Vasoactive intestinal peptide ameliorates intestinal barrier disruption associated with *Citrobacter rodentium*-induced colitis

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Conlin VS, Wu X, Nguyen C, Dai C, Vallance BA, Buchan AM, Boyer L, Jacobson K. Vasoactive intestinal peptide ameliorates intestinal barrier disruption associated with *Citrobacter rodentium*-induced colitis. *Am J Physiol Gastrointest Liver Physiol* 297: G735–G750, 2009. First published August 6, 2009; doi:10.1152/ajpgi.90551.2008.—Attaching and effacing bacterial pathogens attach to the apical surface of epithelial cells and disrupt epithelial barrier function, increasing permeability and allowing luminal contents access to the underlying milieu. Previous in vitro studies demonstrated that the neuroepithelium vasoactive intestinal peptide (VIP) regulates epithelial paracellular permeability, and the high concentrations and close proximity of VIP-containing nerve fibers to intestinal epithelial cells would support such a function in vivo. The aim of this study was to examine whether VIP treatment modulated *Citrobacter rodentium*-induced disruption of intestinal barrier integrity and to identify potential mechanisms of action. Administration of VIP had no effect on bacterial attachment although histopathological scoring demonstrated a VIP-induced amelioration of colitis-induced epithelial damage compared with controls. VIP treatment prevented the infection-induced increase in mannitol flux a measure of paracellular permeability, resulting in levels similar to control mice, and immunohistochemical studies demonstrated that VIP prevented the translocation of tight junction proteins: zona occludens-1, occludin, and claudin-3. Enteropathogenic *Escherichia coli* (EPEC) infection of Caco-2 monolayers confirmed a protective role for VIP on epithelial barrier function. VIP prevented EPEC-induced increase in long myosin light chain kinase (MLCK) expression and myosin light chain phosphorylation (p-MLC). Furthermore, MLCK inhibition significantly attenuated bacterial-induced epithelial damage both in vivo and in vitro. In conclusion, our results indicate that VIP protects the colonic epithelial barrier by minimizing bacterial-induced redistribution of tight junction proteins in part through actions on MLCK and MLC phosphorylation.

**tight junctions; Caco-2 cells; transepithelial resistance**

*Citrobacter rodentium* is the murine equivalent of human enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively). *C. rodentium* and EPEC are noninvasive, gram-negative, attaching and effacing (A/E) bacterial pathogens that infect the colonic epithelium to induce colitis (27, 28). A/E bacteria adhere to the surface of colonic epithelial cells, inject effector proteins and induce accumulation of cytoskeletal proteins underneath the site of attachment, producing the characteristic A/E lesion (15, 47). The injected proteins trigger a broad range of cellular events including alterations in electrolyte secretion, disruption of colonic epithelial barrier function, and mucosal inflammation resulting in colitis (5, 11, 15, 31). Although the bacterial proteins causing the A/E lesions have been extensively studied the mechanism underlying *C. rodentium*-induced intestinal barrier disruption has yet to be determined.

Epithelial barrier integrity is established and maintained by the proteins of the paracellular junction complex. Tight junctions located at the apical end of the paracellular junction complex perform a “gate and fence function”; these proteins control the paracellular passage of ions and solutes while maintaining a physical barrier to the penetration of macromolecular luminal contents. Tight junctions consist of the transmembrane proteins (claudins and occludin) and cytoplasmic plaque proteins such as zonula occludens-1 (ZO-1). The claudin family of proteins appears to be the critical regulator of paracellular permeability in the gastrointestinal epithelium and disruption of these proteins is associated with inflammatory bowel disease in patients and animal models of colitis (19, 34, 38).

In a recent study, EPEC infection of short-term cultures of the human colonic cell line Caco-2 cells was shown to alter epithelial barrier function independent of bacterial-induced host cell death (48). Furthermore, in vitro (12, 17, 34) and in vivo (22, 23) studies have demonstrated that A/E bacteria disrupt tight junction proteins, resulting in a decrease in transepithelial resistance (TER) and increased epithelial paracellular permeability. In vitro, EPEC disrupted the organization of tight junction proteins occludin, ZO-1, and claudins-1, -4, and -5 (34), whereas *C. rodentium* disrupted claudins-4 and -5 (34). In vivo, *C. rodentium*-induced epithelial barrier disruption was associated with disruption of ZO-1 and claudin-3; however, whether this resulted in a drop in TER was not determined (19).

In vitro and in vivo studies indicate involvement of myosin light chain kinase (MLCK) in the maintenance of intestinal epithelial barrier permeability and in EPEC-induced changes in barrier function (52, 53). The activity of myosin is regulated by the actions of MLCK and phosphorylation of myosin light chain (MLC). MLC phosphorylation by MLCK contracts the perijunctional actinomyosin ring, putting tension on tight junction proteins, separating tight junctions, and increasing paracellular permeability (45). Consistent with the ability of EPEC to increase epithelial permeability, MLCK expression and phosphorylation of MLC are increased during EPEC infections (52). Conversely, inhibition of MLCK prevents EPEC-induced MLC phosphorylation (53), attenuating the deleterious effect of EPEC on epithelial barrier TER. Hence MLCK is a key regulator of actinomyosin function (25) and current evidence indicates that the protein plays a key role in the A/E effect of EPEC.
Under both physiological and pathological conditions the enteric nervous system regulates epithelial barrier function (16). Submucosal neurons, and in particular vasoactive intestinal peptide [(VIP)ergic] neurons directly innervate intestinal epithelial crypt cells and regulate intestinal ion and fluid secretion (10, 21) and epithelial barrier homeostasis (26). In vivo and in vitro studies also suggest a role for VIP in the regulation of epithelial paracellular permeability (4, 35) in addition to its anti-inflammatory action during colitis (2, 13, 14). Recent research has revealed a significant increase in the density of submucosal VIP-immunoreactive (VIP-IR) neurons in colonic tissue from pediatric patients with Crohn’s disease (7) and intestinal barrier-induced increase in VIP-IR (32), raising the question of what role, if any, the neuropeptide plays in the physiological processes during colitis.

In this study exogenous VIP was administered to *C. rodentium*-infected mice and the inflammatory response and barrier integrity assessed. Our data indicate that VIP attenuated the adverse consequences of *C. rodentium* infection. In addition, in vitro studies using confluent Caco-2 monolayers reveal that the protective effect of VIP is exerted on the epithelial barrier, protecting TER and preventing redistribution of key tight junction proteins (ZO-1 and occludin). Furthermore, Western blot analysis and MLCK inhibition reveal the protective effect of VIP is mediated in part through actions on MLCK.

**METHODS**

**Mice.** Six to eight-week-old C57BL/6 mice weighing ~18–20 g were purchased from Charles River Laboratories (St. Constant, QC, Canada). Mice were maintained 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 used were approved by the University of British Columbia’s Animal Care Committee and in direct accordance with guidelines drafted by the Canadian Council on the Use of Laboratory Animals.

