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Antisecretory effects of neuropeptide Y in the mouse colon are region-specific and are lost in DSS-induced colitis

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ABSTRACT

Regulation of water movement in the gut is an important homeostatic event that is critical to normal intestinal function. We assessed the effect of neuropeptide Y (NPY) on epithelial ion transport in the normal and inflamed mouse colons. Colitis was induced by dextran sodium sulfate (DSS, 4% wt./vol.) administered in the drinking water for 5 days followed by 3 days of regular water. Segments of proximal and distal colons were excised and short-circuit current (I_{SC}) was measured in Ussing chambers to assess net electrogenic active ion transport. NPY Y_1 receptor (Y_1R) expression was measured by quantitative real-time PCR and immunohistochemistry. Challenge of distal colon from normal mice with NPY (10^{-7} M) evoked a drop in I_{SC} (51.4 ± 9.1 μA/cm^2), which was dependent on Cl^- flux, was insensitive to neural blockade with tetrodotoxin and was mediated primarily through the Y_1R. In contrast, the proximal colon was largely unresponsive to NPY, expressing ~ten-fold less Y_1R mRNA compared to the distal colon. These findings confirm that specific regional regulation of ion transport occurs in the colon. Segments of proximal and distal colons from mice with DDS-induced colitis were virtually unresponsive to NPY, expressed less Y_1R mRNA than tissues from control mice and displayed loss of Y_1R protein expression in the colonic epithelium. This hypo-responsiveness to an antisecretory stimulus adds to the well-documented loss of responsiveness to prosecretory agents during inflammation, attesting to a profound loss of control of active ion transport during enteric inflammatory disease.

1. Introduction

The vectorial movement of cations (Na^+, H^+ and K^+) and anions (Cl^- and HCO_3^-) across the transporting enterocyte is dependent on the asymmetrical distribution of ion channels, co-transporters and exchangers, and the Na^+/K^+/ATPase pump in the basolateral and apical membranes. The coordinated activity of this ion transport machinery, which is typically driven by the mobilization of cAMP and Ca^{2+}, generates electrogenic ion fluxes that are critical homeostatic events, since they create the driving forces for directed water movements [1,2]. The serosal-to-mucosal movement of water hydrates, the epithelial surface for contact digestion, provides the medium for the activity of extracellular digestive enzymes (e.g. those of pancreatic origin) and antimicrobial peptides, and serves to 'wash' pathogens off the enterocytes. Of equal importance is the re-absorption of water, which occurs mainly in the colon, and prevents dehydration. The extreme of either event is diarrhea and constipation, respectively.

External stimuli such as bacterial toxins directly modulate epithelial ion transport, while mediators from the enteric nervous system, endocrine cells and resident or recruited immune cells are major endogenous regulatory elements [3]. Many signals have been identified that exert a prosecretory influence on the enteric epithelium, including acetylcholine (ACh), vasoactive intestinal peptide (VIP), histamine and prostaglandins. Less is known of antisecretory or proabsorptive molecules: the adrenergic system, opiates, somatostatin and the neuropeptide Y (NPY)/peptide YY (PYY) family have been shown to exert antisecretory effects in the mammalian intestine. In the context of the latter, seminal work by Cox et al. [4] has shown that NPY, PYY, and pancreatic poly-peptide (PP) can exert antisecretory effects in human and rodent colons via epithelial Y_1 receptors (Y_1R) and Y_2 receptors expressed on neurons. These neuropeptides directly affect tonic ion transport and the secretory events driven by, for example, direct neuronal activation by the application of VIP to tissues mounted in Ussing chambers [5–10].

Given the importance of intestinal water movements to health and well-being many investigators have assessed epithelial ion transport in tissues from animals with gut disease (e.g. colitis) and from patients with inflammatory disease. Almost universally these studies have shown that the enterocyte is hypo-responsive to prosecretory

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stimuli [11–15]. This reduced responsiveness to secretagogues may be a protective mechanism, serving to safeguard against excessive, inflammation-induced water loss. We showed that colon from mice with dextran sodium sulfate (DSS)-induced colitis responded to the cholinergic agonist carbachol with a decrease in short-circuit current compared to control tissue, which responds with a large transient increase in Cl^- secretion [2,14,16]. Thus, one might speculate that proabsorptive (or antisecretory) events would be enhanced in tissues from individuals with inflammatory disease as a water conserving strategy.

Given the plethora of data on the diminished responses to prosecretory stimuli in tissues from animals or humans with colitis, and the dearth of information on the effectiveness of antisecretory/proabsorptive agents during inflammation, the principal goal of the current study was to compare the effects of NPY in the regulation of colonic active ion transport in tissue from normal and colitic mice. We observed distinct regional differences in the responses of normal mouse colon to NPY, with proximal regions being significantly less responsive than distal segments. Contrary to our hypothesis, the distal colon of mice with DSS-induced colitis was virtually non-responsive to NPY: a physiological event paralleled by reduced expression of Y_1R mRNA and protein.

2. Materials and methods

2.1. Animals and the induction of colitis

Male C57/Bl6, CD1 and BALB/c mice (6–8 weeks old, Charles River, Canada) were housed in isolator cages with *ad libitum* access to standard laboratory chow and tap water. For the induction of colitis, C57/Bl6 mice were treated with 4% (wt./vol.) DSS (M.W. = 36–50 kDa, MP Biomedicals, LLC, Solon, OH, USA) dissolved in tap water, for 5 days, followed by 3 days with tap water [14]. Mice were weighed daily. At sacrifice (isoflurane-induced first deep anesthesia followed by cervical dislocation) the abdomen was opened, the colon removed and measured, and a disease activity score (DAS: max. = 5) based on weight loss (>10%), colon length (>10% shorter colon length compared to controls), loose stools or fluid filled colons, ulceration or fecal blood, and morbidity was calculated [14]. These experiments were performed in accordance with the guidelines on animal experiments of the Canadian Council on Animal Care and the University of Calgary Animal Care Committee.

