Sensory peptide neurotransmitters mediating mucosal and distension evoked neural vasodilator reflexes in guinea pig ileum

D. Patton, M. O’Reilly, and S. Vanner

Gastrointestinal Diseases Research Unit, Queen’s University, Kingston, Ontario, Canada K7L 5G2

Submitted 24 January 2005; accepted in final form 16 June 2005

Patton, D., M. O’Reilly, and S. Vanner. Sensory peptide neurotransmitters mediating mucosal and distension evoked neural vasodilator reflexes in guinea pig ileum. Am J Physiol Gastrointest Liver Physiol 289: G785–G790, 2005. First published June 23, 2005; doi:10.1152/ajpgi.00027.2005.—The aim was to determine the role CGRP and/or tachykinins released from sensory neural mechanisms in enteric neural vasodilator pathways. These pathways project through the myenteric plexus to submucosal vasodilator neurons. Submucosal arterioles were exposed in the distal portion of an in vitro combined submucosal-myenteric guinea pig ileal preparation, and dilation was monitored with videomicroscopy. Vasodilator neural reflexes were activated by gentle stroking the mucosa with a fine brush or by distending a balloon placed beneath the flat-sheet preparation in the proximal portion. Dillations evoked by mucosal stroking were inhibited 64% by the CGRP 8–37 and 37% by NK3 (SR 142801) antagonists. When the two antagonists were combined with hexamethonium, only a small vasodilation persisted. Balloon distension-evoked vasodilations were inhibited by NK3 antagonists (66%) but were not altered by CGRP 8–37. In preparations in which myenteric descending interneurons were directly activated by electrical stimulation, combined application of CGRP 8–37 and the NK antagonists were not altered by CGRP 8–37. In preparations in which myenteric cholinergic vasodilator motoneurons innervating submucosal arterioles; tachykinins; calcitonin gene-related peptide;

intrinsic primary afferent neuron; vasodilation; submucosal plexus; submucosal arterioles; tachykinins; calcitonin gene-related peptide

NEURAL REFLEXES REGULATING gastrointestinal function are mediated by neural pathways that project within and extrinsic to the intestine (5). The intrinsic neural pathways project within the enteric nervous system, providing an important mechanism for precise regulation of intestinal function. This includes motility (8, 34), secretion (2), and blood flow (16), which respond to changes within the lumen and the wall of the intestine. Although the neural components of these reflexes, sensory or intrinsic primary afferent neurons (IPANS), interneurons, and motoneurons, have been characterized in detail in the past few decades, the enteric reflexes regulating blood flow have received relatively less attention (5). Studies have shown that submucosal cholinergic vasodilator neurons are the motoneurons innervating the submucosal arterioles (21), the final resistance vessels controlling mucosal blood flow (9), but little is known about the sensory mechanisms that activate these motoneurons.

IPANs are the first neurons in intrinsic reflexes (5, 6), and several neurotransmitters have been implicated in their actions (5, 6, 11, 22). These neurons exhibit choline acetyltransferase and substance P immunoreactivity in guinea pig (5) and CGRP immunoreactivity in the mouse (7). NK3 receptors are found on most myenteric interneurons and IPANs (15). Their activation appears to underlie the slow excitatory postsynaptic potential (EPSPs) observed in myenteric IPANs and possibly interneurons following mucosal and fiber tract stimulation (5). In vitro functional studies of the peristaltic reflex (11) and electrophysiological studies in the submucosal plexus (22) suggest that mucosal stimulation also releases CGRP from IPANs. Whether one or more of these neurotransmitters underlies enteric vasodilator reflexes is unknown.

Our recent studies (23, 30) have characterized the neural components of vasodilator reflexes activated by mucosal stimulation and distention of the muscle wall of the intestine. Both responses were mediated by enteric neural pathways in the myenteric plexus, which, in turn, activate submucosal cholinergic vasodilator motoneurons innervating submucosal arterioles. Mucosal stimulation resulted in the release of 5-HT-activating neural components, whereas 5-HT played no role in responses evoked by balloon distension (23). These studies set the stage to examine the sensory neurotransmitter(s) that initiate these responses by examining the effects of specific antagonists to CGRP and tachykinins.

