Human ENS regulates the intestinal epithelial barrier permeability and a tight junction-associated protein ZO-1 via VIPergic pathways

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Neunlist, Michel, Férial Toumi, Tsvetelina Oreschkova, Marc Denis, Joel Leborgne, Christian L. Laboisse, Jean-Paul Galmiche, and Anne Jarry. Human ENS regulates the intestinal epithelial barrier permeability and a tight junction-associated protein ZO-1 via VIPergic pathways. Am J Physiol Gastrointest Liver Physiol 285: G1028–G1036, 2003. First published July 24, 2003; 10.1152/ajpgi.00066.2003.—Although the enteric nervous system (ENS) has been shown to regulate various mucosal functions, its role in the physiological control of the human intestinal epithelial barrier is unknown. The aim of this study was to investigate whether the ENS is able to modulate epithelial barrier permeability and a key tight junction-associated protein, zonula occludens-1 (ZO-1). Therefore, we developed a coculture model, consisting of human submucosa containing the submucosal neuronal network and human polarized colonic epithelial monolayers (HT29-Cl.16E or Caco-2). Submucosal neurons were activated by electrical field stimulation (EFS). Permeability was assessed by measuring the flux of paracellular permeability markers (FITC-dextran or FITC-inulin) across epithelial monolayers. Expression of ZO-1 was determined by immunofluorescence, quantitative immunoblot analysis, and real-time RT-PCR. Using the coculture model, we showed that EFS of submucosal neurons resulted in a reduction in FITC-dextran or FITC-inulin fluxes, which was blocked by TTX. In HT29-Cl.16E, the effect of submucosal neuron activation was blocked by a VIP receptor antagonist (VIPra) and reproduced by VIP. Furthermore, ZO-1 expression (mRNA, protein) assessed in HT29-Cl.16E, was significantly increased after submucosal neuron activation by EFS. These effects on ZO-1 expression were blocked by TTX and VIPra and reproduced by VIP. In conclusion, our results strongly suggest a modulatory role of VIPergic submucosal neuronal pathways on intestinal epithelial barrier permeability and ZO-1 expression.

THE INTESTINAL EPITHELIAL barrier located at the interface between the body and the outside environment manages the conflicting tasks of electrolyte and nutrient absorption and protection against potentially harmful antigenic, toxic, or infectious material present in the intestinal lumen (24). The epithelial barrier consists of a continuous monolayer of polarized specialized cells in constant, rapid turnover. These cells are held together by tight junction-associated proteins that control epithelial permeability and polarity (7, 24). Barrier homeostasis, which results in part from coordinated interactions of a wide array of cells within the mucosa, is influenced by various physiological and pathophysiological stimuli such as nutrients, immune mediators, enteric bacteria, and viruses (10, 24, 29). The enteric nervous system (ENS) has recently been shown to play an important role in the pathogenesis of various enteric infections (12), but its physiological role in control of the human epithelial barrier remains largely unknown. The ENS forms a neuronal network embedded within the gastrointestinal wall and is composed of the myenteric and the submucosal plexus. Submucosal neurons innervating the mucosa are composed of intrinsic primary afferent neurons (13) and effector neurons and are key regulators of mucosal functions such as mucosal blood flow, lymphocyte migration, resorption of amino acids, as well as of electrolytes and mucus secretions (8, 32). The human submucosal plexus, in contrast to that of small animals such as the guinea pig or mouse, is composed of three different plexus: 1) the plexus of Meissner located immediately under the mucosa; 2) the intermediary plexus located in the plane of the mucosal blood vessels; and 3) the plexus of Henle, which is in contact with the circular muscle (30). Both in vivo or in vitro approaches have been employed in the few studies that have explored interactions between enteric neurons and the intestinal epithelial barrier. In vivo studies in animal models have suggested that neuromediators such as VIP, substance P, or acetylcholine are involved in the regulation of intestinal permeability (14, 15, 17). Although closely reproducing physiological conditions, these studies make difficult the accurate analysis of the regulatory mechanisms at cellular or molecular levels. In addition, extrapolation of results from animal models to humans is often hazardous. In vitro studies performed on epithelial cell lines or whole mucosa/submucosa have suggested that neuromediators can influence epithelial permeability (5, 14). Nevertheless,