**Bacterial strains and infection of mice.** Colitis was induced with *C. rodentium* by previously described procedures (19, 23, 51). Briefly, mice were infected by oral gavage with 100 μl of Luria Broth (LB) containing 2.5 × 10⁸ colony-forming units of *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 (pi), at which time infection was well established and colitis was evident (28).

Overnight cultures of EPEC in LB broth were grown as previously described (optical density at 660 nm, 0.8; 5 × 10⁸ bacteria/ml) (39). Bacterial suspensions were centrifuged at 10,000 rpm for 2 min and washed 3× in PBS. Bacteria were resuspended in high-glucose DMEM (without antibiotics) and layered onto Caco-2 monolayers at a concentration of 1 × 10⁷ per monolayer.

**VIP treatment.** Individual mice were treated with 0.5, 1, or 5 nmol VIP in saline administered by intraperitoneal (ip) injection daily from...
day 1 to day 10 pi. The dose chosen was in keeping with doses used previously in murine models of colitis (2, 3, 20). Control mice received saline alone by ip injection.

**ML-7 treatment.** Individual mice were treated with 1 mg/kg ML-7 in saline administered by ip injection 1 h prior to infection on day 0 and then daily to day 10 pi. Previous research using ML-7 in short-term small animal models used concentrations ranging from 1 to 2 mg/kg (9, 33). Given the extended duration of the present studies, a more conservative dose was administered. Control mice received saline alone by ip injection.

**Body weight measurements.** Mice were weighed immediately prior to infection and then daily until day 10 pi. Body weight data presented are from one representative experiment of three experiments showing similar results (n = 10). Mice were euthanized on day 10 pi. Colon tissues were removed and prepared for the analyses described below.

**Histopathological scoring.** Histological damage score examination was performed as previously described (6, 51). Briefly, distal colonic segments (0.5 cm) were fixed with 10% neutral buffered formalin and embedded in paraffin. Cuts from the center of the colon (5 µm) were cut and mounted on slides. Tissue sections were stained with hematoxylin and eosin, and cell morphology was viewed via light microscopy. Histological damage scoring was determined with criteria adapted from previous studies (51). Briefly, the damage score consisted of a score for the severity of epithelial injury (graded 0–3, from absent to mild including superficial epithelial injury, moderate including focal erosions, and severe including multifocal erosions), the extent of inflammatory cell infiltrate (graded 0–3, from absent to transmural), and goblet cell depletion (0–1). In each case a numerical score was assigned. Six tissue sections from each animal were coded and examined by two blinded observers to prevent observer bias. Tissue sections were assessed (each separated by at least 500 µm) under a Nikon Eclipse 400 light microscope and averaged to obtain a mean histological damage score. Crypt heights were measured by microscopy, with 10 measurements taken in distal colon sections of each mouse and five mice per group. Only well-oriented crypts and tissue sections with intact muscularis propria were measured.

**Epithelial barrier function.** Murine colonic epithelial barrier function was measured by use of Ussing chambers (DVC 1000, World Precision Instruments, New Haven, CT), as previously described (30, 51). Briefly, distal colonic segments were removed and mounted in Lucite chambers exposing mucosal and serosal surfaces to 10 ml of oxygenated Krebs buffer (in mM: 115 NaCl, 8 KCl, 1.25 CaCl2, 1.2 MgCl2, 2 KH2PO4, 225 NaHCO3; pH 7.35). The buffer was maintained at 37°C by a heated water jacket and circulated with CO2-O2, 95:5. Oxygenation was monitored with criteria adapted from previous studies (51). Briefly, total bacterial counts were assayed by using whole colonic tissues including luminal contents, whereas mucosal associated bacterial counts were performed on colonic tissues after vigorously flushing colon with sterile phosphate-buffered saline (PBS). In both cases, the tissues were homogenized in 1.5 ml of sterile PBS at low speed with a Kinematica tissue homogenizer (Brinkmann). Homogenates were then serially diluted and plated onto MacConkey agar plates selective for gram-negative organisms (PML Microbiologicals). Bacterial colonies were enumerated after over-

### Table 1. Change of body weight of C57BL/6 mice with or without VIP (0.5 nmol/mouse) treatment

<table>
<thead>
<tr>
<th>Day after infection</th>
<th>VIP treated compared with control</th>
<th>CR infected compared with VIP treated</th>
<th>CR infected compared with control</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>16.2±4.2*</td>
<td>15.9±4.2†</td>
<td>31.9±1.9§</td>
</tr>
<tr>
<td>8</td>
<td>6.7±2.2</td>
<td>20.8±2.2†</td>
<td>27.5±1.6§</td>
</tr>
<tr>
<td>10</td>
<td>7.4±2.7</td>
<td>16.7±2.7†</td>
<td>24.0±1.7§</td>
</tr>
</tbody>
</table>

Values are means ± SE. Values are percentage decrease in body weight of vasoactive intestinal peptide (VIP)-treated mice during infection compared with control mice. *P < 0.01, †P < 0.01, ‡P < 0.001. C. rodentium-infected mice compared with VIP treated. §P < 0.001. C. rodentium-infected mice compared with control mice.

**Total and mucosal associated bacterial counts in the colons of C57BL/6 mice with or without VIP treatment at day 10 postinfection**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total Bacterial Counts, 10^8 CFU/g</th>
<th>Mucosal-Associated Bacterial Counts, 10^7 CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. rodentium</td>
<td>8.66±1.13</td>
<td>7.16±1.40</td>
</tr>
<tr>
<td>C. rodentium +</td>
<td>7.76±1.44</td>
<td>7.58±1.40</td>
</tr>
<tr>
<td>VIP (0.5 nmol/mouse)</td>
<td>5.03±1.20</td>
<td>4.88±1.26</td>
</tr>
<tr>
<td>C. rodentium +</td>
<td>4.32±1.47</td>
<td>3.03±0.91</td>
</tr>
<tr>
<td>VIP (5 nmol/mouse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 nmol/mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td></td>
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</tbody>
</table>

Values are means ± SE. CFU, colony-forming unit.
night incubation at 37°C. *C. rodentium* colonies were easily distinguished from colonies derived from commensal flora by their size and appearance. The validity of this approach was previously verified by PCR analysis for LEE genes (46). Bacterial counts are reported as colony-forming units per gram.

**Immunohistochemistry.** Immunofluorescent labeling of colonic tissues was performed as described previously (15, 19, 51). Colonic tissues were rinsed with ice-cold PBS, embedded in Shandon cryomatrix (Thermo Electron, Pittsburgh, PA), frozen with isopentane (Sigma) and liquid N₂, and stored at −80°C. Serial sections were cut...
at a thickness of 4 μm and fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. Tissue sections were directly blocked with 1% bovine serum albumin for 1 h at room temperature, followed by the addition of the rabbit polyclonal anti-ZO-1 or rabbit polyclonal anti-occludin (Zymed Laboratories, South San Francisco, CA). Sections were incubated with secondary antibodies (Alexa 488- or 568-conjugated goat-anti-rabbit IgG, Molecular Probes, Eugene, OR) for 1 h at room temperature.

