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Xenin Augments Duodenal Anion Secretion via Activation of Afferent Neural Pathways

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ABSTRACT

Xenin-25, a neurotensin (NT)-related anorexigenic gut hormone generated mostly in the duodenal mucosa, is believed to increase the rate of duodenal ion secretion, because xenin-induced diarrhea is not present after Roux-en-Y gastric bypass surgery. Because the local effects of xenin on duodenal ion secretion have remained uninvestigated, we thus examined the neural pathways underlying xenin-induced duodenal anion secretion. Intravenous infusion of xenin-8, a bioactive C-terminal fragment of xenin-25, dose dependently increased the rate of duodenal HCO_3^- secretion in perfused duodenal loops of anesthetized rats. Xenin was immunolocalized to a subset of enteroendocrine cells in the rat duodenum. The mRNA of the xenin/NT receptor 1 (NTS1) was predominantly expressed in the enteric plexus, nodose and dorsal root ganglia, and in the lamina propria rather than in the epithelium.

The serosal application of xenin-8 or xenin-25 rapidly and transiently increased short-circuit current in Ussing-chambered mucosa-submucosa preparations in a concentration-dependent manner in the duodenum and jejunum, but less so in the ileum and colon. The selective antagonist for NTS1, substance P (SP) receptor (NK1), or 5-hydroxytryptamine (5-HT)₃, but not NTS2, inhibited the responses to xenin. Xenin-evoked Cl^- secretion was reduced by tetrodotoxin (TTX) or capsaicin-pretreatment, and abolished by the inhibitor of TTX-resistant sodium channel Nav1.8 in combination with TTX, suggesting that peripheral xenin augments duodenal HCO_3^- and Cl^- secretion through NTS1 activation on intrinsic and extrinsic afferent nerves, followed by release of SP and 5-HT. Afferent nerve activation by postprandial, peripherally released xenin may account for its secretory effects in the duodenum.

Introduction

The duodenal mucosa, because of its strategic location between the stomach and the rest of the small intestine, senses luminal nutrients, affects appetite, and regulates duodenal anion secretion, particularly HCO_3^- secretion, which in turn is important for nutrient absorption and mucosal protection from gastric acid (Kaji et al., 2013). We have been investigating the contributions of enteroendocrine cells (EECs) and afferent sensory nerves that link the duodenal mucosa with mucosal defense mechanisms (Akiba and Kaunitz, 2011; Said et al., 2015). As part of this quest, we hypothesized that xenin, a homolog of amphibian xenopsin

and mammalian neurotensin (NT) (Feurle et al., 1992), which is specifically generated in the duodenal mucosa, directly affects duodenal anion secretion.

Xenin as a 25-amino acid peptide is predominantly detected in human gastric and duodenal mucosa in addition to the hypothalamus (Feurle et al., 1992). Some xenin degradation fragments (e.g., xenin 9–25, 11–25, 14–25, or 18–25) are identified in some animal species and an octapeptide xenin-8 (18–25) retains the signal sequence (Hamscher et al., 1995; Martin et al., 2014). The origin of active xenin-25 in the gastric mucosa has been controversial, because xenin can be generated by peptic digestion from its precursor (proxenin), a component of the highly conserved ubiquitous vesicular coat proteins, coatamer protein complex- α (Hamscher et al., 1995). Thus, xenin peptides could be generated in vitro from homogenized gastric mucosa by pepsin (Hamscher et al., 1995).

A specific xenin antibody identified a subset of duodenal EECs that coexpressed xenin with gastric inhibitory peptide (GIP), but not 5-hydroxytryptamine (5-HT) in canine

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ABBREVIATIONS: ACh, acetylcholine; CCK, cholecystokinin; CGRP, calcitonin-gene related peptide; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DRG, dorsal root ganglia; EEC, enteroendocrine cell; Ep, epithelial cells; GIP, gastric inhibitory peptide; GLP, glucagon-like peptide; G_t , tissue conductance; HPLC, high-performance liquid chromatography; I_{sc} , short-circuit current; L-NAME, *N*^ω-nitro-L-arginine methyl ester hydrochloride; LP-SM, lamina propria + submucosa including the submucosal plexus; NG, nodose ganglia; NK1, SP/neurokinin receptor 1; NO, nitric oxide; NT, neurotensin; PBS, phosphate-buffered saline; SP, substance P; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide; VPAC1, VIP/PACAP receptor 1.

duodenum (Anlauf et al., 2000). Because GIP is released in response to luminal nutrients in the regulation of glucose homeostasis, xenin is thought to also be released from EECs by physiologic stimuli. Indeed, intravenous xenin delays gastric emptying, decreases gastric acid secretion, and potentiates insulin secretion when combined with GIP (Feurle, 1998; Wice et al., 2010). Nevertheless, the effects of xenin in the duodenal mucosa have not been previously investigated.

NT, produced by central nervous system and enteroendocrine N cells, is present predominantly in the lower ileum and released by dietary fat. NT inhibits intestinal transit and food intake (Dumoulin et al., 1998; Cooke et al., 2009) and increases electrogenic Cl^- secretion in human colon in vitro (Riegler et al., 2000). The secretory effects of NT were identified in canine duodenum (Konturek et al., 1985), consistent with NT receptor expression in the duodenum (Tanaka et al., 1990). Owing to peptide sequence similarity between NT and the C terminus of xenin and the observation that the NT receptors (NTS) 1 and 2 are activated by xenin with similar affinity to that of NT (Botto et al., 1998), NTS are also thought to be xenin receptors. NTS1 and NTS2 are $\text{G}_{\text{q}/11}$ -coupled G protein-coupled receptors abundantly expressed in neural tissues.

Plasma xenin concentrations are increased by food intake and by sham feeding in humans, suggesting that luminal nutrients and vagal activation release xenin from the duodenal mucosa (Feurle et al., 2003). Because nocturnal workers with high energy intake and high body fat mass have no elevation of plasma xenin after meals (Schiavo-Cardozo et al., 2013) and obese adolescents have significantly higher concentrations of plasma xenin between meals compared with healthy controls (Arslan et al., 2014), xenin-induced satiety signals appear to be disrupted in obesity. Peripheral and central injection of xenin decreases food intake in experimental animals (Alexiou et al., 1998; Cooke et al., 2009), consistent with a satiety function of xenin. The infusion of low concentrations (4–12 pmol/kg) of xenin to humans increases satiety and causes mild diarrhea by an unknown mechanism (Chowdhury et al., 2014; Gault et al., 2015). Because Roux-en-Y gastric bypass surgery diminishes xenin-associated diarrhea (Sterl et al., 2016), the duodenum may significantly contribute to the fluid and ion secretory response to xenin. We thus aimed to characterize the secretory effects of xenin in the duodenum and to investigate the neural pathways underlying xenin-induced duodenal anion secretion.

Materials and Methods

Animals. Male Sprague-Dawley rats weighing 200–250 g (Harlan, San Diego, CA) were fed a pellet diet and water ad libitum. All studies were performed with approval of the Veterans Affairs Institutional Animal Care and Use Committee. Rats were fasted overnight with free access to water before the experiments. Animals were euthanized by terminal exsanguination under deep isoflurane anesthesia, followed by thoracotomy.