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these studies performed on reductionist models, while enabling description of the signaling pathways involved, do not take into account the complexity of cross talk between the epithelium and the underlying neuronal network. Moreover, experiments on whole submucosa/mucosa preparations are not adapted to long-term study of epithelial barrier functions, because colonocytes undergo apoptosis as early as 45 min after incubation in Ussing chambers (16). Thus the effects of ENS activation on barrier functions have not yet been well characterized, and the mechanisms involved in neuromediator-induced modulation of barrier permeability remain unidentified.

In this context, we used an experimental model (31) we recently developed to study cross talk between the intestinal epithelial barrier and enteric neurons, which enables 1) the study of long-term interactions between human submucosal neurons and human polarized intestinal epithelial cells, and 2) mechanistic analysis both at cellular and molecular levels. This coculture was composed of human colonic submucosa containing the enteric neuronal network and human colonic epithelial cell lines. The aim of the study was to determine the effects of electrical activation of the ENS on the regulation of parameters of the intestinal epithelial barrier such as permeability and the expression of zonula occludens-1 (ZO-1), a key tight-junction associated protein.

MATERIALS AND METHODS

Coculture Model

Human submucosa containing submucosal plexus. Colon specimens were obtained from 29 patients undergoing surgery for colon carcinoma (16 males, 13 females, mean age: 66 ± 5 yr, range: 31 to 83 yr). Fragments of human normal colon were taken ~10 cm from the tumor. These tissues were processed according to the Guidelines of the French Ethics Committee for Research on Human Tissues.

Immediately after removal, the tissues (mainly sigmoid colon) were placed in 4°C oxygenated sterile Krebs solution containing (in mM): 117 NaCl, 4.7 KCl, 1.2 MgCl2, 6 H2O, 1.2 NaH2PO4, 25 NaHCO3, 2.5 CaCl2, and 11 glucose. Preparations taken from intertitial regions measured 4–7 cm circumferentially and 4–5 cm longitudinally. The longitudinal and circular muscle layers were carefully removed by microdissection. Mucosa was carefully stripped away, and pieces of submucosa of ~2 × 2 cm were obtained. Submucosa, which contained the three submucosal plexus was then washed four times for 10 min with sterile Krebs solution and pinned back in a sterile Sylgard-coated petri dish (Fig. 1).

Epithelial Cell Lines

The experiments were performed by using two human colonic polarized cell lines with different phenotypes. The HT29-Cl.16E mucus-secreting cell line was grown on porous filters (6-well Transwell Clear, 0.40-μm porosity; Costar) and formed monolayers of polarized mucous cells at postconfluence (3, 18, 19). Cells were seeded at 4.5 × 105 cells per well in DMEM (4.5 g/l glucose; Invitrogen) supplemented with 10% heat-inactivated FCS (Invitrogen), and cultured until postconfluence and full differentiation. Experiments were conducted between days 10 and 20 after seeding (P15–25). The Caco-2 cell line (from the ATCC, which differentiates at confluency into enterocyte-like cells, was grown on porous filters. Cells were seeded at 0.5 × 106 cells per Transwell in DMEM (4.5 g/l glucose), supplemented with 20% heat-inactivated FCS, and cultured until postconfluence and full differentiation. Experiments were conducted between days 18 and 24 (P120–P130) after seeding.

Coculture and Electrical Activation of Enteric Neurons

Epithelial monolayers (HT29-Cl.16E or Caco-2) were grown on filters to ensure the separation of the basolateral and apical compartments. Filters were placed over the submucosa (Fig. 1), and the coculture system was covered with DMEM/Ham’s-F12 (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FCS (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin, 1.1 μg/ml amphotericin B, 20 μg/ml gentamicin (Sigma), 2.4 mM glutamine, and 2.1 g/l NaHCO3.

