 sodium orthovanadate, and 1 sodium fluoride, with 1% Triton X-100, 1% phosphatase inhibitor cocktail (Thermo Scientific), and protease inhibitor cocktail tablets (Roche, Mannheim, Germany)]. Lysates were resolved by 15% and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto 0.2-mm polyvinylidene difluoride membranes (Bio-Rad). Rabbit anti-claudin-4, PKC0, PKCe, and β-actin antibodies were used at 1:1,000 dilution, and secondary anti-rabbit horseradish peroxidase-labeled antibodies were used at 1:2,000 dilution. Blots were visualized by an enhanced chemiluminescence detection system (Perkin Elmer). Incubation with anti-β-actin was used as a loading control. Intensity of the bands was quantified using ImageJ software, and values were normalized against the intensity of β-actin.

**In Vivo Experiments**

Mice. C57BL/6 (BL/6) mice (6–8 wk old, 18–20 g body wt; Charles River Laboratories, St. Constant, QC, Canada) were main-

tained in sterilized, filter-topped cages, handled in tissue culture hoods, and fed autoclaved food and water under specific pathogen-free conditions. Sentinel animals were routinely tested for common pathogens. The protocols were approved by the University of British Columbia Animal Care Committee and in direct accordance with guidelines drafted by the Canadian Council on the Use of Laboratory Animals.

**Bacterial colitis.** Colitis was induced with *C. rodentium* as previously described (12, 24). Briefly, mice were infected by oral gavage with 100 μl of LB containing 2.5 × 10^6 colony-forming units of streptomycin-resistant *C. rodentium* (formerly *C. freundii* biotype 4280), strain DBS100. Control mice received 100 μl of fresh LB by oral gavage. The mice were euthanized on day 10 postinfection, at which point the infection was well established and colitis was histologically evident.

**Treatment of mice with PKCε inhibitor peptide.** Individual mice were treated with PKCe inhibitor peptide (N-Myr-EAVSLKPT, 1,054 mol wt; Genemed Synthesis) in saline (2 mg/kg ip) 1 h prior to infection on day 0 and then daily to day 10 postinfection. In previous research using short-term small-animal models, this PKCe inhibitor peptide was administered at 0.4–10 mg/kg (48, 55). Readouts from inhibitor-treated mice were compared with those from mice receiving saline alone by intraperitoneal injection. Noninfected BL/6 and noninfected PKCe inhibitor-treated BL/6 mice served as uninfected controls. All mice were weighed immediately prior to infection and then daily until day 10 postinfection.

**Histopathological scoring.** Formalin-fixed hematoxylin-eosin-stained colonic tissues were scored semiquantitatively for clinical signs of colitis using a double-blind procedure (50). The tissues were evaluated on the basis of scores for 1) submucosal edema (0 = no change, 1 = mild, 2 = moderate, 3 = profound), 2) crypt hyperplasia (0 = no change, 1 = 1–50% change, 2 = 51–100% change, 3 = >100% change), 3) goblet cell depletion (0 = no depletion, 1 = mild depletion, 2 = moderate depletion, 3 = profound depletion), 4) epithelial integrity (0 = no change, 1 = a few cells sloughed, 2 = rippled epithelial surface, 3 = severely disrupted/damaged), and 5) mucosal edema and submucosal mononuclear cell infiltration [0 = no infiltration, 1 = <20 cells, 2 = 21–50 cells, 3 = 51–70 cells, 4 = >70 cells (per ×10 field)].

**Epithelial barrier function.** As described previously (24), uninfectected mice and mice at day 10 postinfection were orally gavaged with 150 μl of 80 mg/ml FITC-dextran (Sigma) for 4 h. After the mice were anesthetized, blood was collected by cardiac puncture and added to 3% acid-citrate dextrose. Plasma from the blood was measured for FITC quantification using a Perkin Elmer Victor X3 plate reader.