Duodenal Loop Perfusion. Rat duodenal loops were prepared and perfused under isoflurane anesthesia as described previously (Mizumori et al., 2006). In brief, prewarmed saline was infused via the right femoral vein at 1.08 ml/h using a Harvard infusion pump (Harvard Apparatus, Holliston, MA). Under isoflurane anesthesia (2%), a polyethylene tube (diameter 5 mm) was inserted through the forestomach and tied at 0.5 cm caudal from the pyloric ring. Another polyethylene tube was inserted through the distal duodenum and

ligated proximal to the ligament of Treitz. To prevent contamination from bile or pancreatic juice, the pancreaticobiliary duct was ligated just proximal to its insertion into the duodenal wall and cannulated with a PE-10 tube to drain the juice. The resultant closed proximal duodenal loop (perfused length ~2 cm) was perfused with prewarmed saline (pH 7.0) by using a peristaltic pump (Fisher Scientific, Pittsburgh, PA) at 1 ml/min. After stabilization with continuous luminal perfusion of O_2 -bubbled saline (pH 7.0) for ~30 minutes, the time was set as $t = 0$. The duodenal loop was perfused with saline from $t = 0$ minute until $t = 45$ minutes. After intravenous infusion of saline for 15 minutes, xenin-8 in saline was continuously infused at 3 or 10 nmol/kg/h from $t = 15$ minutes to $t = 45$ minutes. Duodenal HCO_3^- secretion was measured using flow-through pH and CO_2 electrodes and expressed as total CO_2 output, calculated from the measured pH and $[\text{CO}_2]$ in the effluent solution, as reported previously (Mizumori et al., 2006).

Immunofluorescence Staining and Real-Time Polymerase Chain Reaction. Small pieces of intestine were immersed in Zamboni's fixative containing 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) overnight for 4°C. The fixed tissues were then submerged in 20% sucrose in phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C, and embedded in optimum cutting temperature (OCT) compound. Frozen sections of 8- μm thickness were placed on aminosilane-coated glass slides (Matsunami Glass USA Inc., Bellingham, WA). Sections were pretreated with 5% normal donkey serum in PBS containing 0.3% Triton X-100 for 1 hour, followed by incubation with primary antibodies: rabbit anti-xenin-25 (Phoenix Pharmaceuticals, Belmont, CA; 1 $\mu\text{g}/\text{ml}$), mouse anti-5-HT (dilution 1:100, MCA3190Z; AbD Serotec, Kidlington, UK), goat anti-GIP (dilution 1:200, sc-23554; Santa Cruz Biotechnology Inc., Santa Cruz, CA), goat anti-cholecystokinin (CCK) (dilution 1:200, sc-21617; Santa Cruz Biotechnology), or goat anti-glucagon-like peptide (GLP)-2 (dilution 1:200, sc-7781; Santa Cruz Biotechnology) overnight at 4°C. To confirm antibody specificity, xenin-25 antibody was preabsorbed with xenin-25 (50 $\mu\text{g}/\text{ml}$) overnight before application to tissue sections (absorption test). After rinsing in PBS, fluorescence-conjugated secondary antibodies were reacted for 2 hours at room temperature. The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and covered with EverBrite mounting medium (Biotium, Hayward, CA). Immunofluorescence was imaged and captured using a confocal laser microscope (LSM710; Carl Zeiss GmbH, Jena, Germany).

The duodenum was separated into the epithelium, interstitium-submucosa, and muscle layers under a stereomicroscope. The tissue was immersed in ice-cold pH 7.0 Krebs buffer containing 10 mM EDTA for 10 minutes, followed by stripping muscle layers by microdissection using fine forceps. The epithelial cells were separated from the interstitium by gentle scraping using a silicone-coated pipette tip. The separated duodenal samples, dorsal root ganglia (DRG), and nodose ganglia (NG) were kept in a RNA stabilization solution (RNAlater, Qiagen, Valencia, CA) at 4°C until use. Real-time polymerase chain reaction was performed as described previously (Akiba et al., 2015) with the following sense and antisense primers, respectively; NTS1 (5'-gtcaaggtcgtcatccaggt-3'; 5'-agaccacaaggaatgacc-3'), NTS2 (5'-ccatcgtggctgtgtatgctc-3'; 5'-agcgtgttggtcaccatgta-3'), NK1 (5'-tctcctcgtccctacatcaac-3'; 5'-tgacctgtgacacgtcctc-3'), NK2 (5'-gtgaaggccatggtactggt-3'; 5'-tcagcctgtcttctcagt-3'), and NK3 (5'-tactcgccttccagaactt-3'; 5'-tccaacgatggttagtgta-3'). β -Actin was used as an internal control. The expression level was presented as the fold induction per 10^3 copies of β -actin by the ΔCt method.

Short-Circuit Current Measurements in Ussing-Chambered Preparations. Mucosa-submucosa preparations were created from the duodenum as described previously (Kaji et al., 2015b). The same preparations from the midjejunum (~20 cm from the pyloric ring), terminal ileum (5 cm from the ileocecal junction), proximal colon (5 cm from the cecum), and distal colon (5 cm from the anus) were used for comparing segmental differences. Each segment was divided longitudinally into two pieces, and each piece was mounted between two

hemislicers with an aperture = 0.3 cm² (Physiologic Instruments, San Diego, CA). The serosal Krebs-Ringer solution contained (in mM) NaCl, 117; KCl, 4.7; MgCl₂, 1.2; NaH₂PO₄, 1.2; CaCl₂, 2.5; NaHCO₃, 25; glucose, 11; and bubbled with 95% O₂-5% CO₂ to maintain pH at 7.4. For HCO₃⁻-free conditions, NaHCO₃ was replaced with NaCl and acetazolamide (0.2 mM) was added into the serosal bath bubbled with 100% O₂. For Cl⁻-free solutions, NaCl, KCl, and CaCl₂ were replaced with sodium gluconate, potassium gluconate, and 8 mM calcium gluconate, respectively. The luminal bathing solution for small intestine contained (in mM) NaCl, 136; KCl, 2.6; CaCl₂, 1.8; HEPES, 10 (pH 7.4); and mannitol, 11; bubbled with 100% O₂. The luminal bathing solution for colon was Krebs-Ringer solution. Measurements of short-circuit current (*I*_{sc}) and tissue conductance (*G*_t) were conducted as described previously (Kaji et al., 2015b). Positive values for *I*_{sc} indicate a negative electrical charge flux from the serosal → luminal bath as a result of anion secretion or cation absorption. Indomethacin (10 μM) was added to the serosal bath for the small intestine or to both baths for the colon to eliminate the effects of endogenous prostaglandin production. The tissues were stabilized for 30–45 minutes before the effects of xenin and other drugs were investigated.

Peptide Synthesis. Xenin-25 (MLTKFETKSARVKGSLFHPKRP-WIL) was synthesized using solid-phase methodology according to the Fmoc-strategy using an automated peptide synthesizer (Model Pioneer, Thermo Fisher Scientific, Waltham, MA). The crude peptide was purified using reverse-phase high performance liquid chromatography (HPLC: Delta 600 HPLC System, Waters, MA) on a column of Develosil ODS-HG-5 (2 × 25 cm, Nomura Chemical Co., Ltd, Seto, Japan). The purity of each peptide was confirmed by analytical HPLC and matrix assisted laser desorption/ionization time of flight and mass spectrometry analysis.

Chemicals. The vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide receptor 1 (VPAC1) antagonist [Ac-His¹, D-Phe², Lys¹⁵, Arg¹⁶, Leu²⁷]-VIP(1–7)-GRF(8–27) was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Xenin-8, A803467, SR48692 (meclinetant), NTRC824, SB268262, CP96345, CP99994, MEN10376, osanetant, PSB603, MRS3777, and GR113808 were purchased from Tocris Bioscience (Pittsburgh, PA). Aprepitant was purchased from Cayman Chemical (Ann Arbor, MI). Levocabastine, ondansetron, suramin, 8-cyclopentyl-1,3-dipropylxanthine (CPX), 8-(3-chlorostyryl) caffeine (8CC), atropine, tetrodotoxin (TTX), lidocaine,

capsaicin, indomethacin, *N*^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), nifedipine, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis. Values are expressed as the mean ± S.E.M. The number of animals in each experimental group was ≥5. Statistical analysis was performed using Prism, ver. 6 (GraphPad Software Inc., La Jolla, CA) with Student's *t* test or analysis of variance followed by Dunnett's or Tukey's multiple comparison depending on the number of experimental groups. *P* < 0.05 was considered statistically significant.