METHODS

All methods were approved by the Animal Care Committee at Queen’s University. Guinea pigs (150–225 g). Animals were anesthetized with isoflurane inhalation and euthanized by cervical transection and exsanguination. Distal ileum was removed, and ileal preparations were dissected as previously described (23). Briefly, the ileum was opened along the mesenteric border and pinned flat with the mucosa facing upward in a Sylgard-lined (Dow-Corning, Midland, MI) Petri dish containing a physiological saline solution. The submucosa was exposed in the distal 5–10 mm of the aboral portion by dissecting off the overlying mucosa. The preparations were subsequently pinned in small organ baths (3–4 ml) with the exposed submucosa facing upward. These organ baths were continuously perfused with a physiological saline solution (in mM: 126 NaCl, 2.5 NaH2PO4, 1.2 MgCl2, 2.5 CaCl2, 5 KCl, 25 NaHCO3, and 11 glucose) that was gassed with 95% O2-5% CO2 and maintained at 35–36°C. Muscle movement was suppressed by adding nifedipine (1 μM) to the bath. Our previous studies suggest this L-type calcium channel blocker does not alter vasodilator responses mediated by submucosal neurons (30) and does not appear to alter synaptic neurotransmission in the enteric nervous system (1).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Vasodilator responses were monitored by continuously measuring the outside diameter of individual submucosal arterioles using a computer-assisted videomicroscopy system (Diamtrak), as previously described (20). Briefly, an Imaging Technology PCVision framegraber board in an IBM PC-AT computer was used to digitize television images of the arteriole. This was converted to an analog signal and stored on a chart recorder. The resolution of the system was <1 μM. The vasodilator responses were monitored by first preconstriciting the arterioles to 80–95% of the maximum they could constrict from their resting diameter using 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin F₂₀. The dilator response was quantified by measuring the area under the formula peak height times width at one-half the peak height, as previously described (33). The magnitude of the dilation was also expressed as the peak amplitude of the dilation in a few cases. This was expressed as a percentage of the potential maximum dilation, which had been defined in previous experiments as ~85% of the preconstriction from the resting diameter (21).

The intestine was stimulated by mechanically distorting the mucosal villi with a hand-held painter’s brush (tip diameter = 5 mm; 1 Hz, duration ~0.5 s) or by distending the intestinal wall with a balloon catheter placed beneath the tissue 1–2 cm from the recording site (distension volumes = 0.4–0.6 ml; 1 Hz, duration ~0.5 s), as previously described (23, 24). The vasodilator studies (23) demonstrated that stroking the mucosa four times or distending the intestinal wall three times reproducibly evoked neurally mediated vasodilator reflexes. In a small series of experiments, the preparation was modified by dissecting off the mucosa, submucosa, and circular muscle in the proximal segment, as previously described (18, 30). This enabled the exposed myenteric plexus to be electrically stimulated with a bipolar tungsten electrode (20 Hz, 6 s). This electrode was modified by bending both feet for ~5 mm so that they were parallel and in contact with the preparation, thus increasing the number of fiber tracts that were activated. In another small series of experiments, a Plexiglass divider was used to isolate the exposed myenteric plexus from the exposed submucosal plexus, as previously described (18, 31). This enabled capsaicin-sensitive afferents to be selectively stimulated with capsaicin in each chamber. The resting outside diameter of submucosal arterioles ranged from 30 to 85 μm (n = 60 preparations, each from a separate animal). Preparations in which a sustained constriction was not obtained or in which the evoked dilation did not return significantly toward baseline (>50%) were excluded from analysis. The videomicroscopy recording sites were 8–14 mm from the nearest point of stimulation with either mucosal stroking or balloon distension.