**Bacterial counts.** Total *C. rodentium* counts were assayed using whole colonic, cecum, mesenteric lymph node, spleen, and liver tissues (24). Briefly, tissues were collected and then homogenized in 1 ml of PBS using a Kinematica tissue homogenizer (Brinkmann) at low speed. Homogenates were serially diluted and plated onto MacConkey agar plates. After overnight incubation at 37°C, plates were evaluated for *C. rodentium* colony-forming units.

**Table 1. Primer sets and conditions for quantitative PCR**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>PCR Cycle Conditions (denature/anneal/extension)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PKCε</strong></td>
<td>5'-GCCCTATGGGCTCTCTCTTATTG-3'</td>
<td>5'-GAAAACAGCTCTCTTGGACAAGG-3'</td>
<td>95°C, 30 s/56°C, 30 s/72°C, 45 s</td>
</tr>
<tr>
<td><strong>PKCβ</strong></td>
<td>5'-GAGGAGACACTACATAGATTTGG-3'</td>
<td>5'-CCAAATTCACAGGACTTTCTCAAT-3'</td>
<td>95°C, 30 s/56°C, 30 s/72°C, 45 s</td>
</tr>
<tr>
<td><strong>PKCγ</strong></td>
<td>5'-GCCGCTTGATTTCCGAGAC-3'</td>
<td>5'-GCCCTGACATGTTTGACTTGGAC-3'</td>
<td>95°C, 30 s/56°C, 30 s/72°C, 45 s</td>
</tr>
<tr>
<td><strong>PKCe</strong></td>
<td>5'-AGTCTGTTCTCAGGGCTCTGCGAAGG-3'</td>
<td>5'-CTTGAGATGTCAGGAGCAGGCGA-3'</td>
<td>95°C, 30 s/55°C, 30 s/72°C, 45 s</td>
</tr>
<tr>
<td><strong>PKCδ</strong></td>
<td>5'-GCATGTGTTGCCAGCTACACAT-3'</td>
<td>5'-GCCCTCTGAAAGCTTCTAGTGG-3'</td>
<td>95°C, 30 s/56°C, 30 s/72°C, 45 s</td>
</tr>
</tbody>
</table>
Inhibitor peptide killing assays. Overnight-cultured EPEC and C. *rodentium* were diluted to 1,000 bacteria/100 μl based on the optical density of the cultures. The bacterial pellet was incubated with PKC0 (50 nM) or PKCε (20 μM) inhibitors or PBS as a control for 2 h at 37°C and then plated on MacConkey agar. Colony-forming units were evaluated after overnight incubation of plates at 37°C.

**Statistical Analysis**

Values are means ± SE of separate experiments. A minimum of four replicates were used for each condition, and the experiments were repeated a minimum of three times, unless otherwise stated. Analyses were conducted with GraphPad Prism 4 statistical software for Windows (GraphPad Software, San Diego, CA). Differences between means were calculated by one- or two-way analysis of variance or paired t-tests where appropriate. Specific differences were tested with the Student-Newman-Keuls test, where *P* < 0.05 was considered statistically significant.

**RESULTS**

**EPEC-Induced Epithelial Barrier Disruption and Rho Kinase and NF-κB Pathways**

Previous studies showed a beneficial effect of inhibition of Rho kinase (ROCK) activity in an in vitro model of *C. rodentium*-induced colitis (18), as well as in the in vivo model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats (49). Therefore, we hypothesized that ROCK activation might be involved in EPEC-induced barrier disruption and tested whether the ROCK inhibitor Y-27632 would protect epithelial barrier function during EPEC infection. Fully differentiated Caco-2 monolayers infected with EPEC alone or pretreated with Y-27632 and then infected with EPEC demonstrated a similar reduction in TEER at 4 h postinfection, suggesting the absence of effect of the inhibitor, at least at the dose used (Fig. 1A). To validate the functionality of the ROCK inhibitor, cultured epithelial cells were immunostained for F-actin filaments in the presence and absence of the inhibitor. As shown in Fig. 1B, cells pretreated with ROCK inhibitor demonstrated reduced focal adhesions and disassembling stress fibers at 4 h postinfection compared with untreated cells infected with EPEC, suggesting functionality of the inhibitor. Taken together, these data suggest that EPEC-induced barrier disruption is at least partially independent of a fully functional ROCK pathway.