Results

Secretory Effect of Xenin-25 and Xenin-8 in Ussing-Chambered Duodenal Mucosa. After a 30-minute incubation in the Ussing chamber, basal *I*_{sc} and *G*_t stabilized at 38.9 ± 4.6 μA/cm² and 41.0 ± 1.3 mS/cm², respectively, in mucosa-submucosa preparations of distal duodenum. Serosal application of xenin-8 immediately increased *I*_{sc} and *G*_t in a dose-dependent manner with EC₅₀ = 0.3 nM (Fig. 1, A and B). A submaximal concentration (1 nM) was subsequently used in experiments to identify the underlying mechanisms. Serosal xenin-25 (1 nM) increased *I*_{sc} (83.2 ± 4.7 μA/cm²; *N* = 5) with a similar time course and peak value to those of xenin-8. Mucosal application of xenin-8 (1 nM) however, did not alter *I*_{sc} or *G*_t, suggesting that xenin receptors are present on the submucosal plexus or epithelial cell basolateral membrane rather than on the apical membrane of duodenocytes.

To confirm the nature of anion secretion in Ussing-chambered duodenal mucosa, the effects of xenin-8 were measured in the presence or absence of HCO₃⁻ or Cl⁻ in the serosal buffer. HCO₃⁻ depletion of the buffer did not alter basal *I*_{sc} or *G*_t (*I*_{sc}, 49.3 ± 13.3 μA/cm²; *G*_t, 49.6 ± 4.1 mS/cm²), or the xenin-8-induced *I*_{sc} increase, compared with normal conditions. Cl⁻ depletion in the serosal bath, however, reduced basal *I*_{sc} and *G*_t (*I*_{sc}, -14.9 ± 28.6 μA/cm²; *G*_t, 22.3 ± 1.6 mS/cm²) and the response to xenin-8 by 80%, suggesting that the xenin-induced *I*_{sc} increase is predominantly electrogenic

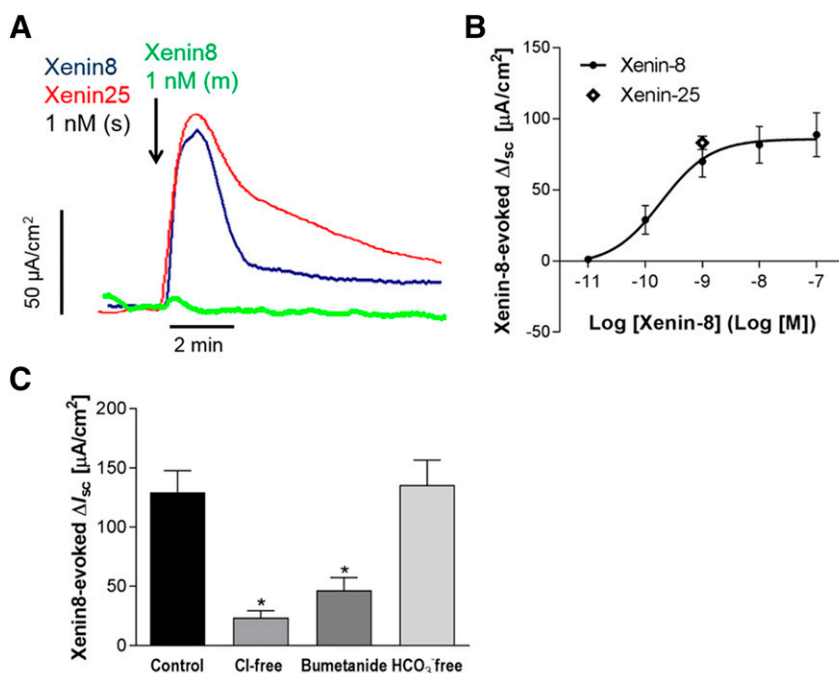


Fig. 1. Effect of xenin on short-circuit current (*I*_{sc}) in mucosa-submucosa preparation of rat duodenum mounted in Ussing chambers. (A) Representative traces of *I*_{sc}. Serosal (s) application of xenin-8 (dark blue line) or xenin-25 (red line) at 1 nM immediately increased *I*_{sc}, whereas mucosal (m) application of xenin-8 (green line) had no effect. (B) Concentration-response curve of serosal xenin-8-induced *I*_{sc} increases. Effect of xenin-25 at 1 nM (unfilled diamond) was similar extent with that of xenin-8. (C) Xenin-8-induced *I*_{sc} increases in the presence or absence of Cl⁻, HCO₃⁻, or bumetanide in the serosal bathing solution. The serosal bath was replaced with each anion-free buffer, or bumetanide (0.1 mM) was added 15 minutes before the application of xenin-8 (1 nM). Each datum represents the mean ± S.E.M. (*n* = 5). **P* < 0.05 versus control group by analysis of variance (ANOVA) followed by Dunnett's test.

Cl⁻ secretion (Fig. 1C). The NKCC1 inhibitor bumetanide reduced xenin-8-evoked I_{sc} , further supporting the presence of Cl⁻ secretion, which depends on basolateral NKCC1 activity.

Segmental Differences in the Response to Xenin-8 in the Ussing Chamber. To compare the extent of secretory response to xenin-8, we measured ion secretion in mucosa-submucosa preparations from proximal duodenum, distal duodenum, midjejunum, terminal ileum, proximal colon, and distal colon. Basal I_{sc} and G_t after stabilization differed among the segments tested (Table 1). Serosal application of xenin-8 (1 nM) increased I_{sc} accompanied by G_t increase to the same extent in the proximal and distal duodenum. The hindgut segments had significantly lower or no response compared with the distal duodenum (Fig. 2A). The viability of the preparations was confirmed by the addition of nicotine (0.1 mM) into the serosal bath after the experiment, which increased I_{sc} to a similar extent in all segments tested (Fig. 2B).

Effects of NTS Antagonists on Xenin-8-Induced Anion Secretion in the Duodenum. To evaluate the contribution of NTS1 and NTS2 toward xenin-8-induced anion secretion, we tested the effects of the selective antagonist SR48692 [2-[[[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)-1Hpyrazol-3-yl]carbonyl]amino]-tricyclo[3.3.1.1^{3,7}]decane-2-carboxylic acid] for NTS1 and levocabastine and NTRC824 for NTS2. SR48692 pretreatment did not alter basal I_{sc} or G_t , but dose dependently inhibited the response to xenin-8 (Fig. 3A), with an IC₅₀ ~30 nM, comparable to the results from in vitro binding assay (Gully et al., 1993). Neither the partial NTS2 agonist levocabastine nor the selective and potent NTS2 antagonist NTRC824 altered basal or xenin-8-induced I_{sc} , suggesting that xenin-8-induced duodenal anion secretion is mediated by NTS1, but not by NTS2.