Paraffin-embedded tissue sections were deparaffinized and rehydrated, followed by antigen retrieval using 0.1 M citric acid monohydrate (Sigma) with 0.05% Tween 20 (pH 6.0) and steam for 45 min. Slides were blocked in PBS with 2% normal horse serum, 1% BSA, 0.1% Triton X-100 for 1 h at room temperature, followed by the addition of rabbit polyclonal anti-claudin-3 (Zymed Laboratories). This was followed by secondary Alexa 568-conjugated goat-anti-rabbit IgG (Molecular Probes). ProLong Gold Antifade (Invitrogen) mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) was used for staining host cell DNA. Tissue sections were viewed at 350, 488, and 594 nm on a Zeiss AxioImager microscope. Images were obtained with a Zeiss AxioImager microscope equipped with an AxioCam HRm camera operating through AxioVision software (version 4.4). Tir staining was performed using rat polyclonal anti-His-tagged Citrobacter Tir sera and Alexa 488-conjugated goat anti-rat IgG (Molecular Probes) as previously described (15, 51).

Quantification of VIPergic nerve fiber density. Control and C. rodentium-infected mice were euthanized at 4 and 10 days pi. Colonos were cut at the anal verge and 4 cm proximal from the anal verge. Colonic tissues were rinsed with ice-cold Krebs solution, fixed in 4% PFA, and embedded in optimal embedding compound. Serial cross sections (40 μm) were treated with ammonia chloride (50 mM) and glycine (50 mM) for 30 min, rinsed 3× in PBS, and blocked with 10% normal horse serum-0.3% Triton X-100 in PBS for 1 h. Tissues were incubated with rabbit anti-mouse VIP for 48 h at 4°C and then with fluorescently conjugated secondary antibodies for 1 h. Tissues were mounted on gelatin-coated glass slides in 2.5% Dabco-PBS-glycerol.

Control tissues were treated similarly except antibody diluent was used in place of primary antibody. Immunomorphometric analysis was performed by use of the Northern Exclipse (V.6), connected to a Nikon E400 epifluorescent microscope. Two adjacent tissue cross sections (40 μm thick) from each mouse were considered for quantification. The entire mucosal area was captured with 6–10 compressed images at 20× magnification (506 μm²). Each compressed image was generated from 10 images collected at 0.2-μm z-axis intervals. A threshold and skeletonization operation was applied in a consistent way to select the mucosal nerve fiber immunoreactivity (IR) on which a 12-μm² grid was superimposed. The number of intersections or “hits” between the grid and IR selection that occurred within six randomly chosen and calculated mucosal areas (comprising 4 crypts each) was determined. The resulting quantity has the units of hits per millimeter squared and represents the IR fiber density in the mucosa. Concurrently, crypt height was determined by measuring the distance between the tip and the base of the crypt. All measurements were carried out by the same investigator.

Epithelial monolayer cell culture. Caco-2 epithelial cells (1 × 10⁶) were seeded in high glucose DMEM growth medium onto 0.33 cm² permeable polyester Transwell filters with 0.4-μm pore size (Costar/Corning). Cells were plated onto the upper surface of the filter and the TER of each well was measured every 48 h over a 21-day period until the monolayers reached a 100% level of confluence, yielding resistances that reached a 100% level of confluence, yielding resistances ranging between 1,200 and 2,000 Ω·cm². The seeding of lower cell densities results in lower levels of resistances (i.e., 3 × 10⁶ cells/filter yields a resistance range of ~400–600 Ω·cm²). All resistance experiments were performed with 21-day-old monolayers.

TER measurements. TER measures passive ion flow (29) and is often used as a description of paracellular permeability. The TER of the Caco-2 epithelial monolayers growing on 0.3-μm, 12-mm-diameter polyester Transwell filters was measured with a voltohmmeter (EVOM, World Precision Instruments). Caco-2 cells (passage 40–50) were grown to full confluence on Transwell filters prior to addition of EPEC and/or 10⁻⁶ M VIP or 10 μM ML-7. Resistance was measured at 0-, 2-, and 4-h time points. After subtracting the resistance of the insert and bathing solution (120 Ω·cm²), we normalized the measured resistance values for the effective growth area of the insert (0.33 cm²) to 1,000 Ω·cm². Filters were then fixed and processed for immunocytochemical analysis.

Immunocytochemistry. Transwell filters from all resistance experiments were fixed 4 h in precooled methanol (−20°C) at 4°C for 30 min, followed by permeabilization with precooled acetone (−20°C) for 3 min at room temperature. Filters were then washed three times in PBS before being blocked with 10% horse serum for 30 min at room temperature. After blocking filters were washed and incubated with the relevant primary antibody (ZO-1 1:150 [BD Transduction Laboratories]; occludin 1:500 [Invitrogen]). After 2 h at room temperature the primary antibodies were removed and the filters washed 3× in PBS prior to incubation in the relevant secondary antibody (Alexa 488 and 594, Molecular Probes) in the dark for 1 h at room temperature. After removal of the secondary antibody filters were washed 3× in PBS and then mounted in Vectashield (Vector Laboratories, Burlington, ON, Canada) on glass slides and screened with a Zeiss microscope. Control incubations included incubation in secondary antibody in the absence of primary antibodies and preabsorption of the antibodies with control antigen when available.

Western blot analysis. Western blot analyses for MLCK, MLC, and phosphorylated myosin light chain (p-MLC) were performed using Caco-2 lysates. Briefly, Caco-2 monolayers were lysed in 300 μl of lysis buffer [in mM: 150 NaCl, 20 Tris pH 7.5, 1 EDTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 PMSF, 1 sodium orthovanadate] and supernatants were collected. Western blots were performed with a 3× dilution of the cell lysate and primary antibodies included rabbit monoclonal anti-MLC (1:100, BD Transduction Laboratories), rabbit polyclonal anti-MLCK (1:1000, Zymed Laboratories); occludin 1:500 (Invitrogen)]. After 2 h at room temperature the primary antibodies were removed and the filters washed 3× in PBS prior to incubation in the relevant secondary antibody (Alexa 488 and 594, Molecular Probes) in the dark for 1 h at room temperature. After removal of the secondary antibody filters were washed 3× in PBS and then mounted in Vectashield (Vector Laboratories, Burlington, ON, Canada) and visualized with a Zeiss microscope. Control incubations included incubation in secondary antibody in the absence of primary antibodies and preabsorption of the antibodies with control antigen when available.