Our previous work revealed that EPEC-induced barrier disruption is mediated in part through activation of MLCK, while VIP prevents MLCK activation and increased phosphorylated...
MLC during EPEC infection (12). NF-κB has been described as the upstream transcription factor responsible for MLCK activation (22). Therefore, we examined the effect of NF-κB inhibition during EPEC infection of Caco-2 monolayers. Inhibition of IκB phosphorylation using the NF-κB inhibitor BAY 11-7082 (20 μM) was not associated with significant differences in TEER compared with controls. Infection with EPEC alone induced a 45% decrease in TEER at 4 h postinfection (P < 0.01, n = 5), comparable to infected cells pretreated with BAY 11-7082 [36% decrease at 2 h postinfection and further decrease to 44% at 4 h postinfection (P < 0.01, n = 5; Fig. 1C)], suggesting that EPEC-induced barrier disruption does not depend on NF-κB activation by 1κB. In addition, we examined the effect of MG132, which is known to inhibit ubiquitin-mediated proteasomal degradation of IκBα protein (20). Compared with controls, TEER in infected cells pretreated with MG132 was reduced 42% by 4 h postinfection (P < 0.01, n = 4), similar to untreated cells infected with EPEC, further suggesting a less important role for NF-κB activation (Fig. 1D).

VIP-Mediated Intestinal Epithelial Barrier Protection Does Not Require the cAMP Pathway

Our previous studies revealed a novel protective action of VIP during EPEC-induced epithelial barrier disruption (12). After confirming our earlier results with VIP treatment (Fig. 2A), we further elucidated the cellular pathways involved by investigating the effects of pharmacological inhibition of signal transduction pathways known to be involved in VIP signaling and TJ formation. cAMP is one such pathway activated by VIP in human intestinal epithelial cells (28). To verify if VIP-mediated barrier protection during EPEC infection was occurring via activation of cAMP, fully differentiated Caco-2 monolayers were treated with the adenylate cyclase inhibitor SQ-22536 (100–400 μM) prior to treatment with VIP and EPEC. As shown in Fig. 2B, infection of cells treated with EPEC alone induced a significant 57% decrease in TEER compared with controls (P < 0.01, n = 8). Inhibition by SQ-22536 prior to VIP treatment and EPEC infection failed to abrogate the protective actions of VIP, yielding no significant decrease in TEER at all concentrations tested, suggesting that VIP, at least in this setting, mediates its protection via pathways independent of cAMP activity. Furthermore, pretreatment of EPEC-infected monolayers with SQ-22536 and 1 μM forskolin (an adenylate cyclase activator) revealed a similar decrease in TEER compared with untreated infected cells (data not shown). In addition, we ruled out participation of calmodulin/Ca2+-dependent protein kinase and phospholipase C pathways, also implicated in VIP signaling, by using their respective inhibitors (data not shown) (5).

EPEC-Induced Disruption of Intestinal Epithelial Barrier Involves Activation of PKC

The ability of EPEC infection to induce PKC activation has been established in several epithelial cell lines (13, 47). Therefore, we used the phorbol ester TPA to examine the effect of PKC activation and inhibition on Caco-2 monolayers. Short-term treatment with TPA is known to activate PKC, while long-term treatment is known to degrade PKC (32, 62). Activation of PKC by short-term treatment with TPA (400 nM) resulted in a 30% decrease in TEER compared with untreated cells, and, interestingly, pretreatment with VIP abrogated this effect, maintaining TEER at levels similar to controls (P < 0.01, n = 4; Fig. 3A). Similarly, monolayers pretreated with VIP were protected from additional epithelial barrier disruption caused by EPEC infection and short-term TPA treatment over a 4-h time course (Fig. 3B). As shown in Fig. 3B, a significant 55% and 40% reduction in TEER was observed at 4 h postinfection in cells infected with TPA and cells infected with only EPEC, and this was completely abolished by addition of 1 μM VIP (P < 0.05, n = 4). Furthermore, permeability assays with FITC-dextran showed a significant increase in epithelial barrier permeability in Caco-2 cells subjected to short-term TPA treatment and/or infected with EPEC for 4 h that was significantly reduced with VIP pretreatment (Fig. 3C). In contrast to the short-term treatment, degradation of PKC by long-term TPA treatment produced a concentration-dependent protection of the epithelial barrier. While...
a significant 46% decrease in TEER was induced by EPEC infection alone, we observed a nonsignificant 41% decrease following long-term incubation with 100 nM TPA (n = 4) and a significant 28% and 17% decrease with 200 nM TPA (P < 0.05, n = 4) and 400 nM TPA (P < 0.01, n = 4; Fig. 3D), respectively. Overall, these results suggest that VIP inhibition of PKC activation is a likely mechanism that protects against EPEC-induced epithelial barrier disruption.