Effects of Neuronal Channel Inhibitors on Xenin-8-Induced Anion Secretion in the Duodenum. To identify the neural pathways mediating the secretory response to xenin, the neural cation channel blockers TTX, A803467 [5-(4-chlorophenyl)-N-(3,5-dimethoxyphenyl)-2-furancarboxamide], lidocaine, and/or capsaicin were used. Serosal pretreatment with TTX decreased basal I_{sc} , indicating that TTX-sensitive submucosal neural activity is involved in basal ion transport under stabilized conditions. Xenin-8-induced anion secretion was inhibited 50% in the presence of TTX (Fig. 3B). Serosal capsaicin (10 μ M) transiently increased I_{sc} only with the first application ($14.0 \pm 2.8 \mu\text{A}/\text{cm}^2$, $N = 6$), suggesting that capsaicin-sensitive afferent nerves innervate duodenal mucosa and contribute to ion secretion. The response to xenin-8 was significantly reduced 15 minutes after capsaicin pretreatment (Cap-t) due to desensitization. The combination of TTX and capsaicin did not additionally inhibit the secretory

response to xenin, suggesting that capsaicin-sensitive afferent nerves interact with submucosal neurons to stimulate secretion. The TTX-resistant sodium channel Nav1.8 is selectively inhibited by A803467 (Jarvis et al., 2007), which was tested for its effect on xenin-8-induced anion secretion. Pretreatment with A803467 (1 μ M) alone had no effect, but the combination of TTX and A803467 abolished xenin-8-induced anion secretion (Fig. 3B). The local anesthetic lidocaine inhibits sodium channels on TTX-resistant nerves and suppresses colonic secretion and inflammation (Yajima, 1988; McCafferty et al., 1994). Serosal, but not mucosal, treatment with lidocaine (0.5 mM) abolished the secretory response to xenin-8. These results suggest that serosal xenin activates TTX-sensitive and -resistant afferent nerve fibers localized in the submucosal plexus. The L-type Ca²⁺ channel blocker nifedipine (0.1 mM) significantly inhibited the response to xenin-8 (Fig. 3C), but did not affect forskolin- or bethanechol-induced secretion (data not shown), supporting our hypothesis that xenin-8 activates nerves in the submucosal plexus, rather than directly activates epithelial cells.

Neurotransmitters Involved in Xenin-8-Induced Anion Secretion. Acetylcholine (ACh) and vasoactive intestinal peptide (VIP) are major and potent neurotransmitters present in submucosal secretomotor neurons, activating muscarinic ACh receptors and VPAC1 on enterocytes, respectively (Xue et al., 2007). Serosal xenin-8-induced secretion was, however, not altered by the muscarinic antagonist atropine (10 μ M) or the

TABLE 1

Segmental differences of basal I_{sc} and G_t in-Ussing chambered intestinal segments

	Basal I_{sc}	Basal G_t
	$\mu\text{A}/\text{cm}^2$	mS/cm^2
Proximal duodenum	39.6 ± 4.5	41.0 ± 1.9
Distal duodenum	37.2 ± 2.0	41.2 ± 0.8
Jejunum	48.2 ± 12.3	52.8 ± 2.8
Terminal ileum	42.2 ± 10.1	28.5 ± 3.4
Proximal colon	55.1 ± 4.65	18.4 ± 0.8
Distal colon	31.7 ± 5.06	11.5 ± 1.6

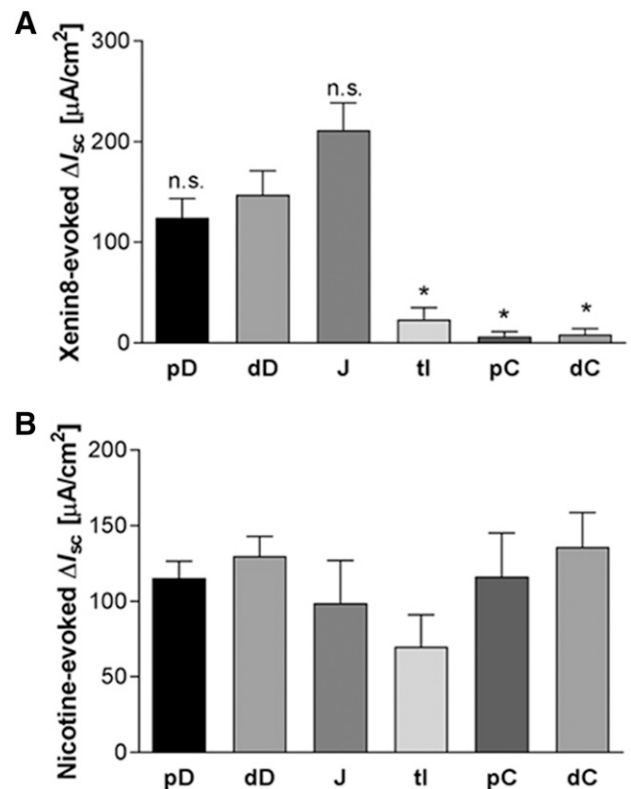


Fig. 2. Segmental heterogeneity in response to xenin-8 (A) and nicotine (B) in Ussing-chambered rat intestinal segments. The I_{sc} peak values in response to serosal xenin-8 (1 nM) or nicotine (0.1 mM) were compared among mucosa-submucosa preparations from proximal duodenum (pD), distal duodenum (dD), mid-jejunum (J), terminal ileum (tl), proximal colon (pC), and distal colon (dC). Each datum represents the mean \pm S.E.M. ($n > 5$). * $P < 0.05$ versus dD, n.s. (not significant) by ANOVA followed by Dunnett's test.