Fig. 1. Schematic representation of the coculture model and electrical field stimulation (EFS) setup. A: human colonic tissue was dissected to expose the submucosa. B: human colonic epithelial cells (HT29-Cl.16E or Caco-2) were grown at postconfluence on a porous filter (the cell line shown is HT29-Cl.16E, stained with alcian blue; original magnification ×200). C: filters were applied to the submucosa in a petri dish and maintained under organotypic culture conditions. Enteric neurons were activated by EFS via electrodes placed at the bottom of the petri dish.
The system was placed in a humidified incubator containing 5% CO₂ and air at 37°C for a period of 3 days. The dishes were placed on a tray shaking at a frequency between 0.25 and 0.5 Hz.

Enteric neurons were activated via a pair of platinum electrodes embedded in the bottom of the Sylgard-covered petri dish (Fig. 1). The electrical field stimulation (EFS) parameters used were trains of pulses (pulse duration: 400 μs; amplitude: 10–12 V; frequency: 20 Hz; train duration: 15 s) applied every 4 min for 64 min. The stimulation protocol was performed twice daily (morning and evening).

Viability of the preparations: lactate dehydrogenase measurement. Epithelial cell death was assessed by measuring lactate dehydrogenase (LDH) activity, by using a standardized kinetic determination (LDH Kit; Enzyline, Biome France). LDH was measured both in the supernatant [extracellular LDH (LDHe)] and in cells adherent to the tissue (intracellular LDH (LDHi)). LDH was measured both in the supernatant and in cells adherent to the tissue at the completion of coculture, epithelial permeability was measured by using a standard protocol of epithelial permeability. At the end of the coculture, epithelial permeability was measured by using FITC-conjugated dextran (4 kDa; 1 mg/ml; Sigma); as previously reported (27), or FITC-conjugated dextran (4 kDa; 1 mg/ml; Sigma) to monitor the intact epithelial barrier. The system was placed in a humidified air atmosphere at 37°C, aliquots of the basolateral medium were taken, and the lactate dehydrogenase (LDH) activity, by using a standardized kinetic determination (LDH Kit; Enzyline, Biome) was measured (measured after 1 freeze-thaw cycle).

Evaluation of epithelial permeability. At the end of the coculture, epithelial permeability was measured by using FITC-conjugated dextran (4 kDa; 1 mg/ml; Sigma), as previously reported (27), or FITC-conjugated inulin (3.5 kDa; 100 μg/ml; Sigma). The Transwell filter of epithelial monolayer was transferred into a six-well culture dish, and FITC-dextran or FITC-inulin (dissolved in culture medium) was added to the apical compartment. After 16- to 24-h incubation at 37°C, aliquots of the basolateral medium were taken, and the fluorescence level was measured by using a fluorimeter (Perkin-Elmer). In coculture, the fluorescence marker flux was normalized to the flux measured in control monolayers not maintained in coculture.

Pharmacological studies. Most drugs were dissolved in sterile deionized water. The following drugs were used: TTX (ICS), VIP (Sigma), VIP receptor antagonist (VIPRa) [VIP(6–28); Sigma], and anti-VIP monoclonal mouse antibody (Bio- genesis). Drugs were applied for at least 30 min before electrical stimulation of enteric neurons in the basolateral medium of the coculture. In some experiments, to assess whether enteric neuromediators act directly or not on epithelial cells, the epithelial monolayer was incubated outside the coculture with VIPRa (1 μM) for 1 h. The monolayer was then placed back in the coculture system, and the ENS was electrically stimulated. This protocol was repeated before each stimulation to block VIP receptors selectively on epithelial cells.

Morphological and immunohistochemical methods. At the completion of coculture, filter-grown HT29-C1.16E cells were fixed and embedded in paraffin. Apoptotic cells were identified on paraffin sections by using immunohistochemistry with the M30 monoclonal antibody (1:50; Roche Diagnostic) followed by a biotin-streptavidin-peroxidase method (LSAB kit; DAKO) using DAB as chromogen and counterstaining with hematoxylin. M30 antibody detects cytokeratin 18 cleaved by caspases and is thus considered as an early marker of apoptosis in epithelial cells (20). The percentage of cells scoring positive for M30 was determined by counting at least 500 nuclei under a microscope at magnification ×400.