**PKCα, PKCδ, and PKCε Isozymes Are Activated During EPEC Infection**

Having established a pivotal role for PKC activation during EPEC infection and a protective action of VIP during TPA-induced PKC activation, we examined the specific PKC isozymes activated during EPEC infection. Using primers specific to PKCα, PKCβ, PKCγ, PKCδ, PKCθ, and PKCε isozymes, we analyzed gene expression on cells infected with EPEC for 4 h. As shown in Fig. 4A, PKCα, PKCδ, and PKCε gene expression was upregulated significantly in EPEC-infected compared with noninfected cells, suggesting a potential role for these PKC isozymes in compromising epithelial barrier integrity during EPEC infection. This finding was further confirmed by immunostaining of infected Caco-2 cells using antibodies specific for the different PKC isoforms. Consistent with the gene expression levels, we found an increase in staining of PKCα, PKCδ, and PKCε isoforms in infected cells (Fig. 4B).

**Activation of PKCε, but not PKCθ, Is Primarily Responsible for Barrier Disruption During EPEC Infections, and VIP Acts by Inhibiting PKC Activation**

As a previous study reported activation of PKCδ and its association with disruption of the E-cadherin/β-catenin complex during EPEC infection (35), we further explored the potential novel roles for PKCθ and PKCε in driving EPEC-induced barrier disruption. We treated Caco-2 monolayers with respective PKC isozyme inhibitors and then subjected the monolayers to short-term TPA treatment and EPEC infection. As shown in Fig. 5A, TEER of cells treated with PKCθ and PKCε inhibitors followed by short-term TPA treatment was maintained at levels similar to controls until 2 h postinfection and then moderately decreased at 3 and 4 h. However, compared with cells treated with TPA only, the inhibitor-treated cells demonstrated a 20% increase in TEER (P < 0.05, n = 4) at 3 and 4 h posttreatment, suggesting that activation of PKCθ
and PKCε plays a role in the barrier disruption induced by TPA. However, when we tested the role of these isozymes during EPEC infection, inhibition of PKC0 had little protective effect on TEER, which was similar to TEER in untreated cells infected by EPEC. Thus PKC0 activation does not appear to be required for epithelial barrier disruption during EPEC infection (Fig. 5B). Conversely, inhibition of PKCε in infected cells resulted in TEER similar to that of uninfected cells, suggesting that PKCε activation is involved in the ability of EPEC to induce barrier disruption (P < 0.05, n = 4; Fig. 5C). Moreover, when EPEC-infected Caco-2 monolayers were pretreated with the PKCε inhibitor and VIP, a significant 70–75% increase in TEER was observed throughout the infection period compared with untreated cells infected by EPEC (P < 0.0001, n = 4; Fig. 5D). The combined effect also led to a 20% increase in TEER compared with cells treated with PKCε inhibitor or VIP independently (P < 0.005, n = 4; Fig. 5, C and D). To determine whether the protective effects of VIP and the PKCε inhibitor during EPEC could be seen in another intestinal epithelial cell line, we tested HT-29 cells. Similar to our results in Caco-2 cells, HT-29 cells infected with EPEC showed a significant drop in TEER at 4 h postinfection. Conversely, TEER in HT-29 cells preincubated with VIP or the PKCε inhibitor was similar to TEER in uninfected cells (data