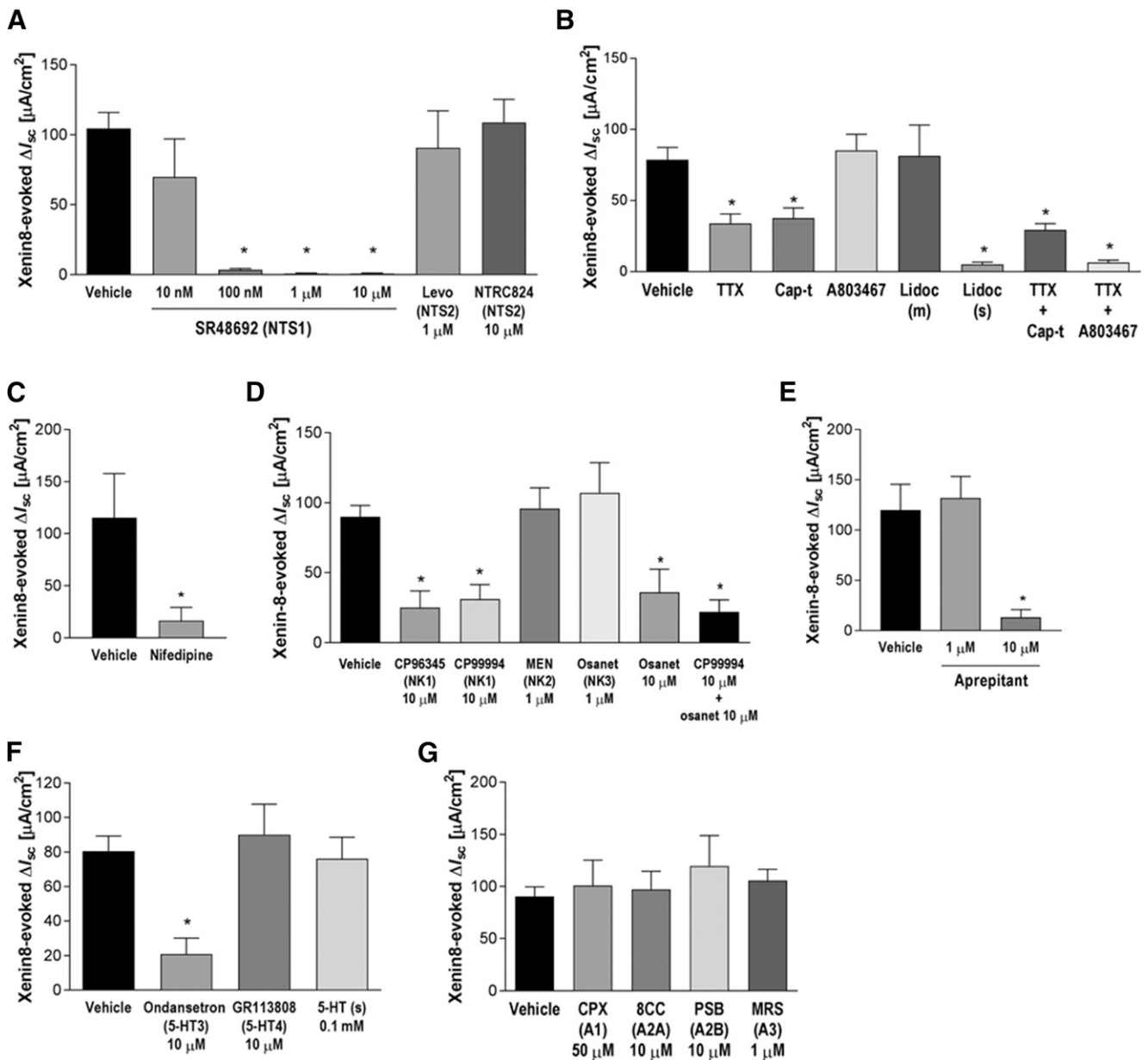


Fig. 3. Effect of selective receptor antagonists and neural sodium channel inhibitors on xenin-8-induced I_{sc} changes. (A) Neurotensin receptor (NTS) 1 antagonist SR48692 or NTS2 antagonists levocabastine (Levo) or NTRC824 was applied to the serosal bath. (B) Tetrodotoxin (TTX, 1 μM), capsaicin (Cap-t, 10 μM), and/or A803467 (1 μM) were applied to the serosal bath. Lidocaine (Lidoc, 0.5 mM) was applied to mucosal (m) or serosal (s) bath. (C) L-type Ca^{2+} channel blocker nifedipine (0.1 mM) was applied to serosal bath. (D) Substance P/tachykinin receptor (NK) 1 antagonist CP96345 or CP99994, NK2 antagonist MEN10376 (MEN), and/or osanetant (Osanet) were applied to the serosal bath. (E) Selective NK1 antagonist aprepitant was applied to the serosal bath. (F) 5-HT₃ receptor antagonist ondansetron, 5-HT₄ antagonist GR113808, or 5-HT was applied to the serosal bath. (G) Adenosine receptor A₁ antagonist (CPX), A_{2A} antagonist (8CC), A_{2B} antagonist PSB603 (PSB), or A₃ antagonist MRS3777 (MRS) was applied to the serosal bath. Each datum represents the mean \pm S.E.M. ($n > 5$). * $P < 0.05$ versus vehicle by paired t test (C) or by ANOVA followed by Dunnett's test (A, B, D–G).

selective VPAC1 antagonist [Ac-His¹, D-Phe², Lys¹⁵, Arg¹⁶, Leu²⁷]-VIP(1–7)-GRF(8–27) (1 μM) (Table 2). Calcitonin-gene related peptide (CGRP) is one of neurotransmitters released from capsaicin-sensitive afferent nerves (Maggi et al., 1986). A selective CGRP receptor 1 antagonist SB268262 [10 μM ; *N*-methyl-*N*-(2-methylphenyl)-3-nitro-4-(2-thiazolylsulfinyl)-benzamide] had no effect on xenin-8-induced I_{sc} increase (Table 2). These results indicated that NTS1-mediated anion secretion was not mediated by ACh, VIP, or CGRP.

Substance P (SP), 5-hydroxytryptamine (5-HT), and nitric oxide (NO) are peptide, monoamine, and gaseous

neurotransmitters, respectively, involved in noncholinergic, nonadrenergic autonomic neurotransmission. To determine the contribution of these transmitters to xenin-induced anion secretion, we tested selective antagonists for tachykinin receptors (NK1, NK2, and NK3), or for 5-HT receptors (5-HT₃ and 5-HT₄), or the NO synthase inhibitor L-NAME for their effects on xenin-8-induced I_{sc} . The NK1-selective antagonists CP96345 [10 μM ; (2*S*,3*S*)-*N*-(2-methoxyphenyl)methyl-2-diphenylmethyl-1-azabicyclo[2.2.2]octan-3-amine] or CP99994 [10 μM ; (2*S*,3*S*)-*N*-[(2-methoxyphenyl)methyl]-2-phenyl-3-piperidinamine dihydrochloride] significantly inhibited

TABLE 2

Xenin-8-evoked I_{sc} increases in the presence or absence of neurotransmitter receptor antagonists or nitric oxide synthase inhibitor

	Vehicle	Treatment
	$\mu A/cm^2$	$\mu A/cm^2$
Atropine	69.5 \pm 7.1	58.7 \pm 7.3
SB268262, 1 μM	88.1 \pm 6.1	111.9 \pm 26.3
SB268262, 10 μM		95.7 \pm 26.6
VPAC1 antagonist	71.4 \pm 15.1	92.1 \pm 22.1
L-NAME	85.0 \pm 20.6	111.2 \pm 24.1

xenin-induced I_{sc} , whereas the NK2-selective antagonist MEN10376 (1 μM ; H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH₂) or the NK3-preferred antagonist osanetant (1 μM) had no effect (Fig. 3D). A high concentration of osanetant (10 μM) or cotreatment with CP99994 and osanetant inhibited xenin-8-induced I_{sc} as same inhibitory extent as CP99994 alone, suggesting that NK1 is mainly involved in xenin-8-induced anion secretion. Another selective NK1 antagonist, aprepitant [5-[[[2R,3S)-2-[(1R)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)-4-morpholinyl]methyl]-1,2-dihydro-3H-1,2,4-triazol-3-one], also abolished the response to xenin-8 at 10 μM (Fig. 3E). Ondansetron (10 μM), a 5-HT₃-selective antagonist, but not the 5-HT₄ selective antagonist GR113808 [10 μM ; 1-methyl-1H-indole-3-carboxylic acid, [1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl]methyl ester], significantly reduced the response to xenin by 65% (Fig. 3F). The response to serosal xenin-8 was not altered by pretreatment with 5-HT (0.1 mM) in the serosal bath, suggesting that 5-HT₃ receptors are not desensitized under these experimental conditions. Although 5-HT mediates neural NO release in guinea pig colon (Kuwahara et al., 1998), serosal pretreatment with L-NAME (0.1 mM) had no effect on the basal and xenin-8-stimulated I_{sc} change (Table 2).

NT-induced Cl⁻ secretion is mediated by adenosine A₁ and A₂ receptors in human colonic mucosa (Riegler et al., 2000), whereas rat duodenal HCO₃⁻ secretion is stimulated by luminal adenosine through A_{2B} receptors (Ham et al., 2010). We thus tested the effect of the purinergic receptor antagonists CPX (50 μM) for A₁ receptors, 8CC (10 μM) for A_{2A} receptors, PSB603 (10 μM) for A_{2B} receptors, and MRS3777 (1 μM) for A₃ receptors on xenin-8-induced Cl⁻ secretion in the Ussing chamber. Using the same concentration of antagonists as in our previous study (Ham et al., 2010), none of these selective antagonists affected the xenin-evoked I_{sc} increase (Fig. 3G). Serosal application of CPX alone increased basal I_{sc} (ΔI_{sc} : 23.6 \pm 4.0 $\mu A/cm^2$), suggesting that A₁ receptors may be activated during stabilization, and an A₁ antagonist unmasks the antisecretory effect of A₁ activation. From these data, we concluded that the adenosine pathway is not involved in xenin-NTS1-mediated Cl⁻ secretion in rat duodenum.

Duodenal HCO₃⁻ Secretion in Response to Intravenous Infusion of Xenin-8. Intravenous infusion of xenin-8 gradually and dose dependently increased the rate of duodenal HCO₃⁻ output (Fig. 4A). Intravenous injection of the NTS1 antagonist SR48692 had no effect on basal HCO₃⁻ output, but inhibited xenin-8-induced HCO₃⁻ secretion (Fig. 4B), suggesting that NTS1 does not contribute to basal HCO₃⁻ secretion in fasted rats, whereas xenin enhances protective duodenal HCO₃⁻ secretion via NTS1 activation in vivo.

