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Effects of neurotransmitters, gut hormones, and inflammatory mediators on mucus discharge in rat colon

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¹*Institut National de la Santé et de la Recherche Médicale U-45, Hôpital Edouard Herriot, 69347 Lyon Cedex 03; and* ²*Laboratoire d'Ecologie et de Physiologie du Système Digestif, Institut National de la Recherche Agronomique, Centre de Recherche de Jouy-en-Josas, 78352 Jouy-en-Josas, France*

Plaisancié, Pascale, Aline Barcelo, Frederic Moro, Jean Claustre, Jean-Alain Chayvialle, and Jean-Claude Cuber. Effects of neurotransmitters, gut hormones, and inflammatory mediators on mucus discharge in rat colon. *Am. J. Physiol.* 275 (Gastrointest. Liver Physiol. 38): G1073–G1084, 1998.—The effect of potential mediators of mucus secretion was investigated in the isolated vascularly perfused rat colon by using a sandwich enzyme-linked immunosorbent assay for rat colonic mucin and by histochemical analysis. Bethanechol (100–200 μ M), bombesin (100 nM), and vasoactive intestinal peptide (VIP, 100 nM) provoked a dramatic mucin discharge (maximal response at 900, 900, and 600% of control loops, respectively). VIP-stimulated mucin secretion was abolished by tetrodotoxin, whereas atropine was without effect. In contrast, both tetrodotoxin and atropine significantly decreased mucin release induced by bombesin. Isoproterenol or calcitonin gene-related peptide was without effect. Serotonin (1–5 μ M) and peptide YY (10 nM) evoked mucin discharge, whereas glucagon-like peptide-1 did not release mucin. Finally, bromolasalocid (20 μ M), interleukin-1 β (0.25 nM), sodium nitroprusside (1 mM), and dimethyl-PGE₂ (2.5 μ M) induced mucus discharge. The results demonstrated a good correlation between the immunological method and histological analysis. In conclusion, these findings suggest a role for the enteric nervous system, the enteroendocrine cells, and resident immune cells in mediation of colonic mucus release.

colonic mucin release; enzyme-linked immunosorbent assay; cavitation; isolated colon

THE INTESTINAL MUCOSA is covered by a mucus gel layer that plays important physiological roles including lubrication, protection against colonization by pathogenic bacteria and their toxins, protection against luminal proteases arising from bacterial and mucosal cells, and constitution of a diffusion barrier for small molecules (11). In the large bowel, mucus also represents a nutrient for the strict anaerobic bacteria. The major structural components in mucus are mucins, which are high molecular-weight glycoproteins produced by goblet cells of the surface epithelium (11). These cells store mucin in apically located granules that are secreted at a slow baseline rate to maintain the mucus coat over the epithelium. In response to stimulation, intestinal goblet cells may dramatically accelerate their discharge of mucins by way of two processes (10, 32). In many mucus cells, a phenomenon of compound exocytosis is induced, resulting in deep apical membrane cavitation that is visible under the microscope. Some intestinal mucus cells can also respond to stimulation by another process, presumably by accelerating single-

granule exocytosis. Although mucus plays a central role in mucosal protection, factors that govern this discharge of mucus are not fully defined, particularly in the colon.

Colonic mucus cells are located in the vicinity of enteric nerves, enteroendocrine cells, and resident immune cells, which suggests a potential role of neurotransmitters, hormonal peptides of the large bowel, and inflammatory mediators. This hypothesis is supported by the observation that cholinergic agents stimulate colonic mucus discharge both in vivo (31) and in mucosal explants (24). Experiments performed with several colonic mucus-secreting cell lines also showed that carbachol (19, 21), vasoactive intestinal peptide (VIP) (21), nitric oxide (NO) (3), interleukin (IL)-1 (18), and PGE₂ (26) stimulate colonic mucin discharge. However, the colonic wall contains many other candidates to influence mucus secretion.

The present study was then undertaken to investigate in detail the influence of various neurotransmitters, hormonal peptides, and inflammatory mediators on colonic mucin secretion. For this purpose, we used the preparation of isolated vascularly perfused rat colon, a new model to study mucin secretion. In this preparation, mucus cells keep their physiological orientation and environment and are submitted to well-defined luminal, neural, and blood-borne stimuli in a manner that eliminates influences potentially encountered in vivo. A sandwich ELISA was developed to measure accurately mucin released from isolated vascularly perfused rat colon. Discharge of mucus granule was also evaluated by histological analysis. These experiments indicate that colonic mucus cells can respond to different classes of mediators produced by nerves, enteroendocrine cells, and the immune system.

MATERIALS AND METHODS

Materials

The following reagents were purchased from Sigma (Saint Quentin-Fallavier, France): guanidinium hydrochloride, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Tris, orcinol, bovine thyroglobulin, gum arabic, proteinase K, biotin disulfide *N*-hydroxysuccinimide ester, polyoxyethylene-sorbitan (Tween 20), α -phenylenediamine dihydrochloride tablets (OPD), bovine albumin (BA, lyophilized powder), avidin-peroxydase conjugate, bethanechol, isoproterenol, calcitonin gene-related peptide (CGRP) (rat CGRP-I), peptide YY (PYY), glucagon-like peptide-1-(7–36) amide, serotonin, VIP, bombesin (BBS), sodium nitroprusside (SNP), IL-1 β , 16,16-dimethyl-PGE₂ (dmPGE₂), TTX, and atropine. EDTA was supplied by Merck (Nogent-sur-Marne, France). Bromola-

salocid was kindly provided by Roche Discovery Welwyn (Welwyn Garden City, England). Electrophoresis products were purchased from Bio-Rad (Ivry sur Seine, France). Cesium chloride (CsCl) and DIG Glycan detection kit were supplied by Boehringer Mannheim (Meylan, France). BSA (30% solution) was purchased from Biovalori (Cassens, France). Hyperamine 25, a mixture of amino acids, was obtained from Braun medical (Boulogne, France).