Fig. 4. Evaluation of colonic paracellular permeability at day 10 pi of control (n = 12), C. rodentium-infected mice (n = 12), and VIP-treated mice (n = 12). Infected mice showed a significant increase in unidirectional mannitol flux compared with control mice (**P < 0.01). In contrast, VIP treatment prevented the infection-induced increase in mannitol flux (*P < 0.05 compared with C. rodentium), resulting in levels similar to those of control mice (P > 0.05). VIP treatment in the absence of infection had no effect on mannitol flux. Error bars represent ± SE. Statistical analyses was performed by ANOVA followed by Student-Newman-Keuls post hoc test where P < 0.05 was considered statistically significant.
date, 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 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for MLCK and by 15% of SDS-PAGE for MLC and p-MLC and then transferred onto 0.2 μm polyvinylidene difluoride, MLC and p-MLC, and nitrocellulose (MLCK) membranes (Bio-Rad). Immunoblotting was performed using mouse anti-MLCK (Abcam), mouse MLC (Sigma) and rabbit anti-phospho-MLC (Cell Signaling Technology) antibodies. Primary antibodies were probed with horseradish peroxidase-conjugated IgG (Jackson Laboratories) and horseradish peroxidase-conjugated IgM (MLC; Abcam, MA). Blots were

![Image](http://ajpgi.physiology.org/)

**Fig. 5.** Immunohistological evaluation at day 10 pi of ZO-1, occludin, and claudin-3 distribution in control, *C. rodentium*-infected, and VIP-treated mice. Immunolabeling of ZO-1 (A–D) and occludin (E–H) revealed a significant loss of both proteins from the lateral membranes of infected mice. The 0.5 nmol VIP treatment preserved the localization and expression of both proteins to the tight junction and lateral membrane. Claudin-3 (I–L) immunoreactivity was delocalized from the lateral membrane into the cytoplasm with punctate aggregates found near the apical region of crypts that had intimately attached *C. rodentium* bacteria. Arrows indicate preservation of tight junction localization. Arrowheads indicate loss of tight junction protein. Blue fluorescence is 4′,6-diamidino-2-phenylindole (DAPI) labeling of double-stranded DNA depicting the nuclei. Scale bar = 50 μm for all images. ×40, ×40 magnification; ×60, ×60 magnification. Images are representative of 5 individual experiments.
visualized by enhanced chemiluminescence detection system (Perkin Elmer). Incubation with mouse anti-β-tubulin antibody (Jackson Laboratories) was used as a loading control. The intensity of bands was quantified with Image J software, and the values were normalized against the intensity of β-tubulin.

Statistical analysis. Results are expressed as means ± SE of separate experiments. Analyses were conducted with Graph Pad Prism 4 statistical software for Windows (GraphPad Software, San Diego, CA). Differences between means were calculated by one-way ANOVA or paired t-tests where appropriate. Specific differences were tested with the Student-Newman-Keuls test where \( P < 0.05 \) was considered statistically significant unless otherwise stated.

RESULTS

C. rodentium infection leads to an increase in mucosal VIP immunoreactivity. Consistent with our initial observations (8), a significant increase in mucosal neuronal VIP density was observed following C. rodentium infection and prior to effective bacterial colonization of the colon (day 4 pi Fig. 1A). A persistent increase in VIP IR was still evident at day 10 pi (Fig. 1, A–C), consistent with VIP exerting a physiological role during enteric infection.

VIP treatment ameliorates C. rodentium-induced morbidity. To evaluate what the role of VIP is during infection, the peptide was exogenously administered at three different doses to C. rodentium-infected mice from day 1 to day 10 pi. Infected mice exhibited overt clinical signs of colitis, including a hunched posture, rapid loss of body weight, and defecation of soft stool. Significant weight loss was evident by day 2 pi \( (P < 0.004, \text{Fig. 2}) \), with a 32 ± 2% \( (P < 0.001) \) decrease in body weight evident by day 4 pi (Fig. 2 and Table 1). Body weight increased marginally from days 4–10 pi; at day 8 and day 10 pi body weights were 28% \( (P < 0.001) \) and 24% \( (P < 0.001) \) lower than that of controls, respectively (Fig. 2 and Table 1). In contrast, VIP treatment significantly attenuated the effect of C. rodentium infection on murine body weight. Treatment of mice with 0.5 nmol VIP proved most effective compared with higher doses; 0.5 nmol VIP treatment reduced the extent of weight loss observed over the initial 4 days of infection \( [a 16\% \pm 4\%; \ P = 0.01] \) compared with a 32% decrease in infected animals. Thereafter, body weight steadily increased until weights were 7% \( \pm 2\% \; \text{P} = \text{not significant (NS)} \) lower than those of control animals by day 8 pi (Fig. 2 and Table 1). Although higher doses (1 and 5 nmol) showed recovery of body weight from day 1 to day 7, further decreases in weight were observed past day 8 pi. This decrease in body weight is consistent with the limited protective anti-inflammatory effect exerted by the higher doses of VIP (subsequently observed histologically). This resulted in significant

![Fig. 6. Immunohistological evaluation at day 10 pi of claudin-3 and Tir distribution in C. rodentium-infected control (A), and C. rodentium-infected, VIP-treated (B) mice. Immunolabeling of claudin-3 (red) and Tir (green) revealed a significant loss of claudin-3 protein from the apical region of crypts that had intimately attached C. rodentium bacteria. VIP treatment preserved claudin-3 expression, revealing a similar pattern of immunoreactivity to that of control tissue. Blue fluorescence is DAPI labeling of double-stranded DNA depicting the nuclei. Arrows indicate preserved claudin-3 distribution. Arrowheads indicated loss of claudin-3 protein. Scale bar = 50 μm.](http://ajpgi.physiology.org/)
VIP does not alter attachment of *C. rodentium* in the mouse colon. To determine whether VIP administration altered the level of bacterial colonization between VIP-treated and untreated mice, the total number of *C. rodentium* in the colon was assessed and compared between groups. As shown in Table 2, treatment with daily doses of VIP revealed no significant decrease in bacterial attachment (1.1- to 2.1-fold decrease in total bacterial counts compared with untreated, infected mice; *P* = NS). In addition, immunolabeling for translocated intimin receptor Tir (which acts as the receptor for bacterial intimin to anchor bacteria to host cells) showed no significant difference between infected mice with or without VIP treatment (data not shown). These findings indicate that VIP attenuates the deleterious effects of *C. rodentium* without altering bacterial adhesion to the epithelial barrier.