Expression of NTS and NK in the Duodenum and Extrinsic Afferent Neurons. Real-time polymerase chain reaction was performed for NTS1, NTS2, NK1, NK2, and NK3 in the isolated duodenal epithelial cells (Ep), in the lamina propria + submucosa including the submucosal plexus (LP-SM), and extrinsic afferent neurons, DRG and NG. NTS1 and NTS2 were detected at very low levels in the epithelial cells, whereas both receptors were expressed in LP-SM containing enteric neural plexuses and in extrinsic afferent neurons (Fig. 5, A and B). In contrast to predominant NTS1-mediated secretion in the Ussing chamber studies, NTS2 expression was abundant in duodenal LP-SM, suggesting that NTS2 may be involved in functions other than anion secretion. All NK receptor subtypes were detected in Ep and LP-SM (Fig. 5, C and D). NK1 receptor expression was significantly higher than NK2 or NK3 in Ep, whereas lower than NK2 in LP-SM (Fig. 5D).

Localization of Xenin in Duodenal Mucosa. Immunohistochemical study of cryostat intestinal sections revealed that a small subset of epithelial cells expressed xenin-25 in the duodenum, but not in the ileum or colon (Fig. 6). Preabsorption, the antibody with xenin-25 abolished the immunoreactivity (data not shown). Xenin-positive cells in the duodenum possessed long narrow apical processes with storage in the basolateral cytosol, suggesting that xenin-25 is expressed in EECs. Double immunostaining demonstrated that xenin-25-immunoreactive cells co-expressed 5-HT, GLP-2, or CCK (Fig. 7, A–C), suggesting that xenin-25 is released from enterochromaffin, L, and/or I cells. Inconsistent with the study in human and canine duodenum (Anlauf et al., 2000), most GIP-containing K cells had no xenin-25 immunoreactivity and vice versa in rat duodenum (Fig. 7D).

Discussion

We demonstrated that the gut peptide hormone xenin induced neurogenic HCO₃⁻ and Cl⁻ secretion through activation of afferent neural reflexes via NTS1, 5-HT₃, and NK1 receptors expressed in rat duodenum. As endogenous xenin was present in a subset of duodenal EECs, postprandial xenin release from the duodenal mucosa may contribute not only to mucosal defense mechanisms but also to satiety signaling via vagal and spinal afferent nerves.

Although ACh and VIP are the major secretagogues for intestinal epithelia in electrically activated submucosal plexus (Krueger et al., 2016), our results showed that serosal xenin failed to activate cholinergic or VIP-ergic neurons. Because the secretory response to xenin was blocked by selective antagonists for NTS1, 5-HT₃, and NK1 receptors and Nav1.8 channels that are generally expressed on the afferent nerves, NTS1 receptors may be specifically expressed on the intrinsic and/or extrinsic primary afferent neurons that release SP. 5-HT-induced duodenal anion secretion is predominantly mediated by epithelial 5-HT₄ in mice (Tuo et al., 2004). Luminal 5-HT also stimulates HCO₃⁻ secretion via 5-HT₄ activation in rat proximal colon (Kaji et al., 2015a). Nevertheless, we here showed that xenin-induced Cl⁻ secretion was partially mediated by 5-HT₃, but not 5-HT₄ activation. In the xenin-evoked neurogenic secretory pathway, 5-HT may not be released from enterochromaffin cells into the lumen as a component of 5-HT₄-mediated secretion. Because 5-HT₃ is expressed in extrinsic afferent neurons (Glatzle et al.,

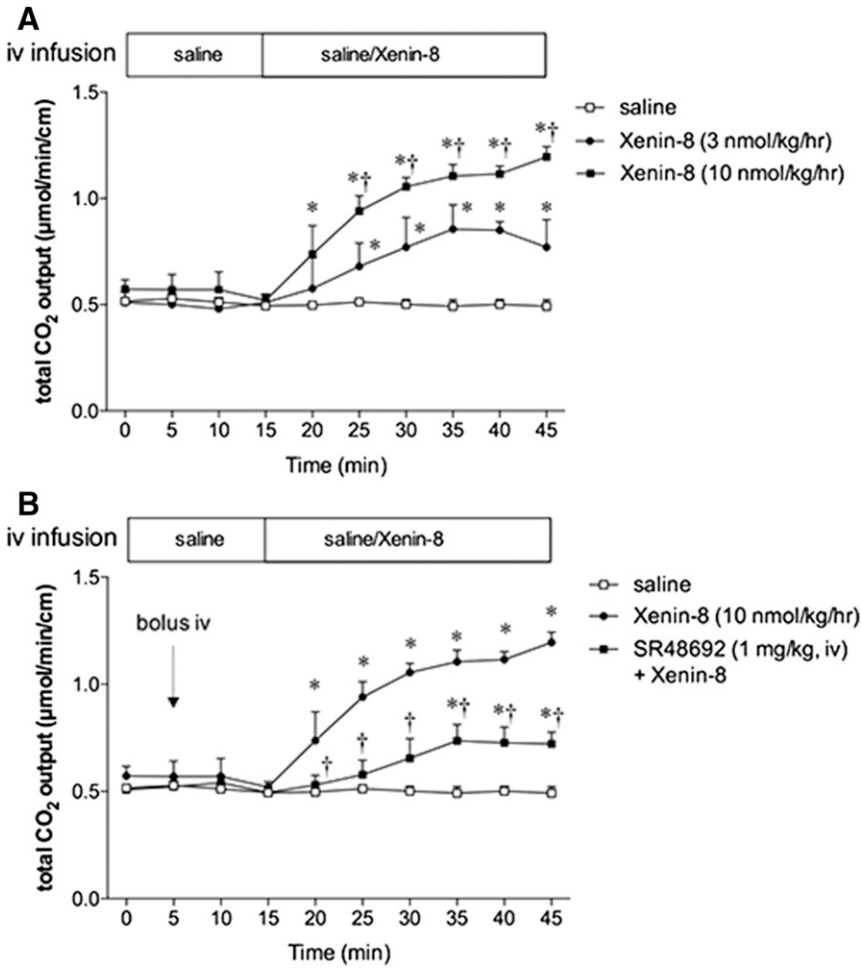


Fig. 4. Effect of intravenous (iv) infusion of xenin-8 and SR48692 on luminal HCO_3^- secretion in the duodenum. A duodenal loop was perfused with pH 7 saline and total CO_2 output was measured in the perfusate with flow-through pH and CO_2 electrodes. (A) Saline with or without xenin-8 was infused into the femoral vein from time = 15 minutes. Intravenous infusion of xenin-8 dose dependently increased total CO_2 output. (B) SR48692 was injected at time = 5 minutes. Bolus injection of SR48692 had no effect on basal HCO_3^- secretion, but significantly inhibited the response to xenin-8. Each data point represents the mean \pm S.E.M. ($n = 6$ rats). * $P < 0.05$ versus saline, † $P < 0.05$ versus another group by two-way ANOVA followed by Tukey's test.