For immunofluorescence detection of ZO-1, whole filters of HT29-C1.16E cells were fixed at room temperature in 4% paraformaldehyde in 0.1 M PBS preincubated for 30 min in PBS/4% horse serum (HS)/0.5% Triton X-100. The monolayers were then exposed to monoclonal mouse anti-ZO-1 antibodies, diluted in PBS/HS/Triton X-100 (1:500; Zymed), for 1 h at room temperature. After the monolayers were washed, they were incubated for 30 min in buffer solution containing anti-mouse antibody conjugated to carboxymethylindocyanine (CY3; 1:500, Beckman Coulter) or Alexa Red 568 (1:200; Molecular Probes, Eugene, OR). Monolayers were mounted and viewed either with an Olympus IX 50 connected to a black and white video camera (model 4910; Cohn) or with a laser confocal microscope (model TCS-SP1; Leica, Heidelberg, Germany) by using the TCS-NT software (Leica) for image processing.

Identification of neurons projecting to the mucosa: neuronal tracing method. The lipophilic neuronal retrograde tracer dye 1,1’didodecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) was applied to identify submucosal neurons innervating normal human mucosa of the sigmoid colon by using a method similar to the one previously described in the guinea pig (22, 23). Briefly, two glass beads (150–200 μm diameter) coated with DiI were carefully applied onto the mucosa of submucosal specimens dissected as previously described from circular and longitudinal muscle (23). After bead application, the tissue was maintained in organ culture for 4 days. To enhance immunoreactivity for paracellular tracer (DiI) and nerve fiber (Neurobiotin), 4% moloney murine leukemia virus reverse transcriptase (40 μM) was added to the medium during the final 14–18 h of culture. After culture, the tissue was fixed for 4–6 h in 4% paraformaldehyde at room temperature. Threeplexes were then dissected under a dissection microscope: the mucosa patch and the remaining circular muscle were removed, the tissue was pinned mucosa up in the dish, and the Meissner plexus was carefully dissected with fine dissection scissors. The intermediary plexus and Henle’s plexus were separated by cutting under the cleavage plane of the vascular bed.

The plexus were permeabilized in successive washes in a PBS glycerol solution with different glycerol concentrations (1 h in 50% glycerol and 6 h in 100% glycerol). After permeabilization, the tissue was incubated in PBS/HS/NaNO₃ at room temperature for 2 h before being incubated for 48 h in PBS/HS/NaNO₃ with mouse anti-VIP (1:1,000; Biogenesis). After incubation with the primary antisera, the tissue was washed with PBS and incubated with 24 h with FITC-conjugated donkey anti-mouse (1:200) or donkey anti-mouse IgG conjugated to 7-amino-4-methyl-coumarin-3-acetate (1:50) (Jackson Labs, purchased from Euromedex).

Immunoblot analysis of ZO-1. Immunoblot analysis of ZO-1 expression was determined after protein extraction with Tri Reagent (Euromedex). Proteins were migrated on 4–7.5% polyacrylamide gels, transferred onto nitrocellulose filters (Bio-Rad, Hercules, CA) by using a Transblot apparatus (Bio-Rad), and immunoblotted with ZO-1 monoclonal antibodies (1:1,000, Zymed). Filters were then incubated with anti-mouse (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies conjugated to peroxidase and processed for enhanced chemiluminescence detection (Roche). A densitometric analysis of immunoblots was performed by using Quantity One software (Bio-Rad).

Real-time RT–PCR analysis of ZO-1 mRNA levels. Total RNA extraction from HT29-C1.16E cells was performed with Tri Reagent (Euromedex) according to the manufacturer’s instructions. For reverse transcription, RNA (5 μg) was combined with 0.5 μg of random hexamers (Promega, Madison, WI), transcription buffer (in mM: 50 Tris–HCl pH 8.3, 75 KCl, 3 MgCl₂, 10 DTT), dNTPs (1 mM each), RINas (50 U; Bioline, London, UK), chloramphenicol (40 μM), reverse transcriptase (200 units; Promega) in a total volume of 25 μl. Incubation was performed at 42°C for 60 min. Amplification conditions of the ZO-1 and GAPDH templates were optimized for the RotorGene 2000 instrument (Ozyme). PCR amplifications were performed by using TITANIUM...
Intestinal Barrier Regulation by Human Enteric Neurons

