Peripheral peptide YY inhibits propulsive colonic motor function through Y2 receptor in conscious mice

Lixin Wang, Guillaume Gourcerol, Pu-Qing Yuan, S. Vincent Wu, Mulugeta Million, Muriel Larauche, and Yvette Taché

CURE/Digestive Diseases Research Center, and Center for Neurobiology of Stress, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine, University of California Los Angeles and VA Greater Los Angeles Healthcare System, Los Angeles, California

Submitted 24 August 2009; accepted in final form 2 November 2009

Wang L, Gourcerol G, Yuan P-Q, Wu SV, Million M, Larauche M, Taché Y. Peripheral peptide YY inhibits propulsive colonic motor function through Y2 receptor in conscious mice. Am J Physiol Gastrointest Liver Physiol 298: G45–G56, 2010. First published November 5, 2009; doi:10.1152/ajpgi.00349.2009.—Peptide YY (YY) antisecretory effect on intestinal epithelia is well established, whereas less is known about its actions to influence colonic motility in conscious animals. We characterized changes in basal function and stimulated colonic motor function induced by YY-related peptides in conscious mice. PYY3–36, YY, and neuropeptide Y (NPY) (8 nmol/kg) injected intraperitoneally inhibited fecal pellet output (FPO) per hour during novel environment stress by 90%, 63%, and 57%, respectively, whereas the Y1-prefering agonists, [Pro34]YY and [Leu31,Pro34]NPY, had no effect. Corticotrophin-releasing factor 2 receptor antagonist did not alter YY3–36 inhibitory action. YY and PYY3–36 significantly reduced restraint-stimulated defecation, and PYY3–36 inhibited high-amplitude distal colonic contractions in restrained conscious mice for 1 h, by intraluminal pressure with the use of a microtransducer. YY suppression of intraperitoneal 5-hydroxytryptophan induced FPO and diarrhea was blocked by the Y2 antagonist, BIIE2046, injected intraperitoneally and mimicked by YY3–36, but not [Leu31,Pro34]NPY. YY3–36 also inhibited bethanechol-stimulated FPO and diarrhea. YY3–36 inhibited basal FPO during nocturnal feeding period and light phase in fasted/refed mice for 2–3 h, whereas the reduction of food intake lasted for only 1 h. YY3–36 delayed gastric emptying after fasting-refeeding by 48% and distal colonic transit time by 104%, whereas [Leu31,Pro34]NPY had no effect. In the proximal and distal colon, higher YY mRNA expression was detected in the mucosa than in muscle layers, and YY immunoreactivity was located in nerve terminals around myenteric neurons. These data established that YY/YY3–36 potently inhibits basal and stress-serotonin/cholinergic-stimulated propulsive colonic motor function in conscious mice, likely via Y2 receptors. PYY3–36; stress; serotonin; bethanechol

PEPTIDE YY (YY) is a 36-amino-acid peptide that was first isolated from porcine gut as containing tyrosine (Y) residues at both amino and carboxyl terminals (59). YY shares structural homology with neuropeptide Y (NPy) and pancreatic polypeptide (PP), and together these comprise the so called PP-fold superfamily (41). Gut YY is produced by endocrine L cells in the small and large intestinal mucosa of mammals including humans (14). The peptide exists in two main bioactive forms, namely PYY1–36 and the truncated form, PYY3–36 (21). YY and related members have distinct affinity to the five cloned receptors Y1, Y2, Y4, Y5, and Y6, which belong to the family of G protein-coupled receptors (31, 41).

The well-established biological actions of YY through interactions with Y1 and/or Y2 receptors primarily relate to the inhibition of gastric and small intestinal motility, pancreatic and intestinal mucosa secretion, and intestinal blood flow (10, 41). The peptide administered peripherally delayed gastric emptying in various species including rodents, guinea pigs, dogs, and humans (8, 33, 54). YY is physiologically released into the systemic circulation after a meal when nutrients reach the small intestine and is a part of the “ileal brake” that postprandially inhibits gastrointestinal motility (44, 50).

Interestingly, the highest concentration of YY is found in the colon of humans, dogs, rats, and mice (14, 44). The gene transcripts for Y1, Y2, Y4, and Y5 and immunoreactivity for Y1 receptors have been detected in human and rat colon although with heterogeneity in expression between species (16, 19, 28, 70). The influence of YY in colonic motor function has been largely performed in vitro in muscle strips (17, 27, 48, 55). Superfusion of YY to colonic muscle strips of guinea pig inhibited the twitch contraction induced by electrical stimulation (55) but increased basal colonic contraction in vitro in rats (16, 17, 48), mice (27), and human (16). In vivo studies on propulsive colonic motor function have been scarce and also yielded divergent results. Initial studies showed that intravenous injection of YY and NPY suppressed colonic motility in anesthetized cats (25, 37), whereas subsequent studies in rats show an increase or decrease in colonic motility (9, 62, 63). In animal studies, receptor subtypes involved in YY or NPY actions on colonic motor function are still to be characterized. In addition, although several NPY and Y receptor genetic models have been developed in mice (34), surprisingly there is no report on the influence of YY and related family members in colonic motor function in conscious mice.