VIP treatment ameliorates *C. rodentium*-induced colitis. Histological analysis of *C. rodentium*-infected mice on day 10 pi revealed superficial epithelial damage, goblet cell depletion, colonic crypt hyperplasia, and the presence of a mild transmural inflammatory infiltrate (comprised of neutrophils and mononuclear cells) (Fig. 3, A and B). VIP treatment attenuated the effect of *C. rodentium*-induced colitis in a dose-dependent manner. The most effective dose of VIP was 0.5 nmol; this dose ameliorated epithelial damage and reduced transmural infiltration of neutrophils and mononuclear cells compared with *C. rodentium* infection alone (Fig. 3C). In contrast, superficial epithelial damage, colonic crypt hyperplasia, and an inflammatory cell infiltrate were observed with 1 mmol and 5 nmol VIP, however to a lesser extent than during *C. rodentium* infection alone (Fig. 3, D and E). Histological changes were more severe in the distal than proximal colon (data not shown), consistent with the predominance of bacterial colonization in the distal colon. Treatment with VIP alone revealed a similar histology to that of control, uninfected mice (Fig. 3F).

Consistent with the histological findings, *C. rodentium* infection was associated with a high histological damage score (10-fold increase compared with controls; *P* < 0.001, Fig. 3G). The 0.5 nmol VIP reduced the damage score by fivefold (*P* < 0.001), resulting in levels similar to controls (*P* = NS). The 1 mmol and 5 nmol VIP reduced the damage score by 1.6-fold and 1.4-fold, respectively (*P* < 0.01); however, levels remained significantly higher than controls (*P* < 0.05). Infected tissues revealed significantly lower numbers of mature goblet cells scattered predominantly at the surface of crypts, in contrast to the typical goblet cell distribution along the length of colonic crypts. The 0.5 nmol VIP abrogated goblet cell depletion, with the majority having a well-differentiated phenotype and containing abundant mucus (data not shown). In addition, 0.5 nmol VIP significantly attenuated *C. rodentium*-associated crypt hyperplasia (*P* < 0.01, Fig. 3H). Consequently all subsequent experiments were performed with 0.5 nmol VIP to determine the underlying protective mechanism of VIP on *C. rodentium*-induced colitis.

VIP treatment attenuates *C. rodentium*-induced disruption of the colonic epithelial barrier. In the following studies, the impact of VIP treatment on colonocyte function during *C. rodentium* infection was examined. The paracellular flux of mannitol across the epithelium of intestinal tissue from control, infected, and VIP-treated mice was determined. Infected mice showed a significant increase in unidirectional mannitol flux compared with control mice (*P* < 0.01; Fig. 4). In contrast, VIP treatment prevented the infection-induced increase in mannitol flux, resulting in levels similar to those of control mice (*P* > 0.05, Fig. 4), whereas VIP treatment in the absence of infection had no effect on mannitol flux (Fig. 4). These studies suggest that VIP plays a pivotal role in maintaining intestinal mucosal barrier integrity during *C. rodentium* infection.

VIP ameliorates *C. rodentium*-induced disruption of tight junctions. We next examined the mechanisms involved in VIP protection of epithelial barrier function, in particular the distribution of the tight junction proteins, ZO-1, occludin, and claudin-3. In control mice, ZO-1 IR localized to the apical region of the intestinal epithelial cells, maintaining consolidated “beltlike” cell-cell contacts and a continuous dense band at the tight junction (Fig. 5, A and B). *C. rodentium* infection correlated with a significant loss of ZO-1 from the cytoplasmic face of the tight junction and disruption to the continuity of the ZO-1 belt (Fig. 5C). In contrast, VIP preserved ZO-1 distribution preventing loss of IR at the tight junction (Fig. 5D). In control mice, occludin localized at the lateral membrane of the tight junction (Fig. 5, E and F). However, *C. rodentium* infection disrupted occludin distribution, resulting in internalization and redistribution of occludin from the tight junctions and into the cytoplasm (Fig. 5G). Similarly to ZO-1, VIP treatment attenuated *C. rodentium*-induced disruption to occludin distribution (Fig. 5H).

![Graph depicting the body weight of control, ML-7 treated, and C. rodentium-infected mice from infection until day 10](https://example.com/graph.png)

**Fig. 7.** Graph depicting the body weight of control, ML-7 treated, and *C. rodentium*-infected mice from infection until day 10. Control mice (*n* = 10) maintain a steady body weight, as do mice treated with ML-7 alone (*n* = 10). *C. rodentium*-infected mice (*n* = 10) revealed a 32% decrease (*P* < 0.001) in body weight from day 0 to day 4 followed by an increase in weight (12%) up to day 10. However, body weights of infected mice remained significantly lower than those of control mice at day 10 pi. ML-7 treatment (1 mg/kg) significantly attenuated the effect of *C. rodentium* infection on murine body weight, revealing a 13% decrease in body weight compared with a 32% decrease in infected mice. The initial 19% attenuation of weight loss by day 4 pi is followed by a steady increase in body weight up to day 10 pi, yielding a final body weight 15% lower than that of controls at the time of euthanasia. Error bars represent ± SE.
In contrast to ZO-1 and occludin distribution, claudin-3 was found to distribute along the entire lateral membrane in control mice (Fig. 5, I and J). Previous studies have found that *C. rodentium* infection disrupts the normal lateral distribution of claudin-3 in intestinal epithelial cells with redistribution into the cytoplasm in the distal region of the colon (19). As expected, *C. rodentium* caused the translocation of claudin-3 from the lateral membrane into the cytoplasm. In addition, punctate aggregates of claudin-3 IR were detected near the apical region of crypts underlying attached bacteria (Figs. 5K and 6A). VIP treatment prevented this disruption to claudin-3, reducing redistribution of protein away from the lateral membrane (Figs. 5L and 6B). VIP treatment alone revealed a similar pattern of IR to that of control mice (results not shown).

To determine whether alterations in ZO-1, occludin, and claudin-3 distribution were accompanied by changes in expression levels of these proteins, we performed Western blots on colonic tissues. ZO-1, occludin, and claudin-3 protein expression was not significantly different between any treatment groups (data not shown). Overall, these results suggest that VIP protects epithelial barrier function by preventing redistribution of ZO-1, occludin, and claudin-3 away from the tight junction complex.

**Inhibition of MLCK ameliorates the effect of *C. rodentium*-induced colitis.** MLCK activation has been demonstrated to regulate tight junction function in vitro. To examine the role of MLCK during *C. rodentium* infection we used the MLCK inhibitor, ML-7. Infected mice exhibited the same overt clinical signs of colitis as previously described. Significant weight loss was evident by day 1 pi (*P < 0.001, Fig. 7), with a 32% (±3%; *P < 0.001) decrease in body weight evident by day 4 pi (Fig. 7). Body weight increased marginally from day 4 to day 10 pi (Fig. 8). Histological evaluation of colonic mucosal morphology in the distal colon on day 10 pi of control (A; *n = 5*), *C. rodentium*-infected (B; *n = 5*), and ML-7-treated (C–D; *n = 5*) mice. Representative hematoxylin-and-eosin-stained specimens demonstrate noticeable alterations in the mucosal architecture of the distal colon in *C. rodentium*-infected mice (B) compared with control mice. This is manifested primarily by superficial epithelial damage, goblet cell depletion, colonic crypt hyperplasia, and the presence of a mild transmural inflammatory infiltrate comprised of neutrophils and mononuclear cells. ML-7 treatment (C) ameliorated epithelial damage, reducing transmural infiltration of neutrophils and mononuclear cells compared with *C. rodentium* infection alone (B). ML-7 treatment alone revealed a mucosal architecture similar to that of controls (D). Scale bar = 100 μm. E: histological damage scores correlated with the histological findings; *C. rodentium* infection was associated with a high histological damage score compared with controls (***P < 0.001). ML-7 attenuated the damage score compared with *C. rodentium* infection alone (*P < 0.05). F: *C. rodentium*-associated crypt hyperplasia was significantly attenuated by ML-7 treatment (*P < 0.05). *Significant difference compared with control; † significant attenuation of damage compared with *C. rodentium*. 