Methods

ELISA for rat colonic mucin. PURIFICATION OF RAT COLONIC MUCIN. Male Wistar rats (Centre d'Élevage Dépré, Saint Doulchard, France) were killed by a lethal dose of pentobarbital sodium. Colonic mucins were then isolated from mucosal scrapings, essentially as previously described by Tytgat et al. (33, 34). Briefly, scrapings were homogenized in the dark for 24 h at 4°C in homogenization buffer, pH 7.5 (6 M guanidinium hydrochloride, 50 mM Tris, 1 mM PMSF, 5 mM EDTA, 100 mM DTT). Sulfhydryl groups were carboxymethylated by adding 250 mM iodoacetamide in the dark for another 24 h. Mucins were then purified by equilibrium centrifugation on three consecutive CsCl density gradients (Centrikon T-2055 ultracentrifuge, TFT 50.38 rotor, 100,000 *g*, 70 h at 12°C). The resulting gradients were fractionated into 12 equal fractions. For analysis, the fractions were dialyzed extensively against distilled water (4°C, 24 h) and then assayed for hexoses using an orcinol assay at 540 nm (12). Protein content was measured by the method of Bradford (Bio-Rad), and nucleic acid detection was performed by absorbance at 260 nm. The high molecular-weight nature of purified mucin was verified by SDS-PAGE and Western blot using an immunological detection system for glycoproteins (DIG Glycan detection kit) (14).

PREPARATION OF POLYCLONAL ANTISERUM AGAINST RAT COLONIC MUCIN. Antisera against rat colonic mucin (RCM), obtained in rabbits after repeated injections of the purified mucin (250 µg), were tested for the presence of anti-rat colonic mucin antibody (anti-RCM) by performing dot-blot immunoassay. Briefly, serial dilutions (from 200 to 6.25 µg/ml) of purified mucin, BA, bovine thyroglobulin, or gum arabic were blotted on nitrocellulose. Blots were incubated with anti-RCM (1:1,000 or 1:5,000). This was followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1,000). Blots were then developed using the electrochemiluminescence method (Pierce, Rockford, IL).

The polyclonal antiserum was then studied for epitope specificity. For this purpose, purified mucin was digested with 0.1 mg/ml proteinase K in 50 mM Tris, pH 7.4 (1 h, 37°C), to test the reactivity toward protease-sensitive epitopes. The digested mucin was blotted on nitrocellulose and processed as described previously.

Determination of specific antibody titer in polyclonal antiserum was performed by dot blot with purified mucin. The highest titer was identified by the intensity of the signal. With the use of the serum with the highest antibody titer (45C), an immunoglobulin-rich fraction was prepared using DEAE-Sephadex A50 (pH 7.2). This was used to coat ELISA plates (as described in ELISA PROCEDURE) and was referred to as coating antibody 45C (c-45C). A part of the immunoglobulin-rich fraction was also used to prepare enzyme-labeled antibody (biotinylated-45C) for the ELISA using a succinimide ester of biotin (13).

IMMUNOHISTOCHEMISTRY. Recognition of mucin by anti-RCM was studied histologically. Sections (5 µm) of paraffin-embedded colonic tissue were incubated with diluted normal blocking serum and then with anti-RCM antiserum (1:1,000 dilution). After washing in PBS, sections were sequentially

exposed to biotinylated goat anti-rabbit antibody, avidin-biotinylated peroxidase complex, and 3,3'-diaminobenzidine solution. All the reagents were purchased from Vector Laboratories, Burlingame, CA. The sections were then counterstained, cleared, and mounted.

ELISA PROCEDURE. Wells of a microtiter plate (NUNC-Immuno Plate; Polyabo Paul block & cie, Strasbourg, France) were coated with 100 µl of diluted c-45C (10 µg/ml in 50 mM phosphate buffer) and incubated 1 h at 37°C and then overnight at 4°C. On the following day, the microtiter plate was washed three times with PBS containing 0.1% Tween (PBS-Tween, pH 7.2). The remaining binding sites in the wells were blocked by addition of 250 µl of PBS-Tween-BA (0.1 g albumin in 100 ml PBS-Tween) for 1 h at room temperature, and the plate was washed again. At this stage, 100 µl of sample diluted in PBS-Tween-BA were added for 1 h at room temperature. Then, after three washes, the wells were incubated with 100 µl of the biotinylated-45C diluted in PBS-Tween-BA (7.5 µg/ml) for 1 h at room temperature. After the wells were washed again, 100 µl of avidin-peroxidase conjugate were added and allowed to bind for 1 h at room temperature. The plate was washed five times. One hundred microliters of the OPD solution were then added to each well, and the color was allowed to develop in the dark for 5–10 min. The reaction was stopped by adding 25 µl of 3 M sulfuric acid to each well. The absorbance was read at 492 nm on a micro-ELISA plate reader. Purified mucin ranging in concentration from 0 to 500 ng/ml was treated in the same way as the test samples to obtain a mucin standard curve. All assays were performed in duplicate.

PREPARATION OF TISSUES FOR CROSS-REACTIVITY STUDIES. Scrapings (stomach, intestine, colon) from rat, mouse, and guinea pig were treated for reduction and carboxymethylation, as described in PURIFICATION OF RAT COLONIC MUCIN. Other rat tissues (lung, kidney, pancreas, liver, spleen, muscle) were first homogenized for 1 min using a homogenizer (Ultra-Turrax, Janke and Kundel) and then treated as mentioned above. After dialysis (4°C, 24 h), homogenates of scrapings and tissues were analyzed for mucin by ELISA and for protein by Bradford assay. Tissue mucin content was expressed according to protein content.