Therefore, the objectives of the study were to characterize the actions of intraperitoneal injection of YY-related peptides on propulsive colonic motility in conscious mice under basal or stress-stimulated conditions, including prototypic YY/NPY agonists with differential affinity for Y-receptor subtypes, namely YY (Y2/Y1/Y5), NPY (Y1/Y2/Y5), [Leu31,Pro34]NPY, [Pro34]YY, (preferential Y1 agonists), and PYY3–36 (Y2 agonist) (41, 49). To address whether YY3–36-induced suppression of the colonic response to a novel environmental stress involves activation of corticotrophin-releasing factor receptor 2 (CRF2) inhibitory pathways (42), we used the CRF2 antagonist, astressin2-B (51). Next, we investigated whether YY3–36 and YY inhibitory action can modulate the prokinetic effect of cholinergic and...
serotonergic transmitters (22, 64) using exogenous serotonin (5-HT) precursor, 5-hydroxytryptophan (5-HTP) that mimics a state of endogenous 5-HT production and release (52) and the long-acting muscarinic antagonist, bethanechol. The role of Y2 in mediating PYY inhibitory action was further assessed pharmacologically with the selective Y2 antagonist, BIIE0246 (3, 12), and potential site of action by assessing the expression of Y2 receptor at the gene and protein levels in the mice colon.

MATERIALS AND METHODS

Animals

Male adult C57BL/6 mice (7–12 wk old, body wt 21–30 g, Harlan, Indianapolis, IN) were in group housing (4/cage) and fed ad libitum with standard rodent chow (Prolab RMH 2500; PMI Nutrition International, Brentwood, MO) and water. All the experiments, except otherwise stated, were performed between 9:00 AM and 12:00 PM in freely fed mice. NIH guidelines were followed in all experimental procedures that were undertaken under the auspices of an OLAW Assurance of Compliance (A3002-01) and performed according to approved protocols (IACUC Committee of the VA Greater Los Angeles Healthcare System, numbers 05058-02 and 04056-06).

Substances

Mouse/rat/porcine PYY, mouse/rat/porcine PYY3-36, mouse/rat/human NPY, mouse [Leu31,Pro34] NPY, and astressin2-B were kindly provided by Dr. J. Rivier (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA), and mouse [Pro30]PYY was provided by Dr. J. Reeve (Peptidomics Core, CURE: Digestive Diseases Research Center, UCLA, Los Angeles, CA). Y2 antagonist, BIIE0246, was purchased from Toctis (Ellisville, MO), bethanechol chloride and 5-HTP [L-2-amino-3-(5-hydroxyindolyl)propionic acid] from Sigma Chemical (St. Louis, MO). Peptides, 5-HTP, and bethanechol in powder form were dissolved in saline, astressin2-B in sterile distilled water, and BIIE0246 in vehicle (10% dimethyl sulfoxide, 5% Tween-80 and 85% saline) immediately before the experiments. The volume of intraperitoneal injection was 0.1 ml/mouse.

Acute Stress

Novel environment. Naive mice were taken out from their home cage (group housing) and placed singly in a clean blue-colored and lubricant was introduced into the distal colon 2 cm past the anus and secured to the tail with tape. The colonic contractions were recorded in conscious mice after placing them in the restraint tube. The pressure transducer was connected to a preamplifier (model 600; Millar Instruments). The signal was then amplified using a transducer amplifier (TBM4; World Precision Instruments, Boca Raton, FL), acquired via a Micro1401 A/D interface (Cambridge Electronic Design, Cambridge, MA) and recorded using Spike 2 version 5 data acquisition software. Abdominal contractions were excluded by smoothing the original trace with a time constant of 2 s. The colonic contractile pressure changes were quantified by measuring the phasic component of the intraluminal pressure trace of the area under the curve (pAUC) using online quantifications for every minute. The phasic component of intracolonic pressure was extracted from the original trace by removing the direct current component with a time constant of 10 s from the 2-s smoothed original trace. In addition, the mean pAUC was calculated over the time periods of 0–15 min because the main contractile colonic pressure occurred usually during the first 20 min, as we recently reported under similar conditions (20). In each trace, the number of high-amplitude contractions (>25 mmHg) was calculated. We previously established that they constituted in more than 50% of the cases in conscious mice under similar conditions of recording and bore characteristics of giant migrating contractions (20). The low-amplitude contractions were defined as amplitude comprised between 10 and 25 mmHg. The colonic motility results were expressed as frequency (number of contractions/h) for the first 15 min and 15–60-min periods as previously stated (20). A total of 439 contractions from 16 h of recording were analyzed.

Gastric and distal colonic transit measurements. Gastric emptying of a solid nutrient meal and distal colonic transit were monitored simultaneously in conscious mice as in our previous studies (40). Overnight (16 h) fasted mice had free access to water and preweighed rodent chow for 1 h and then were briefly anesthetized with isoflurane to insert a 2-mm glass bead into the distal colon at 2 cm from the anus using a glass rod with a polished end lubricated with water to avoid tissue damage. Distal colonic transit was determined by monitoring the time when the glass bead was expelled (bead latency). The percentage of gastric emptying of the ingested meal was assessed 2 h after the 1-h refedding as previously detailed (39), and fecal pellets were also counted for the 2-h postfeeding period.

Food intake. Mice were trained in individual cages with a grid about 3 cm above the cage bottom 4 h/day for 3 days under similar conditions used for food intake tests. Food intake was monitored at 1, 2, and 4 h after intraperitoneal injection by measuring the difference between the preweighed standard chow and the weight of chow and spill at the end of each time point as in our previous studies (65).

Colonic Tissue Processing

RNA isolation. The proximal and distal segments of the colon were dissected from naive mice and the mucosa separated from other layers. Total RNAs were extracted with a phenol-quainidine thiochinate-chloroform method (RNA Bee from Tel-Test, Friendswood, TX).