RESULTS

Viability of the Epithelial Monolayers

After 3 days of coculture, there was no significant difference in LDH released with or without EFS in HT29-CI.16E (3.1 ± 0.4 and 2.9 ± 0.3%, respectively, n = 6) and in Caco-2 [5.7 ± 1.1 (n = 5) and 4.8 ± 1.0% (n = 6), respectively]. These values were similar to the ones measured in control HT29-CI.16E or Caco-2 monolayers cultured alone [1.3 ± 0.2% (n = 6) and 3.0 ± 1.0% (n = 6), respectively]. In addition, M30 antibody, an early marker of apoptosis, labeled <0.1% of HT29-CI.16E monolayer that was maintained in coculture or cultured alone.

Stimulation of the ENS Modulates the Permeability of Two Different Intestinal Epithelial Monolayers

To evaluate the paracellular permeability of epithelial monolayers, the apical-to-basolateral flux of FITC-labeled markers was measured across monolayers. Preliminary experiments revealed that permeability increased starting day 2 of coculture compared with control monolayers not maintained in coculture, and was always observed at day 3. In control monolayers, the FITC-dextran flux of the HT29-CI.16E monolayer was significantly higher (438 ± 53 ng·cm⁻²·h⁻¹, n = 8) than that of Caco-2 cells (7.8 ± 0.9 ng·cm⁻²·h⁻¹, n = 8). When maintained for 3 days in coculture without EFS, the increase in FITC-dextran flux in both HT29-CI.16E and Caco-2 monolayers was 35 ± 6 and 153 ± 55%, respectively, (n = 6 for each cell line), compared with the control monolayers. EFS significantly reduced but did not fully abolish the increase in permeability observed in coculture in the absence of EFS in both cell lines (Fig. 2A). A similar effect of EFS on HT29-CI.16E monolayer permeability was also observed with another standard marker of paracellular permeability, FITC-inulin (Fig. 2B).

In HT29-CI.16E, the effects of EFS on FITC-dextran flux were significantly reduced when EFS was performed in the presence of TTX (Fig. 3A). The addition of TTX to the coculture without EFS did not significantly modify the changes in FITC-dextran flux across the monolayer, compared with the flux measured in the absence of TTX (166 ± 33 and 223 ± 61 ng·cm⁻²·h⁻¹, respectively, P = 0.44, n = 8). We also verified that EFS by itself or TTX did not alter FITC-dextran flux in control monolayers not maintained in coculture (Fig. 3B). In Caco-2 cells placed in coculture, FITC-dextran flux across the monolayer was higher when EFS was performed with than without TTX (data not shown).

Because no major differences were detected between the two cell lines concerning permeability in response to EFS, further mechanistic studies were conducted only in the HT29-CI.16E cell line, which expresses a wide array of well-characterized receptors for neuromediators, known to be coupled to effector systems such as mucus or Cl⁻ secretions (18, 19).

VIP-Positive Neurons Innervate the Human Colonic Mucosa

The analysis of DiI-labeled preparations showed that a total of 71 ± 9 submucosal neurons projecting to the colonic mucosa were labeled when two DiI-coated beads 150–200 μm in diameter were used. These neu-
VIP Reproduces the Effects of EFS on Permeability in HT29-Cl.16E Monolayers

Changes in FITC-dextran flux across the monolayer after EFS was significantly higher in coculture when EFS was performed with than without VIPra (Fig. 4C). A monoclonal VIP antibody added to the coculture as a neutralizing antibody (1:1,000, n = 3) before EFS reproduced the effects of VIPra (data not shown). Furthermore, the addition of VIP (10 nM) in the coculture in the presence of TTX (1 μM; to avoid direct activation of enteric neurons by VIP) induced a significant decrease in FITC-dextran flux compared with the coculture without VIP (Fig. 4D).

Stimulation of the ENS Increases Expression of Tight Junction-Associated Protein ZO-1

Effects of EFS activation on ZO-1 expression were analyzed at protein and mRNA levels by using immunohistochemical detection, densitometric analysis of ZO-1 immunoblots, and real-time quantitative RT-PCR.