Study of mucin secretion and measurement of mucin. SURGICAL PREPARATION. The operative procedure to prepare an isolated vascularly perfused rat colon was previously described (27). Briefly, male Wistar rats (250–350 g) were anesthetized with pentobarbital sodium (50 mg/kg ip). The proximal and transverse colons were freed from their visceral fixations. The ileocolic veins and arteries were tied. Both ends of the colonic loop (10 cm in length) were then equipped with Silastic tubing. A metal cannula and a Silastic one were then quickly inserted into the superior mesenteric artery and portal vein, respectively. The arterial perfusion started immediately at a rate of 2.5 ml/min with a Krebs-Henseleit buffer (2 mM CaCl₂, 6 mM KCl, 3.18 mM NaH₂PO₄, 104 mM NaCl, 1 mM MgSO₄, 41.6 mM NaHCO₃, pH 7.4) containing 25% washed bovine erythrocytes, 3% BSA, 5 mM glucose, and 1% hyperamine 25 (vol/vol). The pressure of perfusion, continuously recorded with a mercury manometer, ranged from 55 to 75 mmHg. The colonic lumen was flushed out with prewarmed isotonic saline and then with air, and both ends of the intestinal loop were ligated. The preparation was then removed and transferred to a bath containing isotonic saline at 37°C.

EXPERIMENTAL PROTOCOL. The experiments consisted of a 10-min equilibration period, followed by a 30-min period of stimulation. Immediately following the equilibration period, the loops were filled by injection with 1.0 ml of prewarmed

saline (37°C). All vascularly perfused drugs and peptides were dissolved in the Krebs-Henseleit buffer supplemented with 3% BSA. They were then delivered at a rate of 0.25 ml/min through a catheter close to the vascular inflow. The drug concentrations represented the final concentrations in the arterial inflow cannula. The control group underwent a 10-min equilibration period, followed by a 30-min period during which Krebs-Henseleit buffer supplemented with 3% BSA was administered to the vascular inflow (0.25 ml/min). The data from the controls were obtained from a separate group of animals. In some experiments, 1 μ M TTX or 10 μ M atropine infusion was started at the equilibration period and then lasted over the 30-min perfusion of secretagogues. Such experiments had a total duration of 40 min. Oxygen saturation, glucose consumption (a measure of metabolic activity), and rate of glucose conversion to lactate (an indicator of anaerobic metabolism) remained constant during the perfusion period (27).

At the end of the experimental period, loops were cut at both ends. Fluid content was carefully collected. To remove mucus adherent to the mucosal surface, loops were carefully emptied by manual massage, subsequently flushed with 15–20 ml air, and drained. Luminal content (fluid content + adherent mucus gel) was weighed, sonicated, and frozen at -20°C for subsequent determination of mucinlike immunoreactivity and luminal DNA content. The adherent mucous gel was taken into account because it represents a substantial part of the secreted immunoreactive material. The empty colonic loops were weighed, measured (cm), homogenized for 1 min in PBS using a homogenizer, and then stored at -20°C . Tissue homogenates were then analyzed for DNA content.

SAMPLE ANALYSIS. Samples of luminal contents were incubated for 24 h with 100 mM DTT for reduction and were then assayed for ELISA reactivity. All luminal assays were performed in three dilutions (1:4,000, 1:8,000, and 1:16,000). Colonic loop homogenates were thawed and briefly homogenized. Aliquots were sonicated for 20 s and then analyzed for DNA content using the fluorimetric method of Hingardner (15). The amount of mucin secreted from each loop was expressed as micrograms of mucin per milligram of tissue DNA. Samples of luminal content were also analyzed for DNA content. This analysis served as an indirect measure of tissue viability, and loops were discarded when luminal DNA content represented more than 2% of the total DNA (tissue DNA plus luminal DNA). Total luminal content (fluid content + adherent mucus gel) recovered from the lumen was expressed as milligrams of luminal content per centimeter of colonic loop.

Histological analysis and goblet cell morphology. HISTOLOGICAL TECHNIQUE. To provide an estimate of depletion of mucus stores from goblet cells, the effect of several pharmacological agents was also monitored by histological analysis, as described previously (28). Briefly, pieces of colon (2–2.5 cm) taken at the end of the isolated colon experiment were fixed into Karnovsky's fixative, processed in Epon, sectioned at 2 μ m, stained with alcian blue (AB, pH 2.5) followed by the periodic acid-Schiff reaction (PAS), and then counterstained with hematoxylin. The AB/PAS method yielded blue color when mostly acidic mucins were present, purple when neutral mucins were also present, and magenta when mainly neutral mucins were present. The corresponding cells were called stained mucus cells.

QUANTITATIVE HISTOLOGY. An estimate of depletion of mucus stores from goblet cells may be provided microscopically (29, 32). In brief, intestinal goblet cells can respond to stimulation either by a process of compound exocytosis resulting in deep apical membrane cavitation or by another mechanism that

decreases the intracellular mucus store but without apical cavitation (10, 32). When the latter mechanism leads to total depletion of mucus granules, goblet cells are no longer stained by AB/PAS and appear indistinguishable from other epithelial cells. Thus the extent of mucus secretion may be quantified both by assessing the number of mucus cells per crypt section and the percentage of cavitated mucus cells per preparation. For this purpose, 15–30 crypts per slide were analyzed, and 500–1,000 mucus cells were counted per sample of colon. A crypt was considered when it was cut along or nearly along the length of the crypt lumen (at least two-thirds of the length of the crypt). Progressing from the crypt base to crypt opening, all stained mucus cells belonging to the epithelium were counted on both sides of the lumen. All mucus cells were evaluated for the presence of cavitation of their apical membrane. A mucus cell with clear apical indentation into the intracellular store of mucus granules was taken as showing signs of the cavitation that accompanies recent compound exocytotic activity. Four to seven rat colons were prepared for each set of experiments. All slides were analyzed by a single investigator who was blinded to the drug treatment.