RT-PCR. First-strand oligo-dT-primed cDNA was synthesized from total RNA (5 μg) of each sample by thermostable SuperScript reverse transcriptase at 55°C for 1 h (Invitrogen, Carlsbad, CA). Oligonucleotide primers for Y2 transcripts were forward 5'-GTCATATCGATGACTTGGATTGTGGAG and reverse 5'-AGGGCACCAAATGGCACAAGGCC (23 bp; accession number GenBank D86238); and house keeping gene, S16 forward 5'- TGCCGATGGTTGACGCCTCTGT and reverse 5'- GCTACCCAGCCCTTTGATGA (309 bp, GenBank accession number M11408). RT-PCR was performed in a final volume of 50 μl containing 1 μl of Red-Taq DNA Polymerase System (Sigma). The reaction mixture was performed under the following conditions: predenaturation at 94°C for 2 min and then cycling 34 times (92°C, 30 s; 59°C, 40 s; 72°C, 1 min 30 s) for amplification, followed by a 5-min elongation at 72°C (MyCycler; Bio-Rad Laboratories, Hercules, CA). The amplified PCR products were fractionated by electrophoresis in 1% agarose gel with ethidium bromide.
Experimental Protocols

Whole-mount preparation of colon enteric plexus. The proximal and distal colonic tissues were collected from naive mice, opened longitudinally along the mesenteric border, and processed as previously described (64). Briefly, the stretched tissue was fixed by immersion with 4% paraformaldehyde and 14% saturated picric acid in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. The submucosal layer with the submucosal plexus was peeled off as whole mount. In addition, another whole-mount preparation consisting of the serosa and longitudinal muscle layer with the myenteric plexus attached to its internal side (longitudinal muscle/myenteric plexus, LMMP) was obtained.

Immunohistochemistry for Y2 receptor in enteric plexus. Free-floating submucosal and LMMP whole mounts of both proximal and distal colon from three naive mice were treated in 10% normal goat serum for 30 min and followed by incubation with polyclonal rabbit anti-Y2 serum diluted at 1:1,000 (Neuromics, Edina, MN) for 2 nights at 4°C. After a thorough rinse, the colon whole mounts were incubated in goat anti-rabbit IgG conjugated with FITC (1:100: Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. The tissues were mounted on slides and sealed by cover slides with anti-fading media (Vector Laboratories, Burlingame, CA). For the antibody specificity, controls were performed by preabsorption of primary antibody with the Y2-imunogenic peptide (TDSFSVEATN-COOh; Neuromics) or omission of the primary antibody. For the preabsorption, the Y2 antibody was diluted at working titer (1:1,000), and 20 μg of Y2 immunogenic peptide was added with an antibody:antigen ratio at 1:20. The solution was incubated for 2 days at 4°C. The procedures for immunostaining were the same as above, except either the primary antibody incubation was replaced by preabsorbed or without primary antibody.

The whole mounts were observed by fluorescent microscopy (Axioskop II; Carl Zeiss, Jena, Germany). Images were acquired by a digital camera (Hamamatsu, Bridgewater, NJ) using the image acquisition system SimplePCI (Hamamatsu, Sewickley, PA).

Experimental Protocols

Effects of PYY3–36 and related peptides on novel environment-induced defecation. The effective dose of PYY3–36 was selected on the basis of a dose-response study using intraperitoneal injection of the peptide at 0.8, 2.5, and 8 nmol/kg compared with intraperitoneal saline. In subsequent studies in naive mice, the related peptides, namely PYY, NPY, [Pro31]PYY and [Leu31,Pro34]NPY were injected intraperitoneally at the equimolar effective dose of PYY3–36 (8 nmol/kg), and controls received intraperitoneal saline. Ten minutes after intraperitoneal injection, mice were placed in a novel environment (monitor box) for 1 h, and fecal pellet output (FPO) was monitored at 15-min intervals. To assess whether CRF2 receptor mediated the PYY3–36 inhibitory effect on stress-induced increased FPO, astressin2B (30 μg/kg) or vehicle (sterile water) was injected intraperitoneally 10 min before the intraperitoneal injection of PYY3–36 (8 nmol/kg), and 10 min later mice were exposed to novel environment for 1 h.

Results of PYY3–36 and PYY on restraint stress-induced defecation and colonic contractile activity. Mice were trained for 1 h/day for 3–4 days before the experiments by handling and placing them singly in the monitor box to avoid novel environment response in the control group. On the experimental day, mice were injected intraperitoneally with saline, PYY, or PYY3–36 (8 nmol/kg), and 10 min later they were placed individually in a restraint tube or the monitor box (controls) for 1 h. FPO was assessed for 1 h at 15-min intervals.

To measure distal colonic contractions, naive mice under brief anesthesia were equipped with a miniature transducer inserted into the distal colon and then injected intraperitoneally with PYY3–36 (8 nmol/kg) or saline. Ten minutes later, conscious mice were placed individually in a restraint tube, and changes in colonic intraluminal pressure were recorded for 1 h.