Fig. 4. A: gene expression of PKCα, PKC0, and PKCε isozymes during 4 h of EPEC infection. mRNA extracted from Caco-2 monolayers was analyzed for expression of PKC isozymes using primers specific for PKCα, PKCβ, PKCγ, PKCδ, PKC0, and PKCε. Primer sequences are shown in Table 1. NI, noninfected. Values are means ± SE; n = 3, performed twice. ***P < 0.0001, *P < 0.05. B: immunohistological staining of uninfected Caco-2 monolayers (control) and monolayers infected with EPEC for 4 h. Cells were fixed with 4% paraformaldehyde and then stained using antibodies specific to the PKC isozymes PKCα, PKCβ, PKCγ, PKCδ, PKC0, and PKCε. Arrows indicate increased positive staining of PKC0 and PKCε following infection with EPEC for 4 h (right) compared with noninfected controls (left). Blue fluorescence is 4′,6-diamidino-2-phenylindole labeling of double-stranded DNA depicting the nucleus of the cell. Scale bar = 10 µm; original magnification ×600.
not shown). To determine if the increase in TEER corresponded to tighter barrier integrity, we analyzed membrane permeability using the paracellular tracer FITC-dextran (4 kDa) at 4 h postinfection. A significant 60% increase in FITC levels was observed in cells infected with EPEC alone or pretreated with PKCθ inhibitor, suggesting severe impairment of epithelial barrier integrity \((P < 0.02, n = 4)\). Conversely, the increase in paracellular permeability typically seen following EPEC infection was not observed in EPEC-infected cells pretreated with VIP alone or in combination with the PKCe inhibitor, which demonstrates FITC levels similar to those in noninfected cells (Fig. 5E). To exclude the possibility that the...
inhibitors impacted the viability of EPEC, the overnight-cultured bacteria were incubated with each inhibitor separately for 2 h before they were plated on agar plates. No significant differences in EPEC colony-forming units were observed between inhibitor- and PBS-treated bacteria, signifying that their impact on barrier function was due to their actions on host cells (Fig. 5F).

EPEC Disrupts the Epithelial Barrier Through Degradation of Claudin-4

Previous studies showed a direct relationship between activation of PKCε and its role in modulating levels of the bijunctional protein claudin-4 (15). We therefore hypothesized that EPEC-induced PKCε activation may be mediating its effects on barrier function by an impact on the expression of claudin-4. TJ fractionation studies revealed an increase in membrane-bound claudin-4 expression in lysates from EPEC-infected Caco-2 cells treated with long-term TPA and VIP, in contrast to the reduced levels in lysates from untreated EPEC-infected cells (Fig. 6A). In monolayers pretreated with PKC9 or PKCε inhibitors alone, we did not observe changes in claudin-4 expression (results not shown). However, when inhibitor-treated monolayers were infected with EPEC for 4 h, membrane-bound claudin-4 levels were maintained in cells treated