Fig. 7. Histological evaluation of colonic mucosal morphology in the distal colon on day 10 pi of control (A; *n = 5*), *C. rodentium*-infected (B; *n = 5*), and ML-7-treated (C–D; *n = 5*) mice. Representative hematoxylin-and-eosin-stained specimens demonstrate noticeable alterations in the mucosal architecture of the distal colon in *C. rodentium*-infected mice (B) compared with control mice. This is manifested primarily by superficial epithelial damage, goblet cell depletion, colonic crypt hyperplasia, and the presence of a mild transmural inflammatory infiltrate comprised of neutrophils and mononuclear cells. ML-7 treatment (C) ameliorated epithelial damage, reducing transmural infiltration of neutrophils and mononuclear cells compared with *C. rodentium* infection alone (B). ML-7 treatment alone revealed a mucosal architecture similar to that of controls (D). Scale bar = 100 μm. E: histological damage scores correlated with the histological findings; *C. rodentium* infection was associated with a high histological damage score compared with controls (***P < 0.001). ML-7 attenuated the damage score compared with *C. rodentium* infection alone (*P < 0.05). F: *C. rodentium*-associated crypt hyperplasia was significantly attenuated by ML-7 treatment (*P < 0.05). *Significant difference compared with control; † significant attenuation of damage compared with *C. rodentium*. 

Fig. 8. Histological evaluation of colonic mucosal morphology in the distal colon on day 10 pi of control (A; *n = 5*), *C. rodentium*-infected (B; *n = 5*), and ML-7-treated (C–D; *n = 5*) mice. Representative hematoxylin-and-eosin-stained specimens demonstrate noticeable alterations in the mucosal architecture of the distal colon in *C. rodentium*-infected mice (B) compared with control mice. This is manifested primarily by superficial epithelial damage, goblet cell depletion, colonic crypt hyperplasia, and the presence of a mild transmural inflammatory infiltrate comprised of neutrophils and mononuclear cells. ML-7 treatment (C) ameliorated epithelial damage, reducing transmural infiltration of neutrophils and mononuclear cells compared with *C. rodentium* infection alone (B). ML-7 treatment alone revealed a mucosal architecture similar to that of controls (D). Scale bar = 100 μm. E: histological damage scores correlated with the histological findings; *C. rodentium* infection was associated with a high histological damage score compared with controls (***P < 0.001). ML-7 attenuated the damage score compared with *C. rodentium* infection alone (*P < 0.05). F: *C. rodentium*-associated crypt hyperplasia was significantly attenuated by ML-7 treatment (*P < 0.05). *Significant difference compared with control; † significant attenuation of damage compared with *C. rodentium*. 

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day 10 pi; at day 8 and day 10 pi body weights were 25% (±2%; P < 0.001) and 20% (±2%; P < 0.001) lower than that of controls, respectively (Fig. 7). In contrast, ML-7 treatment significantly reduced the effect of C. rodentium infection on murine body weight (day 2 = 12.5 ± 4%; day 4 = 13 ± 4%; day 8 = 15 ± 2%; day 10 = 15 ± 3%, decrease in body weight compared with controls).

ML-7 treatment ameliorates C. rodentium-induced colitis. Histological analysis of ML-7 treated tissue at day 10 pi revealed a reduction in superficial epithelial damage, goblet cell depletion, colonic crypt hyperplasia, and transmural infiltration of neutrophils and mononuclear cells compared with C. rodentium infection alone (Fig. 8, A–D). ML-7 treatment significantly reduced the histological damage score (1.5-fold decrease, Fig. 8E, P < 0.05), associated with a significant decrease in cellular infiltrate (Fig. 8E, P < 0.05) and colonic crypt hyperplasia (Fig. 8F, P < 0.05). Treatment with ML-7 alone revealed a similar histology to that of control, uninfected mice (Fig. 8).

VIP treatment attenuates EPEC-induced disruption of the colonic epithelial barrier. Having established that VIP ameliorates the effect of C. rodentium infection in vivo, we investigated whether VIP had a direct effect on epithelial barrier function. Our in vitro results reveal that pretreatment of confluent Caco-2 monolayers with VIP prevents EPEC-induced disruptions to the epithelial barrier (Fig. 9). EPEC induced a 1.5-fold decrease in TER 2 h pi, and by 4 h pi this decrease was 1.7-fold lower than that of control monolayers (P < 0.01, n = 4). In contrast, pretreatment of Caco-2 monolayers with VIP ameliorated the effect of EPEC on epithelial barrier function. Over the course of infection there was no significant difference between VIP-treated monolayers and control monolayers with respect to barrier function (Fig. 9, P = NS, n = 4).

Immunocytochemical analysis 4 h pi revealed EPEC-induced alterations to the transmembrane tight junction protein occludin and the cytoplasmic tight junction plaque protein ZO-1 (Fig. 10). The presence of EPEC resulted in the loss of ZO-1 from the lateral surface of the tight junction resulting in cytoplasmic aggregation of ZO-1 compared with control samples (Fig. 10, A and C). VIP treatment preserved ZO-1 expression at the bilateral tight junction with areas of immunoreactivity concentrated in regions of tricellular contact (Fig. 10D, arrowheads). Similarly, EPEC induced redistribution of occludin from the tight junction and into the cytoplasm compared with control samples (Fig. 10, E and G). In addition, EPEC infection revealed a concentration of occludin protein in regions of tricellular contact in both the absence and presence of VIP (Fig. 10, G and H, arrowheads). VIP treatment of Caco-2 monolayers prevented disruption to both tight junction proteins (Fig. 10, D and H) revealing a similar pattern of immunoreactivity to that of control monolayers (Fig. 10, A and E).