After activation of the ENS, immunofluorescent detection of ZO-1 showed strong continuous pericellular labeling of the epithelial monolayer, which was re-

Fig. 2. Activation of enteric neurons decreases epithelial permeability in HT29-Cl.16E and Caco-2 monolayers. A: measurement of epithelial permeability using FITC-dextran (4 kDa) showed a significant decrease in FITC-dextran flux in both cell lines after EFS (S) compared with nonstimulated (NS) condition. Results normalized to NS conditions are expressed as changes of dextran flux (ΔFITC-dextran flux) compared with control. ΔFITC-dextran flux in NS conditions for HT29-Cl.16E and Caco-2 was 153 ± 56 ng·cm⁻²·h⁻¹ (n = 12) and 10.1 ± 4.3 ng·cm⁻²·h⁻¹ (n = 6), respectively. (P = 0.002; Wilcoxon’s test). All results are expressed as means ± SE. B: measurement of epithelial permeability using FITC-inulin (3.5 kDa) showed a significant decrease in marker flux in HT29-Cl.16E after EFS compared with NS conditions (*P = 0.03; Wilcoxon’s test; n = 4).

Fig. 3. The effects of EFS on FITC-dextran flux across HT29-Cl.16E monolayers are neurally mediated. A: in coculture experiments, EFS (S) significantly reduced FITC-dextran flux compared with coculture without EFS (NS). This effect of EFS was abolished when 1 μM TTX (S + TTX) was added to the bath. Data are means ± SE of 4 experiments in which all 4 conditions were performed (*P = 0.006; 2-way repeated-measure ANOVA followed by Wilcoxon’s test; n = 4). Results normalized to S conditions are expressed as changes of dextran flux compared with control. ΔFITC-dextran flux in NS condition was 100 ± 26 ng·cm⁻²·h⁻¹ (n = 4). B: in HT29-Cl.16E monolayers cultured alone, neither EFS nor TTX per se modified FITC-dextran flux compared with control monolayers (n = 6/7). Dextran flux in control conditions was 438 ± 53 ng·cm⁻²·h⁻¹ (n = 8). All results are expressed as means ± SE.

Fig. 4. Activation of the enteric nervous system (ENS) modulates the FITC-dextran flux across the HT29-Cl.16E monolayer through VIPergic pathways. Neuronal tracing methods showed that a Dil-labeled submucosal neuron (A) innervating the mucosa was immunoreactive for VIP (B, arrow). C: dextran flux was significantly higher on electrical activation of enteric neurons with VIP receptor antagonist (VIPra) (S + VIPra) than without VIPra (S) (P = 0.04; paired t-test, n = 8). Results normalized to S conditions are expressed as ΔFITC-dextran flux compared with control. Changes in dextran flux in S conditions was 144 ± 26 ng·cm⁻²·h⁻¹ (n = 8). D: dextran flux was significantly decreased when VIP was added to the coculture in the presence of TTX (NS + TTX + VIP), compared with coculture with TTX but without VIP (NS + TTX) (P = 0.04; paired t-test, n = 7). Results normalized to NS + TTX conditions are expressed as changes of dextran flux compared with control. Change in NS + TTX conditions was 249 ± 49 ng·cm⁻²·h⁻¹ (n = 7). All results are expressed as means ± SE.
duced in the absence of neuronal activation (Fig. 5, A and B). Densitometric analysis of ZO-1 immunoblots showed a significant increase in protein level when enteric neurons were activated, compared with non-stimulated conditions (Fig. 6A), which suggests that changes in immunofluorescence were not related to redistribution of ZO-1 within the cell but rather to an actual change in protein amount. Furthermore, analysis of ZO-1 mRNA levels showed a significant increase in ZO-1 mRNA levels (67 ± 24%) after EFS, compared with the nonstimulated condition ($P < 0.04$, $n = 7$).