All drugs were added to the bath by superfusion. Antagonists were applied for 3 min before stimulating the preparation to allow equilibration and adequate diffusion in the tissue. The following drugs were used: nifedipine, CGRP 8–37, hexamethonium, capsaicin, CP-99,994 (gift from Pfizer), SR 142801 (gift from Sanofi-Synthelabo Recherche).

The data are expressed as means ± SE. A two-tailed paired Student’s t-test was used to compare data for significant differences. P < 0.05 was considered significant.

RESULTS

Mucosal stroking. After preconstriction of submucosal arterioles with PGF₂₀ (400 nM), mucosal stroking (4 strokes, 1 Hz) consistently evoked vasodilator responses (mean peak dilation = 45%; n = 7), as previously described (23). The mean area of the dilation obtained in the presence of CGRP 8–37 (2 μM) compared with that obtained following a 10-min washout of the antagonist was inhibited by 64% (n = 7, P = 0.037; Fig. 1). Our previous studies have shown that CGRP 8–37 superfused for 3 min at this concentration selectively inhibits maximal CGRP responses in the submucosal plexus evoked by exogenous CGRP application (28). In a separate series of experiments, the mean area of the dilation obtained in the presence of the NK₁ antagonist CP-99,994 (200 nM) and NK₃ antagonist SR 142801 (200 nM) compared with that obtained following a 10-min washout of the antagonists was inhibited ~37% (n = 9; P = 0.034; Fig. 2). Our previous studies (17, 19) have shown that CP-99,994 (100 nM) and SR 142801 (100 nM) completely block maximal neural responses evoked by NK₁ and NK₃ agonists applied exogenously in the submucosal plexus. The NK₁ antagonists CP-99,994 (200 nM) alone had no effect on dilations (Fig. 2C; n = 6).

When the combined actions of the CGRP 8–37 (2 μM) and NK₃ antagonist SR 142801 (300 nM) were studied, a small residual dilation persisted (Fig. 3; n = 6). Similarly, only a small dilation persisted when hexamethonium (200 μM) was added to this combination (Fig. 3; n = 6).

Balloon distension. Balloon distension (3 distensions ~1 Hz) evoked consistent dilations (mean peak amplitude = 46%; n = 8) as previously described (23). The NK₃ antagonist SR
142801 (300 nM) inhibited the mean area of dilations 66% (n = 8) compared with controls obtained following a 10-min washout (Fig. 4, P = 0.047). The balloon distension evoked dilations following a 3-min superfusion of CGRP 8–37 (2 μM; n = 4); however, this did not differ significantly from controls (Fig. 5).

Electrical and capsaicin stimulation of descending myenteric pathways. To test whether actions of the CGRP 8–37 and NK1 and NK3 antagonist could be accounted for by their actions on downstream neurons, i.e., interneurons or motoneurons, we examined the effects of these antagonists on vasodilator responses evoked by electrical stimulation of myenteric ganglia. A modified bipolar stimulating electrode was placed on exposed myenteric ganglia (see METHODS), activating multiple internodal strands and ganglia. Our previous studies (30) showed that electrical stimulation activates cholinergic and noncholinergic myenteric pathways innervating submucosal vasodilator neurons. In the current study, electrically evoked dilations (20 Hz, 6 s) were not inhibited following a 3-min superfusion of the combined antagonists CGRP 8–37 (2 μM), CP-99,994 (200 nM), and SR142801 (200 nM; Fig. 6) compared with controls (n = 5).

The possibility that distension or mucosal stroking activated capsaicin-sensitive nerves was also tested by examining the effects of capsaicin stimulation in the myenteric plexus. We have previously shown that capsaicin (2 μM) activates capsaicin-sensitive nerves innervating enteric neurons and submucosal arterioles (31, 32). In the current study, we employed the exposed myenteric and submucosal preparation shown in Fig. 6 and used a Plexiglass divider placed between these regions to create two chemically isolated chambers, as we have previously described (31). Each chamber was superfused separately, and the arteriole in the submucosal chamber was first preconstricted with PGF2α (400 nM). When capsaicin (2 μM) was superfused into the myenteric chamber, no dilation was observed, but when it was superfused into the submucosal chamber in the same preparation, a characteristic large dilation was measured (mean area = 149.6 ± 19.1 mm², n = 3; data not shown).