Statistical analysis. Data are presented as mean values $\pm$ SE. Data were compared using Student's *t*-test (comparison of 2 means) or an analysis of variance with a Bonferroni test (comparison of >2 means) where appropriate. $P < 0.05$ was considered significant.

RESULTS

Development of Immunoassay for RCM

RCM, used for raising antiserum in rabbits, was isolated from colonic mucosal scrapings by applying three successive CsCl density gradients (Fig. 1). After the third gradient, mucin-containing fractions were pooled and stored lyophilized at -20°C . By immunohistochemistry, the antiserum developed against purified RCM reacted specifically with colonic goblet cells as well as with the mucus blanket (Fig. 2). In a control experiment, purified antigen was preincubated for 30 min at 37°C with the antiserum. Substituting antiserum for the antigen-antiserum preincubation mix showed no reaction toward colonic mucus cells. Similarly, substituting anti-RCM antiserum for PBS produced no reaction toward mucus cells. The antiserum was also tested for mucin specificity and epitope identification by immunodetection on dot blot. Our results showed that the antiserum reacted with the purified mucin. No reaction was observed with BA, bovine thyroglobulin, and gum arabic. SDS-PAGE of the purified mucins and Western blotting with our antibody revealed a single band corresponding to high molecular-weight immunoreactive material (not shown). Finally, proteolytic digestion of the purified mucin with proteinase K resulted in a very strong decrease in reactivity on dot blot (data not shown), thus indicating that protease-sensitive domains in mucins are crucial for binding to the antibodies.

A sandwich ELISA for RCM was then set up using the anti-RCM antiserum. A typical standard curve obtained by using increasing concentrations of purified mucin from 0 to 500 ng/ml is shown in Fig. 3A. Linearity was obtained between 8 and 500 ng mucin/ml. The mucin specificity of the antiserum was further

examined by ELISA of scraping and tissue homogenates. The extracts of rat colon gave the highest response. Rat stomach and small intestine reacted to a lesser extent (Fig. 3B). Homogenates of lung, kidney, pancreas, liver, spleen, muscle, and serum did not react in ELISA. No cross-reactivity was detected with mouse and guinea pig gastric, intestinal, or colonic scrapings. Serial dilutions of luminal samples taken from control preparation or from colonic loop after a 30-min stimulation period with bethanechol or dmPGE₂ gave results in ELISA that could be superimposed on the mucin stan-

Fig. 2. Immunohistochemistry of paraffin-embedded rat colonic tissue after incubation with 1:1,000 anti-rat colonic mucin antiserum. The section is oriented with intestinal lumen at top. Immunolabeling is evident as a brown reaction product. Micrograph shows that only goblet cells and mucus blanket are immunoreactive. Arrows and asterisk indicate examples of immunoreactive goblet cells and mucus blanket, respectively. Bar = 10 μ m.

dard curve, producing parallel slopes. This immunoreactive material was distributed on a CsCl density gradient in a similar way to purified RCM, with a peak at a density of 1.4 g/ml (Fig. 3C), which was characteris-

Fig. 1. Isolation of mucus glycoproteins from rat colon. Distribution of material after first CsCl density gradient is shown in A. Gradient had a starting density of 1.40 g/ml and contained 4 M guanidinium hydrochloride. After centrifugation, tubes were fractionated, leading to collection of 12 fractions. Distribution of hexoses in first gradient showed peak at density of 1.43 g/ml that was characteristic of mucin. Major contaminants in mucin-containing fractions were proteins in top of gradient (fractions 8-12) and nucleic acids in bottom of gradient (fractions 1-6). Mucin-containing fractions indicated by horizontal bar were pooled and loaded on an identical gradient represented in B. Fractions 4-7 of second gradient had a mean density of 1.40 g/ml, corresponding with a peak in hexoses concentration (B). This gradient induced a complete separation of mucin from proteins but not from nucleic acids. Mucin-containing fractions were then pooled and run on third gradient to separate mucin from nucleic acids (C). This gradient had a starting density of 1.50 g/ml and contained 0.2 M guanidinium hydrochloride. In this gradient, DNA was detected in fractions 1-4 whereas hexoses were in fractions 8-10. O.D., optical density.

Table 1. Quantitative histology and ELISA assay from isolated vascularly perfused rat colon after infusion of potential mucus secretagogues

	Quantitative Histology			ELISA	
	Percent of cavitated mucus cells	Number of mucus cells/crypt	n	Mucin, $\mu\text{g}/\text{mg DNA}$	n
Control group	10.8 ± 3.0	29.8 ± 1.5	7	103 ± 14	9
Bethanechol (200 μM)	10.4 ± 2.5	$16.9 \pm 1.3^*$	6	$958 \pm 204^*$	6
BBS (100 nM)	8.8 ± 1.9	$19.2 \pm 1.0^*$	6	$897 \pm 140^*$	9
VIP (100 nM)	$22.3 \pm 4.0^*$	25.1 ± 1.4	4	$574 \pm 76^*$	10
Isoproterenol (1 μM)	7.2 ± 1.0	30.7 ± 1.2	4	119 ± 24	5
PYY (10 nM)	$30.2 \pm 2.8^*$	29.6 ± 1.5	5	$264 \pm 27^*$	6
tGLP-1 (10 nM)	9.1 ± 1.0	32.4 ± 1.3	4	169 ± 45	5
Serotonin (5 μM)	$32.0 \pm 2.7^*$	27.4 ± 1.3	5	$451 \pm 51^*$	6
dmPGE ₂ (2.5 μM)	$35.7 \pm 4.2^*$	$21.4 \pm 1.8^*$	4	$537 \pm 83^*$	5
SNP (1 mM)	19.9 ± 4.5	30.6 ± 1.4	4	$269 \pm 25^*$	6