Fig. 6. Altered claudin-4 levels following infection with EPEC. A–D: Western blot analysis of Caco-2 monolayers infected with EPEC or subjected to short-term treatment with 400 nM TPA for 4 h following long-term preincubation with 400 nM TPA or 1 μM VIP (A), 50 nM PKC9 or 20 μM PKCε inhibitors (B), 50 nM PKC9 or 20 μM PKCε inhibitors (C), and 1 μM VIP or 20 μM PKCε inhibitor (D). Claudin-4 (22 kDa) and PKCε (90 kDa) expression is shown as band density normalized to housekeeping gene β-actin (42 kDa) and analyzed using ImageJ software. Ctrl, control. E: lysates of uninfected and EPEC-infected Caco-2 cells were immunoprecipitated with a claudin-4 antibody, and precipitates were analyzed by Western blotting using an antibody specific for claudin-4 and phosphorylated (serine/threonine) claudin-4 (P Ser/Thr). F: TEER of Caco-2 monolayers pretreated with 200 μM quercetin for 24 h and then infected with EPEC. TEER was not significantly reduced in EPEC-infected cells pretreated with quercetin. **P < 0.005, *P < 0.05.
with the PKCε inhibitor. Conversely, infected cells treated with the PKC0 inhibitor showed decreased claudin-4 levels, similar to those observed in untreated, EPEC-infected cells (Fig. 6B). In parallel to decreased claudin-4 expression in EPEC-infected cell lysates, short-term treatment with TPA also resulted in a similar decrease in claudin-4 expression, suggesting a direct relationship between activation of PKC and reduction of claudin-4 protein (Fig. 6C). To confirm that PKCε is activated during EPEC infection, whole cell lysates were probed with antibodies specific for PKCε. As shown in Fig. 6D, PKCε expression levels were significantly higher in EPEC-infected monolayers with a corresponding decrease in claudin-4 expression. In contrast, cells pretreated with VIP or PKCε inhibitors demonstrated lower PKCε activation than EPEC-infected cells and maintained normal levels of claudin-4 expression (Fig. 6D). To test if PKCε has a role in posttranslational regulation of claudin-4, immunoprecipitation analysis was performed on membrane lysates from noninfected and EPEC-infected cells. Our results showed a reduction in expression of phosphorylated claudin-4 corresponding to a reduction in total claudin-4 in EPEC-infected lysates, suggesting that PKCε-induced barrier disruption is likely mediated in part through degradation of total claudin-4 levels (Fig. 6E). Furthermore, to study if claudin-4 overexpression would enhance the epithelial barrier resistance, quercetin, a flavonoid known to enhance claudin-4 expression (2), was added to cells 24 h before EPEC infection. As shown in Fig. 6F, there was a significant increase in barrier resistance in infected cells treated with quercetin compared with untreated EPEC-infected cells. These results suggest that PKC activation indeed plays an important role in regulating claudin-4 expression and that VIP mediates its protective action during EPEC infection by inhibiting PKCε.

**Inhibition of PKCε Attenuates C. rodentium-Induced Colitis**

We used an infectious colitis model to examine the potential therapeutic benefit of PKCε inhibition in vivo. Since EPEC strains do not efficiently infect mice, we used the well-recognized (and related) mouse pathogen *C. rodentium* to induce colitis in mice. At day 10 postinfection, hematoxylin-eosin staining of formalin-fixed tissues revealed significant protection from *C. rodentium*-induced colitis in mice treated with a PKCε inhibitor peptide. We noted a significant reduction in clinical signs of colonic crypt hyperplasia, submucosal edema, goblet cell depletion, and lymphocyte infiltration in inhibitor-treated mice compared with PBS-treated mice (Fig. 7A). Subsequent semiquantitative histological scoring of colonic tissues revealed a similar significant reduction of clinical manifestations of disease in mice treated with PKCε inhibitor peptide compared with PBS-treated mice (*P* < 0.001, *n* = 5; Fig. 7A). When measured for epithelial barrier leakage, a significant decrease in serum FITC-dextran levels was observed in the PKCε inhibitor-treated mice compared with PBS-treated mice, indicating a tighter epithelial barrier in inhibitor-treated mice (*P* < 0.05, *n* = 5; Fig. 7B). To explore the potential effect of the PKCε inhibitor peptide on colonization of *C. rodentium*, we assessed the total pathogen burdens in the large bowel and systemic tissues of infected mice. Although *C. rodentium* colonization in the cecum and colon was unchanged following treatment with the inhibitor, pathogen burdens in the liver and mesenteric lymph nodes were significantly lower in the PKCε inhibitor-treated mice, confirming a role for PKCε in controlling intestinal epithelial barrier function (Table 2). Antimicrobial assays, which were performed to rule out any direct killing effect of PKCε inhibitor peptide on *C. rodentium*, identified no significant differences in colony-forming units between inhibitor- and PBS-treated *C. rodentium*-infected mice, suggesting that the peptide exerted its effects through actions on the host (Fig. 7C). When mouse colonic tissues were examined for PKCε expression by immunostaining, we observed a significant increase in PKCε expression in the crypt lumen of *C. rodentium*-infected mice that was reduced in mice pretreated with a PKCε inhibitor (Fig. 7D). Finally, when colonic tissues were probed for claudin-4 expression, we also noted low levels of claudin-4 expression in *C. rodentium*-infected mice, whereas mice treated with the PKCε inhibitor peptide maintained claudin-4 expression similar to that of noninfected BL/6 mice (Fig. 7E). These results suggest that inhibition of PKCε results in improved intestinal barrier function, in part by maintaining claudin-4 expression and, thereby, preventing translocation of bacteria into the systemic tissues and, thus, conferring protection to the mice.