Effects of PYY3–36 or related peptides and Y2 antagonist on 5-HTP and bethanechol-induced defeation and diarrhea. All the mice used in these experiments had been trained to be acquainted to the monitor box, thereby avoiding the influence of novelty stress. Mice were injected intraperitoneally with saline, PYY, PYY3–36, or [Leu31,Pro34]NPY (8 nmol/kg) and 10 min later with saline, 5-HTP (10 mg/kg), or bethanechol (5 mg/kg). In a subgroup, the Y2 receptor antagonist, BIIE0246 (5 mg/kg), or its vehicle (10% DMSO, 5% Tween-80 and 85% saline) was injected intraperitoneally 10 min before intraperitoneal injection of PYY (8 nmol/kg), and 10 min thereafter 5-HTP (10 mg/kg ip) or saline was injected. After 5-HTP or bethanechol injection, mice were placed individually in the monitor box for 1 h, and FPO and diarrhea were monitored hourly for 4 h after the intraperitoneal injection of saline or PYY3–36 (8 nmol/kg), either before the onset of dark phase in nonfasted mice (6:00 PM) or around 9:00 AM in overnight fasted, but not water-deprived, mice.

Statistical Analysis

Results are expressed as means ± SE. Comparisons within multiple groups were performed using one-way ANOVA followed by an all pair-wise multiple-comparison test (Tukey’s test). Comparisons between two groups were performed with Student’s t-test. Correlations were analyzed by linear regression. Values of P < 0.05 were considered statistically significant.

RESULTS

PYY3–36 and Related Peptides Inhibited Acute Stress-Induced Propulsive Colonic Motor Function in Conscious Mice

Novel environment stress. Naive mice injected intraperitoneally with vehicle and placed singly in a novel housing environment had increased FPO compared with mice with intraperitoneal vehicle acquainted to the monitoring boxes. The cumulative FPO at 15 and 60 min was 4.5 ± 0.5 and 8.8 ± 1.0 vs. 1.4 ± 0.4 and 4.4 ± 0.9 in trained mice, respectively (n = 16 in each group; P < 0.05), with a peak response at 15 min (Fig. 1A). Compared with trained mice, PYY3–36 injected intraperitoneally at 8 nmol/kg (30 μg/kg, n = 8) completely...
prevented FPO induced by novelty stress throughout the 1-h monitoring period (0.3 ± 0.2 and 0.9 ± 0.5 at 15 min and 1 h, respectively), whereas lower doses (0.8 or 2.5 nmol/kg) had no significant effect at any time point (Fig. 1B).

PYY (8 nmol/kg, n = 13) injected intraperitoneally also blocked the 15-min peak FPO response (number/h) to a novel environment (0.5 ± 0.3) with defecation resuming thereafter although remaining significantly inhibited (4.6 ± 1.3) compared with the saline group (Fig. 1C). NPY (8 nmol/kg ip, n = 13) induced a lesser inhibitory effect than PYY3–36 both at the peak effective time (15 min) and 1 h (FPO: 1.9 ± 0.6 and 4.2 ± 0.8, respectively, n = 13). The Y1-preferring agonists, [Pro34]PYY and [Leu31,Pro34]NPY (8 nmol/kg, n = 7 in each group) had no significant effect compared with saline injection at any time point (Fig. 1C).

Involvement of CRF2 signaling pathways in PYY3–36 inhibitory action in novelty stress-induced FPO was assessed using the peptide CRF2 antagonist, astressin2-B as pretreatment. Astressin2-B (30 μg/kg ip) had a similar tendency to enhance FPO both in saline- and PYY3–36 (8 nmol/kg ip)-treated mice exposed to novelty stress, and there was no modification of PYY3–36 inhibitory effect on novelty stress-stimulated FPO (n = 6–9/group, Fig. 2).

Restraint stress. All mice used in this experiment had been trained and habituated to the monitor box and displayed only a nonstimulated FPO. In intraperitoneal saline-injected mice, restraint induced a peak response within the first 15 min (FPO/15 min: 7.1 ± 1.2 vs. nonrestraint, 0.6 ± 0.3, n = 7/group, P < 0.05), whereas, thereafter, there was a steady increment of ~2 pellet/15 min resulting in 14.1 ± 1.4 FPO for the 1-h restraint period compared with 3.7 ± 0.7 in control group (Fig. 3). PYY3–36 and PYY (8 nmol/kg ip; n = 8/group) inhibited significantly the restraint stress-induced 15-min peak defecation by 52% and 67% and 1-h FPO by 50% and 50%, respectively (Fig. 3). In nonrestraint mice, both PYY and PYY3–36 values were similar to those of saline group (Fig. 3).

Conscious mice injected intraperitoneally with saline (n = 6) and placed in the restraint tubes displayed low- (10–25 mmHg) and high-amplitude (>25 mmHg) distal colonic contractions (Fig. 4A). Analysis of the first 15-min restraint period indicated a robust transient activation of distal colonic contractions (Fig. 4B). There was an increase in frequency (number/h) of contractions with high (30.9 ± 3.3) and low (29.7 ± 5.2) amplitude compared with those during the remaining 15–60-min period (14.8 ± 2.9 and 11.4 ± 1.9, respectively, P < 0.05; Fig. 4, C and D), which were similar to those previously reported in naive mice (20). PYY3–36 (8 nmol/kg, n = 9) injected intraperitoneally before restraint exposure reduced significantly the frequency (number/h) of high-amplitude contractions threefold during the first 15 min of restraint (11.1 ± 2.6 vs. 30.9 ± 3.3 in saline) and twofold during the subsequent 15–50-min period (7.6 ± 1.2 vs. 14.8 ± 2.9 in saline) (Fig. 4C). The frequency (number/h) of low-amplitude contractions did not change either during the 15-min period (PYY3–36: 22.6 ± 4.2 vs. saline: 29.7 ± 5.2) or the subsequent 45 min