2002; Raybould et al., 2003) and because the serosally applied selective 5-HT₃ agonist SR57227 [1-(6-chloro-2-pyridinyl)-4-piperidinamine hydrochloride] or NK1 agonist Sar-Met-SP increased I_{sc} in a 5-HT₃-dependent manner in the duodenum (unpublished observation), endogenous 5-HT may activate afferent axons via 5-HT₃ receptors, potentiating SP release

during NTS1 activation by xenin, implying that SP is an important stimulant for duodenocytes through activation of NK1 on epithelial cells. Although CGRP may be coreleased with SP from extrinsic afferent nerves, xenin-induced neurogenic secretion was not mediated by CGRP receptors in vitro. It is possible that the secretory effect of released CGRP was

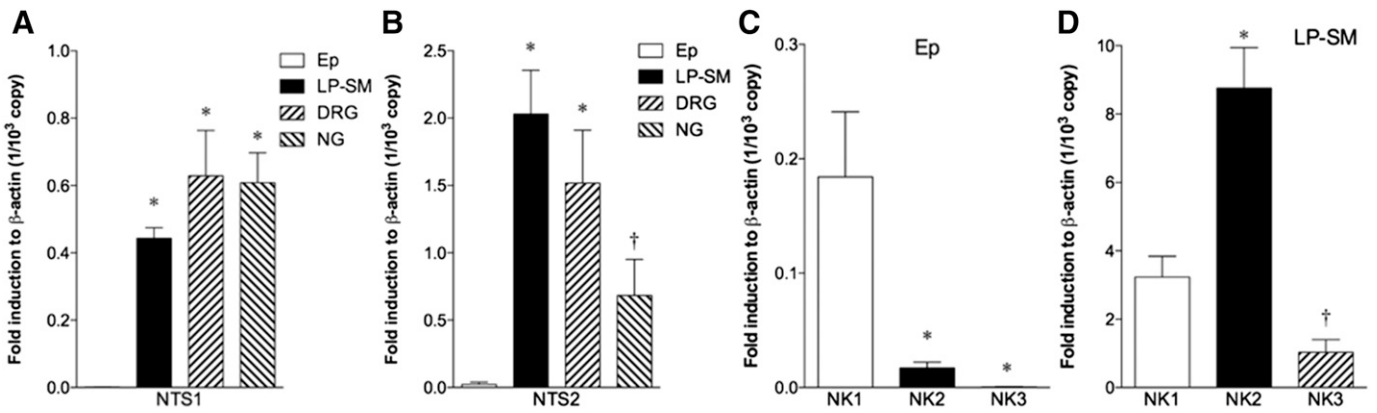


Fig. 5. Expression of mRNA for NTS1, NTS2, and NK receptors in rat tissues determined by real-time polymerase chain reaction. NTS1 (A) and NTS2 (B) expression were compared in isolated duodenal epithelial cells (Ep), lamina propria + submucosa with submucosal plexus from the duodenum (LP-SM), dorsal root ganglia (DRG) and nodose ganglia (NG). * $P < 0.05$ versus Ep, † $P < 0.05$ versus LP-SM by ANOVA followed by Tukey's test. NK1, NK2, and NK3 expressions were compared in Ep (C) and LP-SM (D). * $P < 0.05$ versus NK1, † $P < 0.05$ versus NK2 by ANOVA followed by Tukey's test. Each data represents the mean \pm S.E.M. ($n = 4$).

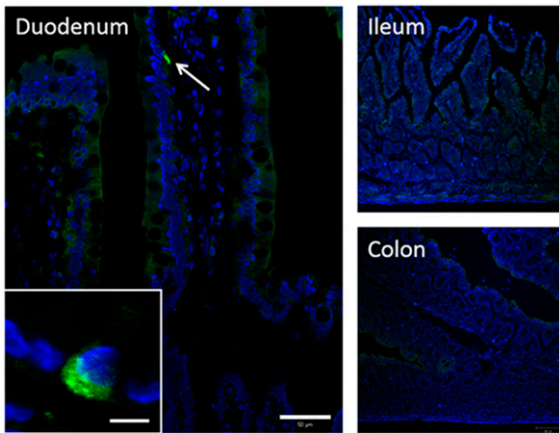


Fig. 6. Immunoreactivity for xenin-25 in frozen sections of rat duodenum, ileum, and proximal colon. Xenin-25 positive cells in duodenal mucosa (arrow and inset, higher magnification: bar, 5 μm) had enteroendocrine-like morphology. Nuclei were counterstained with DAPI (blue). Bar, 50 μm (duodenum) or 100 μm (ileum and colon).

minimal, compared with the effect of released SP in our experimental preparation.

The NK1 selective antagonists CP96345 and CP99994 may directly interact with L-type Ca^{2+} channels (McLean et al., 1993). In addition to these compounds, the L-type Ca^{2+} channel blocker nifedipine also reduced the response to xenin-8, suggesting that L-type Ca^{2+} channel activation in neural tissues mediates xenin-8-evoked anion secretion. Because CP99994 has a considerably lower affinity for Ca^{2+} channels than does CP96345 (McLean et al., 1993), whereas the effects of these two antagonists on the response to xenin were identical, it is likely that the NK1 receptor contributes to xenin-8-induced secretion in the duodenum. Indeed, another type of NK1 antagonist, aprepitant, dose dependently inhibited xenin-8-induced secretion, suggesting that SP-NK1 is downstream of NTS1 activation in xenin-evoked duodenal anion secretion. Although SP is a well-characterized intracellular Ca^{2+} -mediated secretagogue present in intrinsic afferent neurons (Mitsui, 2010), the identity of the physiologic stimulus that releases SP has not yet been identified. Our study revealed that a physiologic concentration of xenin (<1 nM) strongly activated NK1-mediated anion secretion through TTX-sensitive and -resistant pathways, suggesting that NTS1 activation by xenin releases SP from the afferent nerves. In the myenteric plexus, SP is involved in the regulation of motility mediated by cholinergic excitatory motor neurons (Furness et al., 2015). Nonetheless, xenin-induced secretion was independent of the cholinergic secretomotor pathway. The neural circuits of secretomotor and muscle motor functions may differ, although the same neurotransmitter is involved. NK1, as well as NK2 and NK3, are expressed in enteric neurons (Grady et al., 1996), although isolated duodenocytes predominantly expressed NK1, consistent with the functional expression of NK1 in isolated colonocytes (Southwell and Furness, 2001; Hosoda et al., 2002). Although xenin-positive EECs are a tiny subset of all EECs, local SP release may be stimulated by SP itself via NK1 receptors present on the afferent nerves (Mitsui, 2010) and may potentiate the secretory response.

Our immunohistochemical data demonstrated that xenin-25 was present in a part of 5-HT/CCK- or GLP-2-expressing

EECs, respectively termed I cells and L cells. Xenin-25 and GIP immunoreactivities were mostly detected in distinct cells; only few GIP-containing K cells coexpressed xenin-25. The concept of EEC classification was recently reconsidered. Egerod et al. (2012) reported that CCK, secretin, GIP, GLP-1, PYY, and NT but not somatostatin were coexpressed in a novel group of EECs, according to the endocrine cell lineage. Cho et al. (2014) reported that 5-HT and CCK were often coexpressed in EECs of mouse duodenum. Therefore, our observation suggests that EECs expressing multiple gut hormones also express xenin-25, particularly in the duodenum. Further studies are required to fully categorize and identify the particular nutrient receptors expressed in these multihormone cells. As the flow rate of luminal contents is quite rapid in the duodenum (Quon et al., 1989), it is reasonable to hypothesize that multiple gut hormones are released at once in response to luminal nutrients to promptly activate postprandial physiologic responses, including mucosally protective ion secretion, metabolic responses, and satiety signals via hormonal and neural effectors.

Nav1.8 is functionally expressed in extrinsic afferent neurons of the NG and DRG, whereas it is rarely identified in enteric neurons (Miranda-Morales et al., 2010; Gautron et al., 2011). Interestingly, although Nav1.8 inhibition alone had no effect, in combination with TTX, Nav1.8 inhibition abolished xenin-8-evoked anion secretion. Furthermore, TTX or capsaicin pretreatment alone similarly inhibited xenin-8-induced secretion, whereas cotreatment with TTX and capsaicin had no additional effect. These results suggest that xenin-8-induced anion secretion is primarily neurogenic, possibly involving the synergistic activation of TTX-sensitive and -resistant afferent nerves. Our study did not distinguish intrinsic afferent activation from extrinsic afferent activation. Because the capsaicin receptor transient receptor potential vanilloid 1 is expressed on extrinsic afferent nerves and because the secretomotor cholinergic pathway was not involved, our results further suggest that xenin-NTS1-SP pathway mainly involves extrinsic afferent nerve activation. These results suggest that intact extrinsic nerve innervation is required to exert full the effect of xenin.