**DISCUSSION**

While it is well accepted that IBD results from a dysregulated mucosal immune response to environmental factors in genetically susceptible hosts, there is increasing evidence that epithelial barrier dysfunction plays an important role in disease onset and pathogenesis (19, 37). Altered barrier permeability has been observed in a subset (10–54%) of first-degree relatives of patients with IBD and in many murine models of IBD (19, 34, 43). A number of studies of mouse models of colitis have shown that protecting the epithelial barrier and decreasing epithelial permeability reduce the onset and severity of disease (4, 12). Having previously established a protective role for VIP in epithelial barrier homeostasis in infectious models of colitis (12), we explored mechanistic pathways involved in EPEC-induced disruption of the epithelial barrier with the aim of determining the protective mechanism(s) of action of VIP.
We have shown, for the first time, that pretreatment of Caco-2 monolayers with VIP prevents EPEC-induced PKC activation and subsequent disruption to the epithelial barrier. Conversely, degradation of PKC via long-term treatment of Caco-2 monolayers with TPA protected epithelial barrier function during EPEC infection in a dose-dependent manner. Short-term TPA exposure mimics the effect of EPEC infection on epithelial barrier function, while VIP prevents these deleterious

![Image of histopathological score and FITC-Dextran (µg/ml) charts]

**Figure A:** BL/6 CR vs. BL/6 CR-PKCε In

**Figure B:** Bar graph showing FITC-Dextran concentration (µg/ml) and C. rodentium CFU counts.

**Figure C:** G.399 VIP PREVENTS PKC-INDUCED BARRIER DISRUPTION DURING EPEC INFECTION

**Figure D:** BL/6 CR vs. BL/6 CR-PKCε In

**Figure E:** Western blots showing Claudin-4 and β-actin expression.
actions during TPA treatment. Conversely, inhibition of PKC revealed a concentration-dependent protective effect on epithelial barrier function during EPEC infection. The protective actions of PKC inhibition correlated with the increased expression of membrane-bound claudin-4 during TPA treatment and EPEC infection compared with EPEC infection alone.

Several studies have described VIP as a regulator of immune cells by inhibition of proinflammatory cytokines (17), as well as a factor that is neuroprotective against LPS-induced neurodegradation (16) and protective against endotoxemia (58) and TNBS-induced colonic inflammation (1). However, few studies have evaluated the immune regulatory role of VIP during infectious conditions (12, 54). VIP and its structurally similar pituitary adenylate cyclase-activating peptide have been shown to inhibit TNF-α signaling by regulating cAMP and NF-κB pathways (17).

Our results with intestinal epithelial cell lines infected with EPEC suggest that several established pathways of epithelial barrier disruption, namely, cAMP, ROCK, and NF-κB, are less functionally involved (17). However, in agreement with earlier findings (14), we have shown that EPEC causes epithelial barrier disruption through activation of PKC enzymes. Early studies with phorbol esters such as TPA and phorbol 12-myristate 13-acetate revealed downregulation of phorbol ester-binding sites on leukemic cells upon chronic exposure to TPA (53). Subsequent studies revealed degradation of PKC within the cytoplasm, inhibiting PKC function in several cell types upon long-term (≥18 h) incubation with TPA (6, 62). In contrast, short-term (1–6 h) exposure to TPA correlates with membrane translocation of PKCε, characterized by a rapid decrease in TEER (46). Using short- and long-term TPA exposures, we have shown, for the first time, that pretreatment of Caco-2 monolayers with VIP prevents PKC-induced disruption of the epithelial barrier function during EPEC infection. Activation of PKC by short-term TPA treatment resulted in a reduction of TEER similar to that observed with EPEC infection. Conversely, pretreatment with VIP protected against EPEC-induced infectious insult to the epithelial barrier by maintaining TEER at a level similar to that observed in cells subjected to long-term TPA treatment with inhibitory PKC function. These findings suggest that protective action of VIP on the intestinal epithelial barrier is likely due to the inhibition of PKC enzyme activation.