**Fig. 1.** Intraperitoneal peptide YY (PYY) and PYY3–36 inhibit fecal pellet output (FPO) response to novelty environmental stress for 1 h in mice. A: novelty increased defecation in naive mice (n = 16) exposed singly to a novel environment (box) compared with trained mice (n = 16) accustomed singly to the box for 1 h/day for 3–4 days prior. B: dose-response of PYY3–36 on novelty stress-induced defecation. PYY3–36 was injected intraperitoneally 10 min before the stress (n = 7–8/group). C: effect of intraperitoneal injection of neuropeptide Y (NPY)/PYY agonists with different Y receptor selectivity on novelty stress-induced defecation. Peptides (8.0 nmol/kg ip) or saline were injected 10 min before the stress. FPO was monitored every 15 min for 1 h. Data are means ± SE of n = 7–13/group. *P < 0.05 vs. saline at the corresponding time.
(PYY$_{3-36}$: 10.1 ± 3.2 vs. saline: 11.4 ± 1.9; Fig. 4D). The pAUC of distal colonic motility during the first 15 min period was reduced by 46.5% (131.6 ± 19.2 vs. saline: 232.1 ± 26.3 mmHg · min, respectively, $P < 0.05$). The 1-h FPO monitored at the same time as the recording of distal intracolonic pressure was correlated to the number of high ($r^2 = 0.48, P < 0.05$; Fig. 4E) but not to the low-amplitude contractions ($r^2 = 0.08, P > 0.05$, data not shown).

**PYY$_{3-36}$-inhibited Colonic Motor Response to 5-HTP and Bethanechol**

All mice used in these experiments had been acclimated to the monitor boxes to avoid the response to novelty stress. The intraperitoneal injection of 5-HTP (10 mg/kg) alone induced a peak stimulation of FPO within the first 15 min postinjection as we previously described (64). Therefore, results are presented as the cumulative first 30-min response. In saline-pretreated mice, 5-HTP induced a significant increase in FPO/30 min, reaching 10.7 ± 1.3 (n = 10) compared with 3.7 ± 1.0 in saline plus saline-treated group (n = 9, $P < 0.05$). Both PYY$_{3-36}$ and PYY (8 nmol/kg ip) completely blocked the defecation response to 5-HTP (3.1 ± 0.7 and 1.6 ± 0.4, respectively, n = 8 or 7 in each group; P < 0.05 vs. saline plus 5-HTP), whereas the Y$_1$-preferring agonist, [Leu$^{31}$,Pro$^{34}$]NPY, had no effect (number/30 min: 12.4 ± 2.0, n = 7; $P < 0.05$; Fig. 5A). All mice treated with 5-HTP developed diarrhea that occurred mostly within the first 15 min after the injection (Fig. 5B). PYY and PYY$_{3-36}$ both abolished the diarrhea, whereas [Leu$^{31}$,Pro$^{34}$]NPY had no effect (Fig. 5B). The Y$_2$ antagonist, BIIE0246 (5 mg/kg ip), completely prevented intraperitoneal PYY-induced blockade of the 30-min FPO increase (Fig. 5C) and diarrhea (data not shown) in response to intraperitoneal 5-HTP. BIIE0246 injected before 5-HTP resulted in a 32–35% significant reduction of FPO compared with vehicle plus 5-HTP group.

Bethanechol injected (5 mg/kg ip) in saline-pretreated mice induced diarrhea and increased significantly FPO in the first 15 min compared with saline plus vehicle (8.6 ± 1.1 vs. 0.9 ± 0.3 pellets, n = 7 in each group; $P < 0.05$, Fig. 6). The FPO reached a plateau response at 30 min (Fig. 6A). PYY$_{3-36}$ (8 and 25 nmol/kg ip) pretreatment resulted in a dose-related suppression of bethanechol-induced stimulation of propulsive colonic motor function (pellets/15 min: 5.9 ± 1.4 and 3.5 ± 0.6, respectively, n = 7 or 8 in each group), and there was a 100% inhibition of FPO and diarrhea response at 1 h postinjection with the highest dose of PYY$_{3-36}$ (Fig. 6).

**Time Course of PYY$_{3-36}$ Inhibitory Effects on Food Intake and Gut Motor Function**

*Food intake and FPO.* PYY$_{3-36}$ (8 nmol/kg) injected intraperitoneally inhibited food intake response to an overnight fast by 27.0% only for the first hour compared with saline and no longer during the subsequent 3-h period (Fig. 7A). By contrast, the peptide significantly inhibited FPO/h monitored simultaneously by 100%, 87.5%, and 82.8% during the first 3 h postinjection, respectively, with a return to control values during the fourth hour (Fig. 7C). Likewise, PYY$_{3-36}$ injected (8 nmol/kg ip) in freely fed mice at the onset of the dark phase significantly reduced food intake by 36.4% during the first hour only, whereas there was a significant 77.9% and 57.7% inhibition of FPO during the first and second hour periods postinjection, respectively (Fig. 7, B and D).