VIP prevents the increase in MLCK expression induced by EPEC in intestinal epithelial cells. To determine whether MLCK is responsible for the observed decrease in intestinal epithelial barrier MLCK protein expression was examined in vitro. Long MLCK expression was significantly increased (1.5-fold, n = 3, P < 0.01) in EPEC-infected Caco-2 monolayers 4 h pi (Fig. 11, A and B). VIP treatment of EPEC infected monolayers significantly decreased MLCK expression levels to that of control monolayers (P < 0.001 compared with infected, n = 3), whereas VIP treatment alone had no effect on MLCK expression levels compared with controls (Fig. 11, A and B).

VIP decreases EPEC-induced phosphorylation of MLC. Our data demonstrated that intestinal epithelial MLCK protein expression was increased during EPEC infection. To assess in vitro MLCK activity, we measured phosphorylation of the endogenous MLCK substrate, MLC and expressed the results as a ratio of phosphorylated MLC against total MLC. Quantitative analysis by Western blot revealed that EPEC increased phosphorylation of MLC in intestinal epithelial cells by 1.4-fold compared with control monolayers (Fig. 12, A and B, P < 0.01, n = 3). VIP treatment prevented EPEC-induced increases in MLC phosphorylation, resulting in levels similar to that of controls (Fig. 12, A and B). VIP treatment alone did not significantly change the level of phosphorylated MLC compared with control monolayers.

MLCK inhibition abrogates EPEC-induced epithelial barrier disruption. To further assess the role of MLCK in intestinal epithelial barrier disruption we pretreated Caco-2 monolayers prior to EPEC infection with an MLCK inhibitor, ML-7. EPEC infection resulted in a 24 and 27% decrease in resistance 2 and 4 h pi, respectively. In contrast, ML-7 treated monolayers revealed a similar level of TER to that of control monolayers over the initial 2-h infection period; however, ML-7 treatment failed to protect TER levels between 2–4 h pi, revealing an 18% decrease in resistance compared with control monolayers (Fig. 13). Higher concentrations of ML-7 (15 and 20 μM) proved cytotoxic to the monolayers. Bacterial counts taken at all time points revealed no significant difference in bacterial load (data not shown).

MLCK inhibition prevents EPEC-induced disruption of tight junction proteins. Immunocytochemical analysis of control monolayers revealed ZO-1 distribution was evenly localized to the tight junction in a “honeycomb” pattern of IR with no evidence of tricellular IR (Fig. 14A). EPEC revealed a loss of bilateral ZO-1 expression and concentrated expression at tricellular tight junctions 4 h pi (Fig. 14C). ML-7 treated mono-
layers revealed a partial preservation of ZO-1 protein at bilateral tight junctions compared with infected monolayers, with select regions denuded of ZO-1 expression (Fig. 14D, arrows). ZO-1 expression in regions of tricellular contact was also observed (Fig. 14D, arrowheads).

Occludin distribution was localized along the entire lateral membrane in control and ML-7-treated monolayers (Fig. 14, E and F). EPEC infection resulted in the loss of occludin from bilateral tight junctions (Fig. 14G, arrows) and a concentration of protein at tricellular tight junctions (Fig. 14G, arrowheads). ML-7 preserved occludin expression at bilateral tight junctions and minimized the concentration at regions of tricellular contact (Fig. 14H, arrowheads).

**ML-7 decreases EPEC-induced phosphorylation of MLC.** Western blot analyses were performed on protein lysates from the ML-7 experiments. EPEC induced a 1.7-fold increase in MLCK expression compared with control monolayers ($P < 0.01$, $n = 3$, Fig. 15, A and B). In contrast, ML-7 treatment reduced MLCK expression to levels similar to control monolayers ($P = 0.52$, $n = 3$, Fig. 15, A and B). Determining the ratio of phosphorylated MLC to total MLC protein assessed MLCK activity. EPEC induced a 1.43-fold increase in phosphorylated MLC expression relative to total MLC protein compared with control monolayers ($P < 0.05$, $n = 3$, Fig. 15, C and D). ML-7 treatment prevented an increase in phosphorylated MLC expression levels revealing expression levels sim-
DISCUSSION

This study reveals for the first time that VIP has a protective effect on intestinal barrier function in Citrobacter and EPEC model systems. Although the anti-inflammatory properties of VIP have been previously reported (1), these studies were designed to investigate whether VIP acted as a modulator of epithelial barrier integrity during bacterial infection.

Previous studies of infectious colitis (22, 51) reveal that C. rodentium infection causes a marked disruption of the colonic epithelial barrier structure and function, which is particularly pronounced in the distal colon. Routinely, C. rodentium infection is associated with superficial epithelial damage, goblet cell depletion, colonic crypt hyperplasia, and the presence of a mild transmural inflammatory infiltrate comprised of neutrophils and mononuclear cells. The present study expands on previous research revealing that C. rodentium alters the distribution of pivotal tight junction proteins, ZO-1, occludin, and claudin-3.

Reports on the efficacy of VIP as a potential therapeutic strategy for intestinal inflammatory disease have revealed conflicting results (2, 3, 20, 36). In contrast to the negative effect of VIP treatment demonstrated by Newman et al. (36), our present C. rodentium study corroborated previous investigations revealing that VIP treatment reduced the severity of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (2, 3, 20). Our data show that at concentrations tested, exogenous VIP significantly reduced the initial lipopolysaccharide-associated weight loss (30). Interestingly, after colonic colonization, infected mice treated with the higher concentrations of VIP showed a decrease in body weight and higher histological

Fig. 11. A: Western blot analysis revealed an EPEC-induced increase in myosin light chain kinase (MLCK) expression. B: densitometry analysis of MLCK protein expression levels revealed that EPEC (n = 3) increased MLCK expression in Caco-2 epithelial cells by 1.5-fold compared with controls (**P < 0.01). VIP treatment of EPEC-infected monolayers (n = 3) significantly decreased MLCK expression levels to that of controls (**P < 0.001). Blot is representative of 3 individual experiments; within each experiment individual parameters were assessed by use of 6 Caco-2 monolayers per condition.

Fig. 12. Increased myosin light chain phosphorylation (p-MLC) activity in EPEC-infected Caco-2 monolayers 4 h pi. A: EPEC (n = 3) increased phosphorylation of MLC in intestinal epithelial cells by 1.4-fold compared with control monolayers (**P < 0.01). VIP treatment (n = 3) prevented EPEC-induced increase in MLC phosphorylation, resulting in levels similar to that of control monolayers (n = 3). VIP treatment alone (n = 3) did not significantly change the level of phosphorylated MLC compared with control monolayers. B: densitometry analysis of MLC-to-p-MLC ratio of expression. Statistical analyses was performed by ANOVA followed by Student-Newman-Keuls post hoc test where **P < 0.01 was considered statistically significant.