Values are means $\pm$ SE; n = no. of animals. * $P < 0.05$ compared with control group. Control group underwent a 10-min equilibration period followed by a 30-min period during which Krebs-Henseleit buffer supplemented with 3% BSA was administered to the vascular inflow. A reduced number of mucus cells indicated a release of mucin from colonic mucosa, and cavitation of mucus cells was the hallmark of accelerated mucus discharge by a process of compound exocytosis. BBS, bombesin; VIP, vasoactive intestinal peptide; PYY, peptide YY; tGLP, truncated glucagon-like peptide; dmPGE₂, dimethyl-PGE₂; SNP, sodium nitroprusside.

tic of mucins (34). No immunoreactive material was present on the top or on the bottom of the gradient.

Release of Colonic Mucus by Cholinergic and Adrenergic Agonists

Intra-arterial infusion of 200 μM bethanechol in the isolated vascularly perfused rat colon evoked a sharp increase in mucin secretion (Table 1). As shown in Fig. 4, the effect of bethanechol was concentration-dependent over the range 10–200 μM . The first significant response was obtained with 100 μM bethanechol. This mucin discharge was associated with a decrease in the number of stained mucus cells per crypt section as compared with the control group (Table 1, Fig. 5). Isoproterenol (1 μM) did not induce any significant increase in the release of colonic mucus (119 ± 24 $\mu\text{g}/\text{mg DNA}$, $n = 5$, $P > 0.05$).

Total luminal contents in response to bethanechol and isoproterenol are shown in Table 2. Bethanechol

Fig. 3. Standard curve, cross-reactivity of prepared mucin antiserum with rat gastrointestinal scrapings, and CsCl gradient density profile of colonic luminal content. A: calibration curve of ELISA. Mucin was diluted in PBS-Tween-bovine albumin buffer to contain 0–500 ng mucin/ml. ELISA was performed as described in MATERIALS AND METHODS. Points represent mean of 2 replicate samples for each concentration of purified mucin. B: cross-reactivity of prepared mucin antiserum with rat gastrointestinal scrapings. Scrapings were obtained as described in MATERIALS AND METHODS and analyzed for mucin reactivity. C: CsCl gradient density profile of colonic luminal content. Samples were first homogenized in homogenization buffer and alkylated by addition of 0.25 M iodoacetamide. They were then centrifuged at 100,000 g for 70 h at 4°C, separated into fractions, and dialyzed against water before assay. Starting density of CsCl gradient was 1.40 g/ml, and gradient contained 4 M guanidinium hydrochloride.

Fig. 4. Effects of increasing doses of bethanechol on mucin secretion in isolated vascularly perfused rat colon. Data are means $\pm$ SE. * $P < 0.05$ vs. control group.

(200 μ M) led to a significant increase in luminal content recovered from the lumen, but luminal DNA content was similar to that observed in the control group (Table 2). On the contrary, isoproterenol (1 μ M) did not modify the luminal content.

Neuropeptides and Mucin Release

Intra-arterial administration of 100 nM VIP induced colonic mucin discharge (Table 1). On stimulation with 10 nM VIP, the rise in mucin release was less pronounced (Fig. 6A). A greater proportion of cavitated goblet cells was found in loops submitted to intra-arterial infusion of VIP as compared with controls (Table 1). The effects of TTX (1 μ M) and atropine (10 μ M) were tested on intra-arterial infusion of 100 nM VIP. As shown in Fig. 6A, pretreatment with TTX abolished VIP-induced mucin release ($P < 0.05$ vs. VIP alone). In contrast, atropine did not inhibit the mucin secretory response. In control experiments (without VIP), TTX (1 μ M) or atropine (10 μ M) alone had no effect on mucin secretion (110 ± 8 μ g/mg DNA, $n = 6$, $P > 0.05$, and 97 ± 12 μ g/mg DNA, $n = 6$, $P > 0.05$, respectively). As shown in Table 2, VIP (100 nM) induced a small increase in luminal content that was not associated with a significant rise in luminal DNA content.

BBS (100 nM) produced a sharp rise in the discharge of immunoreactive material (Table 1). Thin section of tissue demonstrated that colonic preparations exposed to 100 nM BBS for 30 min exhibited a decrease in the number of stained mucus cells compared with control preparations (Fig. 5, Table 1). In contrast, BBS infusion did not modify the percentage of cavitated mucus cells. As shown in Fig. 6B, mucin secretion induced by BBS

was completely abolished by pretreatment with 1 μ M TTX. Atropine (10 μ M) decreased BBS-stimulated colonic mucin secretion by 60% (Fig. 6B).

CGRP, at final concentrations up to 5 nM, did not elicit any release of mucin (91 ± 10 μ g/mg DNA, $n = 6$, $P > 0.05$), whereas a significant increase in total luminal content was observed (Table 2).