2.2. Measurement of active epithelial ion transport

In the majority of studies, 2.5 cm portions of tissue either side of the mid-point of the colon were excised and designated the proximal and distal colons (in other studies the 2.5 cm pieces of tissue directly aboral from the cecum and directly oral from the anus were used). These full-thickness tissues were opened along the mesenteric border and mounted in modified Ussing chambers with an exposed surface area of 0.6 cm^2 [17]. Tissues from DSS-treated mice displaying obvious gross macroscopic ulceration were not used in this study, since the expected lack of epithelium on such tissues would impact ion transport. Also, <10% of tissues were discarded due to damage caused during the process of mounting in the Ussing chamber. Tissues were bathed in oxygenated 37 °C Krebs buffer (in mM: NaCl (115.0), KCl (8.0), $CaCl_2$ (1.25), $MgCl_2$ (1.2), KH_2PO_4 (2.0), $NaHCO_3$ (25.0)) containing glucose (10 mM) at pH 7.35 ± 0.3 and the spontaneous potential difference (PD, measured in mV) was held at zero volts by the continual injection of an external current (EVC4000-4, World Precision Instruments, Mississauga, ON, Canada): the short-circuit current (I_{SC}, measured in μA). I_{SC} indicates net electrogenic active ion transport across the tissue and was continuously recorded (Lab-Scribe2 Software, iWorkx, Dover, NH, USA); it should be noted that electroneutral fluxes go undetected with this procedure. In some experiments, a Cl^--free Krebs buffer was used (in mM: isethionic acid sodium salt (114.8), KH_2PO_4 (2.0), gluconic acid (2.4), Ca^{2+} gluconate (0.65), $NaHCO_3$ (25) and K^+ gluconate (8.0) [18]). Following a 15 min equilibration period, baseline I_{SC} and PD were recorded and tissue conductance (G in mSiemens) was calculated using Ohm's law. Conductance was also measured at the end of each experiment, when all tissues were challenged with forskolin (FSK, 10^{-5} M; Sigma-Aldrich, Oakville, ON, Canada) and the maximum change in I_{SC} (ΔI_{SC}) to occur with 5 min of challenge was recorded. Only tissues with starting G = 10–50 mS/cm^2 and that did not vary by more than 20% between the beginning and end of the experiment were included in the electrophysiological analyses. In addition, all tissues responded to forskolin (see below) [17].

The effects of the following reagents on I_{SC} was recorded as the maximum ΔI_{SC} to occur within 5 min of their addition to the buffer bathing the serosal surface of the colonic tissue: neuropeptide (NPY, 10^{-9}–10^{-6} M), the neurotoxin, tetrodotoxin (TTX, 10^{-6} M [14]), the Na^+ channel blocker, amiloride (10^{-5} M, [19]), VIP (10^{-6} M; all from Sigma-Aldrich), the Y_1R antagonist, BIB03304 (3×10^{-7} M [9]), the Y_1R agonist, (Pro^{34})NPY (10^{-7} M [20]) and the Y_2R agonist, NPY(3–36) (10^{-7} M [6]; all from Bachem Inc., Torrance, CA, U.S.A). Negative values indicate a drop in I_{SC}.

2.3. RNA isolation

mRNA was isolated from a 1 cm segment of full-thickness tissue or mucosal scrapings from the proximal and distal regions of the colon or mouse brain samples. Mucosal scrapings were grouped together from 3 mice. Samples were snap-frozen in liquid N_2 and RNA was isolated using TRIzol (Invitrogen Canada Inc., Burlington, ON, Canada) as per the manufacturer's instructions. One micrograms of RNA was reverse transcribed using an iScript™ cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada) and the premixed buffer (dNTP, $MgCl_2$, stabilizers and oligo(dT)primers). Quantitative, real-time PCR was conducted using the iQ™ SYBR Green Supermix system (dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green 1 and 20 nM of fluorescein), using gene specific primers for the Y_1R and β-actin (see Table 1). Each cycle of PCR involved a 95 °C denaturing step, 55 °C annealing step and a 68 °C extension stage. Fluorescence was measured at each step by the Eppendorf Mastercycler ep-realplex^2 real-time PCR system.

mRNA levels in whole-thickness tissues were assessed using the paired ΔΔ cycle threshold (C_T) method [21], in which the fold-change in Y_1R expression between the distal and proximal colon was determined for each mouse. Mucosal scraping mRNA content was quantified relative to expression levels in brain tissue; a standard curve was generated using three and 10-fold serial dilutions of brain cDNA, and the Y_1R mRNA and β-actin mRNA were amplified from the brain. Mucosal scraping samples were quantified for the Y_1R and β-actin by comparison with the standard curve. Standardized Y_1R expression was then divided by standardized β-actin expression to give a Y_1R relative to β-actin.

2.4. Immunohistochemistry

Samples of colon from control and DSS-treated mice were fixed in Zamboni's fixative overnight and then transferred to 20% sucrose in

Table 1
Primer sequences used in this study.

Gene	Product size (bp)	Forward primer (5′–3′)	Reverse primer (5′–3′)
Y_1 receptor	206	CTGATGGACCACTGGGTCTT	GAAGAAGCCACTGCAAGGAC
β-actin	228	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA

bp, base pairs.