Fig. 13. Graph depicting the TER of EPEC-infected confluent Caco-2 monolayers. Control (●), infected (▲), and ML-7-treated (● and ■) monolayers were monitored for TER over 4 h. Resistances were measured prior to infection and then at 2-h intervals. Control and ML-7 treated monolayers showed no significant changes in TER over 4 h. EPEC-infected monolayers revealed 24 and 27% decreases in TER at 2 and 4 h pi, respectively. ML-7 treatment prevented the EPEC-induced decrease in TER up to 2 h pi, revealing resistance measurements similar to those of control monolayers; however, at 4 h pi ML-7-treated monolayers revealed an 18% decrease in TER compared with controls. Each bar represents ± SE. *P < 0.05 and **P < 0.01. Graph is representative of 4 individual experiments.
damage scores compared with 0.5 nmol VIP. Consistent with this observation, Abad et al. (2) demonstrated a worsening of TNBS-induced colitis associated with the higher dosing of VIP. In addition, higher concentrations of VIP in vitro were associated with impaired barrier function (data not shown), supporting the adverse consequence of high-dose VIP observed in vivo. Although the mechanism(s) of action remain unclear, high-dose long-term exogenous VIP administration in *C. rodentium*-induced colitis is less efficacious, possibly because of downregulation of receptors (37).

The bacterial colony counts and immunolabeling results show that the continued beneficial effect of VIP, at 0.5 nmol, after day 8 is not attributable to decreased bacterial attachment to host colonic epithelial cells. Interestingly, *C. rodentium* infection disrupts claudin-3 distribution only in areas of intense bacterial colonization, suggesting a bacterial effect on claudin-3. In contrast ZO-1 and occludin disruption is also observed in areas of limited colonization, possibly due to the effect of MLCK on the perijunctional actinomyosin ring (40). Treatment with exogenous VIP prevents the disruption to tight junction proteins, ZO-1, occludin, and claudin-3 while attenuating bacterial-induced alterations to epithelial barrier permeability. Our in vitro studies reveal that VIP protects the epithelial barrier from EPEC-induced damage to tight junction architecture. Preservation of barrier integrity establishes a novel protective role for VIP beyond the anti-inflammatory effects (35).

**Fig. 14.** Immunocytochemical analysis of tight junction proteins. EPEC-infected monolayers were fixed 4 h pi and labeled for ZO-1 (A–D) and occludin (E–H). A: control monolayers reveal a honeycomb pattern of ZO-1 expression at the cytoplasmic face of the tight junction. B: ML-7 treatment alone has no effect on ZO-1 distribution. C: EPEC infection resulted in a loss of ZO-1 protein from the cytoplasmic face of the bilateral tight junctions and concentrated ZO-1 in regions of tricellular contact (arrowheads). D: ML-7 treatment partially prevents disruption of ZO-1. Whereas a limited loss of ZO-1 expression at bilateral tight junctions is seen at 4 h pi (arrows), ML-7 treatment prevents a total loss of protein yielding a similar pattern of immunoreactivity to that of control monolayers. Immunoreactivity is also concentrated in regions of tricellular contact (arrowheads). E: control monolayers reveal a honeycomb pattern of occludin expression. B: ML-7 treatment alone has no effect on occludin distribution. G: EPEC infection resulted in a loss of occludin from the bilateral tight junctions (arrows) and a concentration of occludin at tricellular tight junctions (arrowheads). H: in contrast, ML-7 treatment prevents loss of occludin at the bilateral tight junctions while maintaining a similar pattern of tricellular immunoreactivity to that of infected monolayers (arrowheads). Blue fluorescence is DAPI labeling of double-stranded DNA depicting the nuclei. Scale bar = 10 µm (×60 magnification). Images are representative of 5 individual experiments.
by ANOVA followed by Student-Newman-Keuls post hoc test where * represents the level of p-MLC protein compared with control monolayers. Blots are representative of 3 individual experiments. Statistical analyses were performed using ANOVA followed by Student-Newman-Keuls post hoc test. P values < 0.05 and ** P values < 0.01 were considered statistically significant.

Fig. 15. Decreased MLCK and p-MLC levels in ML-7-treated Caco-2 monolayers 4 h pi. A: Western blot analysis revealed an EPEC-induced increase in MLCK expression. B: densitometry analysis of MLCK protein expression levels revealed that EPEC (n = 3) increased MLCK expression in Caco-2 epithelial cells by 1.7-fold compared with controls (**P < 0.01). ML-7 treatment (n = 3) prevented an EPEC-induced increase in MLCK expression, resulting in levels similar to that of control monolayers (n = 3). ML-7 treatment alone (n = 3) revealed a slight decrease in the level of MLCK protein compared with control monolayers. C: Western blot analysis revealed an EPEC-induced increase in p-MCL levels without altering the expression level of MLC. D: densitometry analysis of p-MCL expression levels revealed that EPEC (n = 3) increased expression in Caco-2 epithelial cells by 1.43-fold compared with controls (*P < 0.05). ML-7 treatment (n = 3) prevented an EPEC-induced increase in p-MCL levels, resulting in levels similar to that of control monolayers (n = 3). ML-7 treatment alone (n = 3) did not significantly alter the level of p-MCL protein compared with control monolayers. Blots are representative of 3 individual experiments. Statistical analyses were performed by ANOVA followed by Student-Newman-Keuls post hoc test where *P < 0.05 and **P < 0.01 were considered statistically significant.

tribution of ZO-1 and occludin at bilateral tight junctions. Small regions devoid of both proteins were still evident after ML-7 treatment 4 h pi, explaining the observed decrease in TER. However, ML-7 reduces the frequency of disorganized ZO-1 and occludin expression at tricellular tight junctions. Interestingly the organization of tight junctions at regions of tricellular contact differs from bicellular contact zones (18, 43, 49, 50). Analysis of the structure of tight junctions (42) in tricellular regions has revealed the presence of cylindrical central sealing elements, and a protein, tricellulin (24), is implicated in the regulation of these elements, which is essential to the functional effectiveness of the barrier (24). Bacterial disruption to these regions likely underlies bacterial-induced disruption to epithelial barrier function.

Our in vitro mechanistic studies reveal that increased MLCK protein expression and activity correlate to altered barrier function during bacterial infection, which is consistent with previous in vitro EPEC studies (52). Our MLC phosphorylation data provide further evidence for the role of MLCK during epithelial barrier disruption. Although VIP treatment was more efficacious in vivo and provided a complete protective effect to the epithelial barrier during EPEC infections, the effect of ML-7 was limited. Further studies are required to examine the added beneficial effect of VIP over ML-7 through actions on signal transduction pathways integral to epithelial barrier function (17). VIP protection of epithelial barrier function may include formation of VIP-calmodulin complexes (41); activation of PKC (44); and stability, function, and expression of tight junction proteins (35).

In summary, our results demonstrate a novel protective action of VIP on the colonic epithelial barrier structure and function during C. rodentium and EPEC infection. The ability of VIP to suppress MLCK protein expression and activity during infection suggests a potential novel role for VIP in the treatment of patients with intestinal infectious and inflammatory disease.

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