*Gastric and distal colonic transit.* In overnight-fasted and 1-h freely refed mice, PYY$_{3-36}$ injected intraperitoneally (8 nmol/kg) reduced by 48% the amount of food emptied from the stomach (Fig. 8A) and slowed the distal colonic transit time as shown by the 104% increase in the time at which the intracolonic bead was expelled compared with saline (Fig. 8B). By contrast, [Leu$^{31}$,Pro$^{34}$]NPY had no significant effect on gastric and colonic transit under the same conditions (Fig. 8, A and B). There was also a significant 77% reduction of 2-h FPO induced by PYY$_{3-36}$ that was not observed with the Y$_1$-preferring agonist (Fig. 8C).
Y2 Receptor Expression in Mouse Colon

Y2 receptor transcript was detected by RT-PCR both in the mucosa layer and submucosa plus muscle layers with a higher signal in the mucosa than in the other layers in both the proximal and distal colon, as well as a higher signal in the distal than proximal colon (Fig. 9, A and B). Immunofluorescent staining revealed the localization of Y2 receptor in the submucosal (Fig. 9 D) and myenteric plexi (Fig. 9 E) in whole-mount submucosal layer and LMMP preparations of both proximal and distal colon. The labeling was found mainly in nerve terminals shown as varicosity around neurons (Fig. 9, D and E) and running between ganglia. Incubation of whole-mount preparation of proximal and distal colon with primary antiserum preadsorbed with the immunogenic Y2 receptor peptide resulted in complete disappearance of immunostaining (Fig. 9 C), as well as when the Y2 antibody was omitted (data not shown).

DISCUSSION

The present study demonstrates that mouse PYY3–36 injected intraperitoneally completely suppressed the stimulated colonic propulsive response to the stress of a novel housing environment or restraint and the secretory-motor responses to peripheral injection of 5-HTP and cholinergic agonist (bethanechol) as assessed by monitoring defecation, diarrhea, distal colonic contractions, or distal colonic transit in conscious mice. PYY3–36 injected intraperitoneally also inhibited basal defecation occurring during the nocturnal feeding period and the refeeding following an overnight fast. To our knowledge, the present findings provide the first evidence of an inhibitory action of PYY3–36 on propulsive colonic motor function in conscious mice. There was only one in vivo study that reported reduced basal colonic myoelectrical activity in conscious rats after intravenous injection of PYY as assessed by the decrease in the total number of spike bursts compared with controls (9). In our study, PYY3–36 injected intraperitoneally significantly reduced the frequency of high-amplitude contractions in the distal colon throughout the 1-h restraint conditions in conscious mice monitored using a novel noninvasive manometric method (20). We recently characterized these high-amplitude contractions to be giant migrating contractions (20) that were found in the present study to be correlated with fecal output. By contrast, other studies in anesthetized rats and rabbits showed that intravenous injection of NPY or PYY increased colonic contractile activity (38, 63). The existing and present data may indicate that the state of the animal conditions, conscious vs. anesthetized, influences PYY- and NPY-induced colonic motility alterations, resulting in an inhibitory or stimulatory effect, respectively.

We compared the inhibition of colonic motor function induced by intraperitoneal PYY3–36 with its well-established inhibitory effects on upper gut transit and food intake (4, 8, 30, 66). We found that PYY3–36 injected intraperitoneally at 8 nmol/kg resulted in a more robust suppression of propulsive colonic than gastric motor function or food consumption as monitored simultaneously in conscious mice. This was shown by the greater magnitude (78%-58%) inhibition of defecation occurring during the first 2 h of the nocturnal free feeding,
A. 30-min FPO after PYY, Y1 agonist, [Leu31,Pro34]NPY, and Y2 agonist, PYY3–36 (8 nmol/kg), or saline were injected intraperitoneally 10 min before 5-HTP (10 mg/kg ip); n = 7–10/group. *P < 0.05 vs. saline-saline; #P < 0.05 vs. saline-5-HTP. B: diarrhea score from the same mice in B. *P < 0.05 vs. vehicle-saline-5-HTP; #P < 0.05 vs. vehicle-saline-5-HTP. C: PYY-induced suppression of 30-min stimulated defecation induced by intraperitoneal 5-HTP in mice, with reversal by the Y2 antagonist, BIIE0246. BIIE0246 (5 mg/kg) or vehicle was injected intraperitoneally at 20 min before and PYY3–36 (8 nmol/kg) 10 min before 5-HTP (10 mg/kg) or saline in mice (n = 6–10/group). Data are means ± SE. *P < 0.05 vs. vehicle-saline-5-HTP; #P < 0.05 vs. vehicle-saline-5-HTP.

whereas the decrease in food intake was 36% and lasts only the first hour after PYY3–36 injection. Moreover, concurrent monitoring of distal colonic transit, defecation, and gastric emptying of solid food in conscious mice showed that intraperitoneal PYY3–36 induced a higher percentage of inhibition of distal colonic transit (104%) and defecation (77%) than gastric emptying (48%). In addition, there was a long-lasting suppression of giant migrating contractions in the distal colon induced by intraperitoneal PYY3–36 throughout the 1-h restraint period compared with saline group. The intraperitoneally effective dose (8 nmol/kg) at which PYY3–36 completely suppressed basal- and stress-stimulated colonic motor function was lower that the reported dose (50 μg/kg, about 12 nmol/kg) inducing maximal activation of medullary brain neurons and reduction of dark phase food intake in mice (23, 66).