Release of Colonic Mucin by Hormones of the Distal Gut

Intra-arterial infusion of PYY (10 nM) in the isolated vascularly perfused rat colon induced release of colonic mucin (Table 1). As shown in Fig. 7A, the responses were related to the concentration of PYY over the range 0.01–10 nM. Numerous goblet cells became deeply cavitated after 30 min of 10 nM PYY stimulation (Table 1).

In contrast, intra-arterial truncated glucagon-like peptide-1 (10 nM) did not induce a significant increase in mucin release (169 ± 45 μ g/mg DNA, $n = 5$, $P > 0.05$ vs. control group).

The effect of serotonin on mucin secretion is shown in Fig. 7B. The mucinlike immunoreactivity was 451 ± 51 μ g/mg DNA, $n = 6$, $P < 0.05$, and 245 ± 24 μ g/mg DNA, $n = 6$, $P < 0.05$, after 5 and 1 μ M serotonin, respectively. Stained mucus cells with apical membrane cavitation were very numerous after administration of 5 μ M serotonin (Table 1, Fig. 5). The response to 5 μ M serotonin was unaffected by intra-arterial TTX (1 μ M) (373 ± 32 μ g/mg DNA, $n = 6$, $P > 0.05$ vs. 5 μ M serotonin alone).

Release of Colonic Mucin by Mediators of the Immune System

Intra-arterial perfusion of bromosalocid (20 μ M), a degranulator of connective and mucosal mast cells, produced a modest increase in mucin release: 254 ± 64 μ g/mg DNA ($n = 5$, $P < 0.05$ vs. control group). In contrast, luminal administration of 20 μ M bromosalocid failed to induce significantly mucin discharge: 120 ± 15 μ g/mg DNA ($n = 6$, $P > 0.05$ vs. control group).

Intra-arterial administration of a stable analog of PGE₂ (dmPGE₂, 2.5 μ M) in the isolated vascularly perfused rat colon led to a fivefold increase in mucin secretion (Table 1, Fig. 8). By light microscopy, a rise in the percentage of cavitated mucus cells was observed. The number of stained mucus cells per crypt section was also significantly reduced (Table 1).

As can be seen from Fig. 8, IL-1 β (0.25 nM) provoked a modest rise in the secretion of mucin. The mucinlike immunoreactivity release was 200 ± 35 μ g/mg DNA ($n = 6$, $P < 0.05$ vs. control group) after administration of IL-1 β .

SNP (1 mM), an NO generator, increased mucin secretion (Table 1, Fig. 8). By morphological analysis, SNP showed a tendency to increase the percentage of cavitated mucus cells, although this value did not achieve statistical significance (Table 1).

DISCUSSION

To measure mucus released from isolated vascularly perfused rat preparation, we developed a sandwich

Fig. 5. Thin-section histology of perfused rat colon. Bar = 10 μ m. *A*: crypt of a control preparation. This colonic loop underwent 10-min equilibration period, followed by 30-min period during which Krebs-Henseleit buffer supplemented with 3% BSA was administered to the vascular inflow. Goblet cells (alcian blue/periodic acid-Schiff stained) are filled with mucus. Arrows indicate examples of stained mucus cells. *B*: crypts of perfused rat colon after 30-min perfusion with 200 μ M bethanechol. Few stained mucus cells (arrows) were observed along the length of the crypt. *C*: colonic mucosa after administration of 100 nM bombesin (BBS) for 30 min. Few stained mucus cells were observed along the length of the crypt. *D*: colonic crypt after 30-min perfusion with 5 μ M serotonin. Numerous mucus cells with deep cavitation of apical cell surface were easily observed (arrows).

ELISA for RCMs. For this purpose, RCMs were purified using a validated procedure (33), and these purified glycoproteins were then used to elicit a polyclonal antiserum. Because glycan structures are also present in glycolipids and in other glycoproteins, it was necessary to obtain a polyclonal antiserum that reacts primarily with the protein part of RCM. Our data demonstrated that anti-RCM antiserum no longer recognized RCM after proteinase K digestion, thus indicat-

ing that antigenic determinants were localized to a nonglycosylated segment of colonic mucins. In agreement with Satchithanandam et al. (30), our study also showed that the anti-RCM antiserum is highly specific for antigenic determinants found only in scrapings of rat digestive tract. The anti-RCM antiserum detected only mucus blanket and intracellular mucin granules in goblet cells by immunohistochemistry. Furthermore, the immunoreactive material released from isolated

Table 2. Total luminal content (fluid content + adherent mucus gel) and luminal DNA content after infusion of potential mucus secretagogues in isolated vascularly perfused rat colon

	Luminal Content, mg/cm	Luminal DNA Content, % of total DNA	n
Control group	129 ± 07	0.65 ± 0.10	9
Bethanechol 200 µM	176 ± 16*	0.44 ± 0.10	6
Bethanechol 100 µM	154 ± 04	0.60 ± 0.10	5
Bethanechol 20 µM	157 ± 09	0.57 ± 0.08	5
Bethanechol 10 µM	134 ± 08	0.40 ± 0.10	6
Isoproterenol 1 µM	117 ± 23	0.45 ± 0.08	5
BBS 100 nM	150 ± 13	0.80 ± 0.20	9
BBS 10 nM	146 ± 07	0.62 ± 0.12	5
VIP 100 nM	167 ± 11*	0.68 ± 0.06	10
VIP 10 nM	151 ± 03	0.56 ± 0.10	6
CGRP 5 nM	163 ± 09*	0.48 ± 0.07	6
PYY 10 nM	113 ± 13	0.70 ± 0.13	6
PYY 1 nM	130 ± 08	0.64 ± 0.05	6
PYY 0.1 nM	134 ± 01	0.76 ± 0.10	4
tGLP-1 10 nM	125 ± 18	0.50 ± 0.15	5
Serotonin 5 µM	153 ± 10	0.43 ± 0.10	6
Serotonin 1 µM	121 ± 10	0.41 ± 0.10	6
dmPGE ₂ 2.5 µM	166 ± 10*	0.56 ± 0.14	5
SNP 1 mM	197 ± 04*	0.83 ± 0.14	6
SNP 0.1 mM	142 ± 16	0.55 ± 0.15	5
IL-1β	151 ± 08	0.40 ± 0.07	6
Bromosalasolod 20 µM	164 ± 11*	0.45 ± 0.10	5