To further investigate the possible specific PKC isozymes involved in EPEC infection, a family of six PKC novel isoforms was analyzed. A previous study reported activation of PKCα and its association with disruption of the E-cadherin/β-catenin complex during EPEC infection (35). We found evidence of PKCθ and PKCε isozyme activation/upregulation upon EPEC infection at 4 h postinfection. While it has been shown that PKCθ plays an important role in activation of autoreactive T cells (27), PKCε was shown to be involved in regulation of the TJ barrier protein claudin-4 (15). Using specific inhibitors of PKCθ and PKCε, we showed that EPEC infection-induced epithelial damage is a result of PKCε activation modulating the expression of claudin-4 and that VIP protects against this barrier disruption by inhibiting PKCε activation. Inhibition of PKCε activation also ameliorated the disruption in TJ morphology of zonula occludens-1 and occludin (data not shown).

The beneficial effects of PKCε inhibition in C. rodentium-induced colitis are intriguing and open a potential avenue for targeted therapeutic intervention. Indeed, previous studies have shown that PKC inhibitors reduce inflammation in TNBS-induced colitis (7, 9) and in other models of experimental inflammation such as phorbol 12-myristate 13-acetate-induced mouse ear edema (30). However, PKC isozymes differ in their mechanisms of activation, underlying conditions of activation, expression, and downstream effects, suggesting that a targeted approach is more suitable. Taken together with previous observations, our data suggest that the beneficial effects of PKCε inhibition are mediated through anti-inflammatory mechanisms and direct effects on the epithelial barrier. Indeed, it has been shown that nitric oxide-induced cell injury is associated with an increase in the level of PKCε and is accompanied by PKCε redistribution from the cytosol to cell membrane, with disruption of tubulin assembly, microtubule disarray, and disruption of the intestinal barrier (56). Furthermore, our data suggest that TJ redistribution is mediated through PKCε activation. PKCε may also mediate TNFα-induced cytotoxicity and apoptosis contributing to barrier disruption (10). Moreover, there is evidence that this isoform modulates mucin gene expression in epithelial cells (26). The discrepancies between our study and a recently published study using a stress model demonstrating VIP-mast cell interaction in the regulation of rat and human ileal barrier function can be associated with the two different models used to induce barrier disruption. In the stress-induced model, mast cells are known to infiltrate in high numbers, whereas C. rodentium is an inflammatory model where mast cells have very little role. It is possible that, depending on the host’s hostile environment, VIP may function via different mechanisms to mediate epithelial barrier protection (23, 29).

In summary, our results demonstrate a novel protective mechanism of VIP during EPEC-induced epithelial barrier disruption and function. The ability of VIP to inhibit PKCε activation and maintain TJ protein levels suggests a potential role for VIP as a therapeutic agent in treatment of patients with intestinal infectious diseases and inflammatory conditions.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

---

**Table 2. Citrobacter rodentium CFU at day 10 postinfection**

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>C. rodentium CFU, log_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
</tr>
<tr>
<td>Colon</td>
<td>8.7 ± 0.19</td>
</tr>
<tr>
<td>Cecum</td>
<td>8.67 ± 0.16</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.45 ± 0.32</td>
</tr>
<tr>
<td>Liver</td>
<td>3.3 ± 0.31</td>
</tr>
<tr>
<td>Mesenteric lymph nodes</td>
<td>3.48 ± 0.32</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 5). CFU, colony-forming units; PKCε In, PKCε inhibitor; NS, not significant.
REFERENCES


VIP PREVENTS PKC-INDUCED BARRIER DISRUPTION DURING EPEC INFECTION