Effects of EFS on ZO-1 protein expression were neurally mediated, because ZO-1 protein level was significantly decreased when EFS was performed in the presence of TTX (1 μM; Fig. 6B). In addition, EFS by itself or TTX applied to monolayer alone did not modify ZO-1 protein expression compared with control conditions (98 ± 18 and 106 ± 16%, respectively, $n = 5$). TTX (1 μM) added to the coculture without EFS, did not modify ZO-1 protein level compared with coculture without EFS ($n = 5$).

VIP Increases ZO-1 Expression, in Part, Via Direct Action on HT29-Cl.16E Monolayers

After coculture, a significant decrease in ZO-1 protein level was observed when EFS was performed with VIP-ra compared with without VIP-ra (Fig. 6C). In addition, a significantly greater amount of ZO-1 protein was observed after the addition of VIP (1−10 nM) to the coculture compared with coculture without VIP (Fig. 6D).

To further confirm that VIP can directly regulate ZO-1 expression, experiments were performed on HT29-Cl.16E monolayers cultured alone. VIP (0.01 to 1 nM) added to the basolateral side of HT29-Cl.16E monolayers increased the level of ZO-1 protein ($n = 4$, Fig. 7A). In addition, VIP (0.01 to 1 nM) increased ZO-1 mRNA levels (Fig. 7, B and C). This VIP-induced increase in ZO-1 mRNA level peaked at 15 h and returned to basal level at 24 h (Fig. 7B). GAPDH mRNA levels were constant over time in the absence or presence of VIP (data not shown).

**Fig. 6.** Activation of the ENS and VIPergic pathways modulate ZO-1 protein expression. A: densitometric analysis of ZO-1 immunoblots from HT29-Cl.16E monolayers maintained in coculture revealed that activation of enteric neurons (S) increased ZO-1 protein expression significantly compared with NS conditions ($^{*}P = 0.01$; paired t-test; $n = 12$). Data are normalized to ZO-1 expression measured in NS conditions. B: ZO-1 protein levels were significantly decreased when enteric neurons were stimulated in the presence of TTX (S + TTX), compared with stimulation of enteric neurons without TTX (S) ($^{*}P < 0.05$; paired t-test; $n = 5$). Data are normalized to ZO-1 expression measured in S conditions. C: immunoblot followed by densitometric analysis showed a decrease in ZO-1 expression after electrical activation of enteric neurons in the presence of VIPra (1 μM) (S + VIPra), compared with neuronal activation alone (S) ($^{*}P = 0.002$; paired t-test; $n = 5$). Data are normalized to ZO-1 expression measured in S conditions. D: immunoblot analysis of ZO-1 expression showed that the addition of VIP (10 nM) in coculture medium without neuronal activation (NS + TTX + VIP) led to an increase in ZO-1 expression compared with coculture in the absence of VIP (NS + TTX) ($^{*}P = 0.036$; paired t-test; $n = 6$). Data are normalized to ZO-1 expression measured in NS + TTX conditions. All results are expressed as means ± SE.

**Fig. 5.** Activation of the ENS modulates zonula occludens-1 (ZO-1) protein expression in HT29-Cl.16E monolayers. Immunofluorescence staining followed by confocal microscopy observation (original magnification, ×630) showed that ZO-1 expression was stronger after coculture with EFS (A) than without EFS (B).
DISCUSSION

This study used a novel two-compartment human coculture model to show that the ENS can influence barrier permeability in two phenotypically distinct colonic cell lines, HT29-Cl.16E (mucus secreting) and Caco-2 (enterocyte-like). Additional pharmacological and molecular experiments performed on HT29-Cl.16E showed that VIPergic pathways are involved in the control of permeability and the regulation of ZO-1, a key tight junction-associated protein.

A major finding of this study is that activation by EFS of human submucosal neurons regulates flux of paracellular markers across monolayers of two human colonic epithelial cell lines (HT29-Cl.16E and Caco-2). Both FITC-dextran and FITC-inulin are considered as standard markers of paracellular permeability (4, 27). Effects of EFS were shown to be neurally mediated in both cell lines, because they were significantly reduced by TTX. In addition, in HT29-Cl.16E, we showed that EFS by itself did not modify the permeability of the monolayer. For further detailed pharmacological and molecular studies aimed at identifying the putative mediators involved in neuronal regulation of the epithelial permeability, we decided to focus on HT29-Cl.16E. Indeed, the HT29-Cl.16E cell line displays several functional receptors for neuromediators with characteristics similar to those usually expressed by normal human colonocytes (3, 19, 26). In particular, the VIP receptors present on HT29-Cl.16E cells are coupled to effector systems such as mucin exocytosis and Cl⁻ transport (18, 19).