Values are means ± SE. CGRP, calcitonin gene-related peptide; IL, interleukin. * $P < 0.05$ compared with control group.

vascularly perfused rat colon after administration of two well-known mucus secretagogues (bethanechol or dmPGE₂) (20, 25) had the same affinity with the antiserum as the purified mucin. Overall, these data suggest a selective recognition of epitopes belonging to the mucus fraction of intestinal contents by the present antiserum. The sandwich ELISA developed in this study is then suitable for specific and quantitative determination of colonic mucin in rats. The major advantages of this sandwich method are that the assays are accurate and are carried out in 5 h with precoated plates and that samples do not need to be purified before use. Consequently, we used it in studies of mucus secretion to measure mucins in diluted and unpurified samples. The interference of other glycoproteins or proteins is negligible.

Studies providing direct evidence for mucin discharge from the colonic epithelium by neurotransmitters and neuropeptides are scarce. Tumoral mucus-secreting cell lines were instead used to answer these questions. In the course of characterizing the effect of endogenous factors on colonic mucin secretion, we found that the β-adrenergic agonist isoproterenol was not a stimulant of mucin discharge in the isolated vascularly perfused rat colon. Similarly, studies performed in organ culture showed that α- and β-adrenergic agonists did not result in accelerated secretion of mucin from rabbit colon (24). Taken together, adrenergic transmitters do not appear to be major determinants in the regulation of colonic mucin discharge.

The possibility that gastrointestinal neuropeptides may induce colonic mucin secretion has not been clearly

explored previously. The present study demonstrated that VIP caused a sharp release of colonic mucus by compound exocytosis. In contrast, Neutra et al. (24) reported that VIP did not accelerate mucin discharge in rabbit small and large intestine in organ culture. In another study consistent with the former investigation

Fig. 6. Effect of 2 neuropeptides [vasoactive intestinal peptide (VIP) and BBS] on mucin secretion in rat colonic preparations. A: dose-related effect of VIP on mucin discharge and effect of TTX (1 µM) or atropine (10 µM) on secretory response induced by 100 nM VIP. Colonic preparations were pretreated for 10 min with TTX or atropine before being challenged for 30 min with 100 nM VIP. Values are means ± SE. * $P < 0.05$ vs. control group. † $P < 0.05$ vs. 100 nM VIP alone. B: dose-related effect of BBS on mucin secretion and effect of TTX or atropine on secretory response induced by 100 nM BBS. Data are means ± SE. * $P < 0.05$ vs. control group. † $P < 0.05$ vs. 100 nM BBS alone.

Fig. 7. Effects of peptide YY (PYY) and serotonin on mucin release. *A*: luminal mucin secretion in response to PYY. Values are means $\pm$ SE. * $P < 0.05$ vs. control group. *B*: luminal mucin secretion in response to serotonin, the major product of the enterochromaffin cells. Values are means $\pm$ SE. * $P < 0.05$ vs. control group.

(19), VIP (1 μ M) did not alter basal mucin secretion in the HT-29-CL.16E colonic cell line, despite the presence of functional VIP-receptors. An attractive hypothesis that could explain the difference between these results is that VIP stimulates mucin release indirectly through the activation of the enteric nervous system, which is left intact in the isolated vascularly perfused rat preparation. This hypothesis is supported by the observation that pretreatment with the neurotoxin TTX completely

abolished VIP-evoked mucin secretion from rat colon but had no effect on baseline mucin discharge. This result is in agreement with early observations indicating that enteric nerves may be involved in VIP-stimulated intestinal secretion (16). Indeed, it has been pointed out that the VIP effect on bicarbonate secretion is mediated by the enteric nervous system in rabbit proximal duodenum (16). In our study, the possibility that muscarinic receptors participate in the VIP-evoked mucin secretion may be excluded, because

Fig. 8. Effects of bromolaserol, dimethyl-PGE₂ (dmPGE₂), interleukin (IL)-1 β , (A) and sodium nitroprusside (B) on mucin secretion in isolated vascularly perfused rat colon. * $P < 0.05$ vs. control group.

atropine did not alter the secretagogue-induced effect on mucin discharge.

The neuropeptide gastrin-releasing peptide (GRP)/BBS is another neuropeptide found in mammalian gastrointestinal tract. GRP/BBS is thought to stimulate secretion from endocrine cells in the stomach and intestine (7). The present study showed for the first time that BBS markedly increased the discharge of colonic mucin. Similarly, Hootman and De Ondarza (17) were able to show that, in isolated pancreatic duct, BBS stimulated goblet cell degranulation. Together, these results suggest another secretory function for BBS in the digestive tract. Our morphometric analysis additionally indicated that BBS induced mucus secretion by a mechanism that did not result in the cavitation of apical membrane. This histological picture resembles the aspect of colonic crypts after 30-min perfusion with bethanechol. To gain an insight in the mechanism underlying the BBS-evoked mucus secretion in the isolated vascularly perfused rat colon, TTX or atropine was administered intra-arterially before BBS infusion. Mucin secretion elicited by BBS was completely inhibited by TTX. Interestingly, atropine reduced by ~60% the BBS-induced mucin response, suggesting a partial cholinergic mediation. Together, these data indicate that BBS evokes mucin discharge through the activation of enteric nerves, part of which involves the participation of muscarinic receptors.