We used a pharmacological approach to ascertain the role of Y2 receptors in mediating PYY3–36 inhibitory actions on the colon. Convergent data support such a contention. First, binding studies indicate that PYY3–36 is a relatively selective agonist for the Y2 receptor (13, 31, 41). Second, differential results were obtained with prototypic NPY/PYY agonists with differential binding affinity to the Y2 receptors when peptides were injected intraperitoneally at the effective equimolar dose of PYY3–36. In particular, we showed that the preferential Y1 agonists, [Leu31,Pro34]NPY or [Pro34]PYY (41, 47), were unable to elicit any significant changes in distal colonic transit and defecation during the refeeding response after a fast and in the stimulated defecation induced by 5-HTP and a novel environment. In contrast, PYY that displays Y2/Y1/Y5 receptor affinity (31, 41, 57) inhibited defecation stimulated by exposure to a novel environment, restraint, and peripheral injection of 5-HTP. NPY, which also displays binding affinity to the Y2 receptor (31, 47), significantly inhibited the colonic response to novel environment although less prominently than PYY or PYY3–36, which display PYY-prefering profile (31, 47) with enhanced Y2 receptor effect in many tissues (18). Third, the highly selective nonpeptide Y2 antagonist, BIIE0246 (3, 12), injected intraperitoneally blocked PYY-induced defecation in response to 5-HTP. There was a 32–35% decrease in fecal output after BIIE0246 treatment in 5-HTP-treated mice, which could reflect a partial agonistic effect. Fourth, we found that PYY and PYY3–36 blocked 5-HTP- and bethanechol-induced diarrhea, whereas [Leu31,Pro34]NPY under the same conditions was not able to affect the diarrhea response to 5-HTP. Moreover, the selective Y2 antagonist, BIIE0246 (12), blocked PYY-induced suppression of 5-HTP-related diarrhea. Taken together, these data suggest that the activation of Y2 receptor is the predominant Y receptor subtype involved in PYY- and PYY3–36-induced suppression of basal- and stress-stimulated colonic motor function and diarrhea induced by muscarinic and serotonergic activation in conscious mice.

Previous studies in mice established that the decrease in gastric emptying and food intake induced by intraperitoneal injection of PYY3–36 is Y2 mediated, respectively, through vagal-dependent and -independent recruitment of specific brain circuitries (23, 58, 66). Nonaka et al. (43) have reported that PYY3–36 crosses the blood-brain barrier by nonsaturable process, and PYY3–36 injected intraperitoneally at a similar dose as used in the present experiment induced neuronal activation in some brainstem nuclei of mice (23). However, in the present study, functional and neuroanatomical evidence supports that the suppression of colonic function induced by intraperitoneal PYY3–36 may be exerted through a direct effect on Y2 receptor expressed in the colon. Previous in vitro studies using preparations of mouse colonic mucosa with intact submucosal...
plexus innervation showed that PYY3–36 inhibited electrogenic ion secretion stimulated by vasoactive intestinal peptide, and the response was completely abolished by the Y2 antagonist, BIIE0246, in wild-type mice and not observed in colonic mucosal tissue from Y2 knockout mice (27). Additionally, in this isolated colonic mucosa/submucosal plexus preparation, the Y2 receptor antisecretory action is neurally mediated predominantly through modulation of submucosal enteric transmission by prejunctional Y2 receptor and, to a lesser extent, Y2 receptors on noncholinergic submucosal neurons (26, 27). Consistent with the functional evidence of the presence of Y2 receptor in mice colon, we showed the expression of Y2 receptors at the gene and protein levels that was not established before in the mice colon. In particular, we found that Y2 receptor mRNA expression in mucosa and submucosa/muscle layers displayed a more prominent density in the distal than proximal colon. Immunohistochemistry of colonic whole mount revealed Y2 labeling in nerve fibers and terminals forming varicosities around enteric neurons along with a suggestive localization within enteric neurons. The Y2 immuno-

Fig. 6. Dose-related action of intraperitoneal PYY3–36 on bethanechol-induced stimulation of FPO (A) and diarrhea (B) in mice. PYY3–36 (8 and 25 nmol/kg) or saline was injected intraperitoneally 10 min before intraperitoneal bethanechol chloride (5 mg/kg) or saline. FPO was monitored every 15 min for 1 h. Data are means ± SE of n = 7/group. *P < 0.05 vs. saline-saline; #P < 0.05 vs. saline-bethanechol.

Fig. 7. Time course of PYY3–36-induced inhibition of food intake (A and B) and fecal output (C and D) monitored simultaneously hourly for 4 h in fasted/refed mice (A and C) or in response to dark phase in freely fed mice (B and D). Data are means ± SE of n = 8/group. *P < 0.05 vs. saline.
CRF₁ receptor-mediated activation of colonic myenteric neurons contributes to stress-related defecation response, whereas activation of CRF₂ receptors by intraperitoneal injection of the endogenous CRF₂ ligand, urocortin 2, blocked novel environment stress-induced defecation in rodents (42). However, the CRF₂ antagonist, astressin₂-B, injected intraperitoneally, at a dose previously established to completely prevent gut motor alterations induced by intraperitoneal urocortin 1 in mice (51), did not alter the magnitude of PYY₃–₃₆ inhibitory effect on novel environment-induced defecation. These data indicate that activation of Y₂ receptors by intraperitoneal PYY₃–₃₆ does not recruit CRF₂ inhibitory signaling pathways in the colon under stress.