Luminal adenosine induces duodenal HCO_3^- secretion through the activation of $\text{A}_{2\text{B}}$ receptors expressed on the apical membrane of duodenocytes, whereas A_1 receptors are only expressed in enteric neurons (Ham et al., 2010). Serosal xenin-8-induced Cl^- secretion was not altered by any serosal antagonist for A_1 , $\text{A}_{2\text{A}}$, $\text{A}_{2\text{B}}$, or A_3 receptors, indicating that adenosine receptors expressed on submucosal neurons were not involved in NTS1-mediated Cl^- secretion. Consistent with the previous reports in the colon (Hancock and Coupar, 1995; Cooke et al., 1999), A_1 receptor inhibition increased I_{sc} , suggesting that adenosine or its precursor ATP/ADP/AMP is released in the submucosal plexus and suppresses basal electrogenic secretion in the duodenum. Therefore, luminal and basolateral stimuli may separately and locally regulate epithelial secretory function, by the same mediator via different receptor activation.

Although the precise $t_{1/2}$ of xenin-25 in the circulation is unknown, we speculate that xenin activity as a gut hormone may last longer than other gut peptides, because xenin-derived peptide fragments of varying length have similar affinities to its receptor (Martin et al., 2014). Therefore, xenin is not rapidly deactivated by proteolytic cleavage, implying a

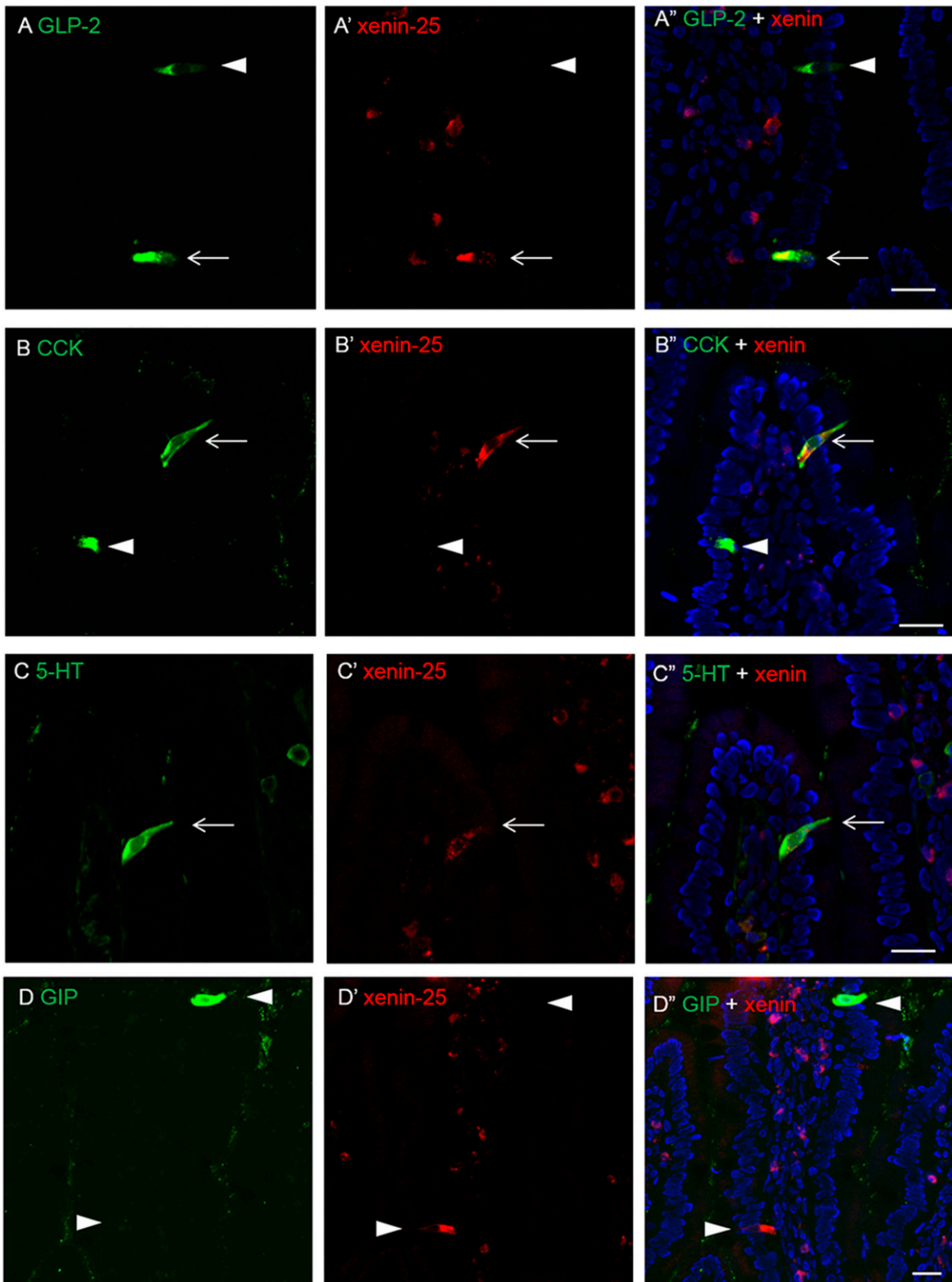


Fig. 7. Double immunostaining for xenin-25 and enteroendocrine cell (EEC) markers in frozen sections of rat duodenum. A part of glucagon-like peptide 2 (GLP-2)-, cholecystokinin (CCK)-, or serotonin (5-HT)-containing cells coexpressed xenin-25 (arrows). Most of gastric inhibitory peptide (GIP)-containing cells were xenin-25-negative. Arrowheads indicate EECs, which are stained with a single marker. Nuclei were counterstained with DAPI (blue). Bar, 20 μ m.

much-extended physiologic $t_{1/2}$, which in turn may influence its function in other tissues, such as the central nervous system and pancreas, in addition to the duodenum. Despite

the presence of NTS1 expression and NT-containing N cells, the secretory response to xenin was significantly lower in the lower intestine compared with the duodenum or jejunum,

suggesting that NTS1 activation is less involved in the secretory response in the lower intestine. NTS1 expression is upregulated in colonocytes obtained from inflamed mucosa (Bossard et al., 2007); furthermore, the expression of NK1 and 5-HT₃ is increased in inflamed mucosal afferent nerves (Utsumi et al., 2016). Segmental differences in NTS1-mediated secretory responses suggest varying function of xenin and NT in the upper and lower intestine, respectively.

Consistent with xenin localization in the upper intestine, the presence of xenin signaling in the duodenum for stimulating mucosal protective HCO₃⁻ secretion further suggests that the xenin-NTS1 pathway, independent of the cholinergic or VIP-ergic pathways, is the alternative pathway that enhances duodenal mucosal defenses. NTS1 activation is implicated in the suppression of tonic pain, consistent with the abundance of NTS1 expression in DRG neurons (Roussy et al., 2008). Long-lasting xenin fragments may be useful for duodenal mucosal protection, appetite control, and antinociceptive therapeutics as a potent, selective agonist for NTS1 receptors on intrinsic/extrinsic afferent neurons.

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Authorship Contributions

Participated in research design: Kaji, Akiba, and Kaunitz.

Conducted experiments and analyzed data: Kaji, Akiba, and Maruta.

Contributed new reagents: Kato and Kuwahara.

Wrote or contributed to the writing of the manuscript: Kaji, Akiba, Kato, Kuwahara, Maruta, and Kaunitz.

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