CGRP-containing nerves are found in the submucosal layer of the gut wall. The present study showed that CGRP did not induce mucin output from the isolated vascularly perfused rat colon. Another interesting fact is that luminal DNA content was not modified, although CGRP significantly increased fluid accumulation. Overall, it is likely that the moderate colonic fluid distension induced by fluid accumulation in our model was not sufficient by itself to induce mucosal damage and mucin discharge. This hypothesis is strengthened by the observation that the most potent fluid secretagogues administered in this report (bethanechol, VIP, SNP) did not significantly modify luminal DNA content. Furthermore, histological analysis of the stimulated preparations did not reveal any damage of the epithelium, suggesting that mucin secretion obtained after fluid secretagogues was not induced by nonspecific breakdown of the epithelial barrier and mucosal damage. The conclusion to be drawn from these results is that mucin release from our model is the result of a true secretory response induced by secretagogues and was not the consequence of colonic loop distension and pressure necrosis.

Besides a neural mechanism, the secretion of colonic mucin may also implicate hormonal or local paracrine pathways. Supporting this hypothesis, El-Sahli et al. (8) were able to observe that enteroendocrine L cells in human colon emit long cytoplasmic processes to the neighboring goblet cells, suggesting a paracrine action on mucus secretion. This hypothesis was therefore tested in the present study. L cells produce and secrete PYY, a 36-amino-acid polypeptide hormone that has a variety of biological effects on the gastrointestinal

tract, including inhibition of gastric and pancreatic secretion and of gastric emptying and intestinal motility (35). The present study showed that intra-arterial infusion of PYY (10 nM) produced a threefold increase in mucin release. This result is in agreement with our previous finding that PYY (10 nM) induced cavitation of the apical granule mass of goblet cells in the isolated vascularly perfused rat colon (28). In the present study, intra-arterial infusion of PYY mimicking a physiological concentration of the circulating peptide (0.1 nM) (1, 2) induced a modest rise in mucin secretion, thus excluding a major hormonal action of this peptide. However, the possibility that PYY exerted a local paracrine action on mucin release cannot be ruled out. Consequently, it is conceivable that PYY, released by intraluminal stimuli, could act locally on mucus cells to protect the colonic mucosa.

The other prominent enteroendocrine cell in the colon is the enterochromaffin cell, which synthesizes the biogenic amine serotonin. Serotonin stimulated mucin secretion from the dog gastric antrum (22). Moore et al. (23) have also demonstrated that serotonin (3 and 5 μ M) induces the release of mucin in an isolated loop of rat small intestine. Used at the same concentrations, serotonin also induced a clear rise in colonic mucin secretion in our model. The present study also showed for the first time that this mucus discharge was mediated by compound exocytosis. In contrast, serotonin was without effect on mucin release in rabbit small and large intestine in organ culture (24). It is likely that these discrepancies are related to the different models used.

The accelerated discharge of mucus may also involve resident immune cells of the normal colon, which consist of lymphocytes, macrophages, and mast cells. Several studies performed with isolated gastric mucous cells and the human goblet cell line HT-29-CL.16E showed that mucin was released by several mediators secreted by immune cells (3, 4, 18). Similarly, the present study demonstrates that several immunoregulatory mediators induce a rise in mucus discharge from colonic goblet cells in rats. Two different populations of mast cells have been identified in the colon: connective tissue mast cells and mucosa mast cells that are found in large number. The present study demonstrated that the degranulator compound bromolasalocid, which acts on both types of mast cells (9), induced a small increase in mucin secretion. Furthermore, luminal administration of bromolasalocid did not induce mucin discharge, thus suggesting that this drug did not directly degranulate goblet cells.

NO is a mediator released by mast cells, but also by macrophages, neutrophils, and enteric nerves. With a model of isolated rat gastric mucous cells, Brown et al. (4) showed that NO donors induced mucin secretion. Subsequently, a specific effect of NO donors to stimulate mucus secretion was described in the goblet cell line HT-29-CL.16E (3). Similarly, the present study demonstrated that NO liberated from SNP caused a concentration-dependent increase in colonic mucin release. Taken together, these results suggest that NO

may be implicated in the control of gastrointestinal mucin release.

The immunoregulatory cytokine IL-1 is more specifically secreted by macrophages (6). IL-1 has been demonstrated to induce a rise in the release of mucin in explant culture of mouse duodenum (5) and in the goblet cell line HT-29-CL.16E (18). Our observation that IL and SNP elicited an increase in mucin release into the colonic lumen must be tempered because the magnitude of the response was relatively low. It represented at most a doubling of basal secretion calculated from data obtained in a different animal group. Because baseline responses may vary among animal groups, the significance of the results is questionable when rather weak stimulants are tested.

In conclusion, we have provided evidence that the sandwich ELISA is useful to measure accurately mucin secretion in the isolated vascularly perfused rat colon. This approach already led to the demonstration that colonic mucin secretion may be induced by several factors, such as a classical neurotransmitter, two gut neuropeptides, an hormonal peptide of the distal gut, a biogenic amine, and several mediators of the immune system. Additionally, the morphological method enabled us to show that accelerated secretion of mucus is accomplished by two different mechanisms according to the nature of secretagogues. Several factors, including VIP, PYY, and serotonin, induced a phenomenon of compound exocytosis, resulting in deep cavitation of apical membrane surface. On the contrary, bethanechol and BBS provoked mucus secretion by another process, presumably by accelerating single-granule exocytosis.

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