Another finding of our study is that VIPergic submucosal pathways were involved in the control of the intestinal epithelial permeability. Using neuronal tracing methods, we showed that the majority (80%) of enteric neurons innervating the human sigmoid colon mucosa contained VIP. This proportion was higher than that observed in submucosal neurons innervating the mucosa in the human transverse and proximal colon (25). This difference could be due to the use of colchicine in our study to further enhance VIP immunoreactivity and detection sensitivity. Based on pharmacological studies, we then showed that VIP released after activation of enteric neurons decreased paracellular permeability. Although the majority of VIP is probably of neuronal origin, one cannot exclude the finding that VIP can also be released by other cells such as lymphocytes (21). Our results obtained after activation of human submucosal neurons are reinforced by an in vivo study showing that VIP was able to reduce the increase in paracellular permeability induced by substance P in the rat (14). VIP has also been reported to decrease transepithelial resistance in Caco-2 and HT29-Cl.19A cell lines (5). In our coculture model, other neuromediators besides VIP may be involved in the neuronal regulation of epithelial permeability. In fact, in the human colon and rectum, submucosal neurons colocalize various mediators other than VIP, such as acetylcholine (1, 2, 28), which has been shown to alter epithelial permeability in colonic cell lines (5).

This study also provides the first evidence that the activation of the ENS and that VIP were able to regulate the expression of ZO-1 in intestinal epithelial cells, both at the mRNA and protein level. This action on ZO-1, which is a key regulator of tight junctions (7), could explain, in part, the regulatory effect of submucosal neurons on epithelial permeability. Effects of VIP released after activation of submucosal neurons on ZO-1 expression were at least partly mediated by a direct action of VIP on epithelial cells. Indeed, selective blockade of the VIP receptors on HT29-Cl.16E mono-

Fig. 7. VIP increased ZO-1 protein and mRNA levels in HT29-Cl.16E monolayers. A: densitometric analysis of ZO-1 immunoblots showed that VIP induced a significant increase in ZO-1 protein amount compared with control monolayers at a concentration of 1 nM VIP (24-h incubation). Means ± SE of 4 different experiments. *P < 0.05 compared with other VIP concentrations (repeated ANOVA on ranks followed by Dunnett’s test). B: real-time quantitative RT-PCR analysis of ZO-1 mRNA levels normalized to GAPDH showed a significant increase in mRNA levels 15 h after the addition of VIP (1 nM), compared with control monolayers (*P < 0.05; repeated ANOVA on ranks followed by Dunnett’s test; n = 4). C: at 15 h after the addition of VIP, this increase was significant at 1 nM VIP compared with 0.01 and 0.1 nM (*P < 0.05; n = 4).
layers before EFS led to a significant decrease in ZO-1 protein level. In addition, VIP increased ZO-1 protein and mRNA levels significantly in HT29-C1.16E monolayers cultured alone. The mechanisms of ZO-1 regulation by submucosal VIPergic pathways could involve a cAMP-dependent pathway, because 1) VIP increases intracellular cAMP level in HT29-C1.16E (19), and 2) cAMP increases ZO-1 expression, in human placental microvascular endothelial cells (11).

In conclusion, our study suggests a novel role for enteric neurons and especially for VIPergic submucosal pathways in the control of parameters regulating the human intestinal epithelial barrier homeostasis such as paracellular permeability and tight junction proteins. This novel role of the ENS is reinforced by the recent observations that another major component of the ENS, i.e., enteric glial cells, plays a key role in the maintenance of intestinal barrier integrity (6, 9). Furthermore, our study suggests that changes in neurotransmitter content occurring during various intestinal infectious or inflammatory diseases could be directly involved in the disturbances of intestinal barrier functions observed.

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