There is indisputable evidence that the activity of myenteric neurons is essential for induction of motility patterns associated with propulsive motor function (67). Indirect evidence indicates that PYY₃–₃₆ inhibitory action on colonic motor function may involve modulation of serotonergic and muscarinic enteric transmission. A direct action on colonic smooth muscles is unlikely based on a report that, in an isolated mouse colonic longitudinal smooth muscle preparation, PYY₃–₃₆ and NPY exclusively elicit a Y₂-mediated tetrodotoxin-resistant excitatory contractile response (27). We previously characterized in conscious mice that the rapid onset defecation and diarrhea induced by intraperitoneal 5-HT₄ agonists involves a 5-HT₄ receptor-mediated activation of colonic cholinergic and nitric myenteric neurons (64). Restraint stress in mice also activates colonic myenteric neurons; the stimulation of motility is atropine sensitive (20), and defecation response is blocked by 5-HT₄ antagonists (53). The prokinetic effect of 5-HT₄ receptor activation in mice is suggested to be linked with presynaptic inhibition or strengthening of excitatory neurotransmission (35). In the present study, we showed Y₂ receptor labeling on colonic myenteric/submucosal nerve terminals around enteric nerves, consistent with a presynaptic site of action for the Y₂-mediated inhibitory effects. Supportive of such a contention, electrophysiological studies indicate that PYY and Y₂ agonist exert profound presynaptic inhibition of cholinergic transmission in myenteric neurons of guinea pig colon (5). Lastly, we found that PYY₃–₃₆ suppressed the betahanechol-induced diarrhea and increased fecal output in mice. Although the defecation and diarrhea score induced by betahanechol and 5-HTP were of similar magnitude, a threefold higher dose of PYY₃–₃₆ was required to achieve full blockade. These data suggest that Y₂ inhibitory mechanisms have a higher sensitivity toward 5-HT₄ than muscarinic receptor-initiated stimulation of colonic secretory motor function. In addition, the data indicate that PYY₃–₃₆ is able to interfere with muscarinic receptor activation of secretory motor processes, which are known to be mediated by both enteric and direct action on colonic epithelial cells (11). However, it cannot be ruled out that PYY- or PYY₃–₃₆-induced suppression of colonic motility also encompasses a more complex neural circuitry involving a primary action on sensory afferents, bearing similarity with that established in small intestinal transit inhibition (61).

In PYY₃–₃₆ doses ranging from 0.8 to 8.0 nmol, the inhibition of stimulated defecation induced by novel environment was not dose related, as shown by the lack of effect of 10-fold or threefold lower doses than the maximal effective dose. Likewise, other reports showed that PYY₃–₃₆ injected intra-
peritoneally at 3–4 doses ranging from 5 to 240 nmol/kg did not result in a clear dose-response suppression of food intake in mice (1, 24, 66), indicative that PYY3–36 may induce all-or-none actions to some extent. The intraperitoneal effective dose most likely results in supraphysiological PYY levels in the circulation, as postprandial plasma concentration of PYY in rats varies from 12 to 93 pmol and postprandial levels are reproduced by the intravenous infusion of PYY at 50–100 pmol/kg/h in rat (29, 44). However, it cannot be ascertained whether PYY3–36 at 8 nmol/kg injected intraperitoneally results in colonic concentrations similar to those induced by local postprandial release of PYY, which is highly expressed in colonic L cells (44), along with the Y2 immunoreactivity in the submucosal and myenteric plexi, with sympathetic nerve stimulation in the colon near Y2 receptors (70). Although the pathophysiological significance of these observations still remains to be established, it can be speculated that the postprandial colonic release of PYY (44) may play a role in dampening the gastrocolic peristaltic reflex. Of interest are also clinical studies showing that patients with chronic idiopathic slow-transit constipation display an increase of PYY cells in the colon, which was commented to be potentially one of the causes of the disease (15).

In conclusion, using pharmacological approaches we demonstrated that intraperitoneal administration of PYY3–36 or PYY exerts Y2 receptor-mediated sustained inhibitory effects on basal- and stress-stimulated colonic propulsive motor function in conscious mice. We found that Y2 activation resulted in the suppression of high-amplitude, but not low-amplitude, contractions in the distal colon that we previously characterized to be giant migrating contractions related to the colonic propulsion and defecation (20). PYY3–36 also inhibited defecation and diarrhea induced by exogenous activation of serotonergic and muscarinic receptors. The inhibitory effect on colonic transit and defecation was of greater magnitude and duration compared with that of gastric emptying or food intake monitored simultaneously. The demonstration of gene expression of Y2 receptor in both proximal and distal colonic mucosal and submucosal and muscle layers, along with the Y2 immunoreactivity in the submucosal and myenteric plexi, suggests a direct action in the colon that may involve modulation of enteric neuronal transmission although reflex circuitry cannot be ruled out. Peripheral injection of PYY3–36 in mice may provide a new model of constipation-like disorders to investigate underlying mechanisms of decreased bowel motor function. The mice and human colon contain the highest expression of PYY compared with other gut segments (2). It remains to be established whether the PYY-Y2 receptor signaling has relevance in modulating the gastrocolic reflex and chronic idiopathic slow-transit constipation associated with increased colonic PYY cells (15) or other functional pathology linked with alterations of colonic secretory motor function.

ACKNOWLEDGMENTS

We thank Mrs. Honghui Liang for expert technical assistance, Dr. Joseph Reeve (Peptidomics Core, CURE, UCLA, Los Angeles, CA) for the generous supply of [Pro34]PYY, and Dr. Jean Rivier (Salk Institute, Peptide Laboratories, La Jolla, CA) for the generous gift of the other peptides.

GRANTS

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases R01 grant DK-57238 (Y. Taché), Center grant DK 41301 (Peptidomics core and Animal Model core), and DK078676 (M. Million), and VA Senior Career Scientist Award (Y. Taché).

DISCLOSURES

No conflicts of interest are declared by the author(s).
REFERENCES