**DISCUSSION**

This study examined the role of CGRP and/or tachykinins in enteric vasodilator reflexes using selective antagonists to these putative neurotransmitters. Our previous studies (23) have shown that mucosal stroking and balloon distension can activate these reflexes but that different mechanisms were involved, because 5-HT antagonists blocked mucosal responses but had no effect on balloon distensions. These studies also characterized the interneuronal pathways in the myenteric plexus that project to the submucosal plexus. Within the submucosal plexus, they activate cholinergic vasodilator neurons, the final common pathway in these reflexes. Extrinsic...
capsaicin-sensitive vasodilator nerves were not involved in these reflexes. We have shown in the current study that capsaicin stimulation in the myenteric plexus failed to elicit the vasodilator reflexes. In addition, in our previous data, we demonstrated that capsaicin evoked neural responses in the submucosal plexus were confined to short distances in the submucosal plexus, i.e., single-parent arteriole (23, 31). In the present study, CGRP antagonists and, to a lesser degree, NK3 antagonists significantly blocked dilations activated by mucosal stimulation, but only NK3 antagonists inhibited dilations evoked by balloon distension. The implications are that CGRP and tachykinins underlie sensory neurotransmission in mucosal-activated reflexes, whereas tachykinins are predominantly involved in distension-evoked responses.

The interpretation of the results of this study rely in part on the selectivity and inhibitory concentration of the antagonists employed. We have previously shown that CGRP 8–37 (2 µM) blocks maximal CGRP-evoked vasodilator (29) responses but does not alter tachykinin or muscarinic responses. Similarly, we have shown that CP-99,994 and SR 142801 in the 100 nM range block maximal NK1 and NK3 agonist-evoked vasodilator (29) and secretomotor responses (17) and remain selective in their actions in this range. Thus, in the current study, these antagonists were employed at concentrations near or at their maximal effect, yet they maintain their selectivity. This selectivity is also supported by the absence of effect in the electrical stimulation experiments (see Fig. 5) of interneurons and motoneurons. It is unlikely that diffusion barriers account for the absence of effect with the CGRP 8–37 antagonist in the

**Fig. 4.** Dilations evoked by balloon distension were inhibited by tachykinin but not CGRP antagonists. A: schematic drawing of the in vitro balloon distension preparation showing position of balloon beneath the serosa and intact myenteric and submucosal neural plexus above. Outside diameter of submucosal arteriole was monitored with videomicroscopy (parallel black bars). B: representative traces from a single preparation showing dilation evoked by balloon distension (3 distensions, −1 Hz) were inhibited by SR 142801 (300 nM) compared with the response obtained following a 10-min washout of the antagonist. Vessels were preconstricted, as described in Fig. 1. Resting outside diameter = 62 µm. C: summary of mean dilation area in the presence of SR 142801 (300 nM) and following a 10-min washout, which were evoked by balloon distension (3 distensions, −1 Hz; n = 8). Mean dilator area was inhibited by ~66%. *P = 0.047.

**Fig. 5.** Dilations evoked by balloon distension were not blocked by CGRP antagonists. Summary of mean area of the dilations in the presence of CGRP 8–37 (2 µM) and following a 10-min washout evoked by balloon distension (3 distensions, −1 Hz; n = 4). CGRP 8–37 had no effect on mean dilation area.

**Fig. 6.** Dilations evoked by electrical stimulation of descending myenteric pathways were not inhibited by combined application of CGRP 8–37 (2 µM), CP-99,994 (200 nM), and SR 142801 (200 nM). A: schematic drawing showing the position of the bipolar stimulating electrode. B: representative trace showing dilation evoked by electrical stimulation (20 Hz, 6 s; left) is not inhibited when repeated in the presence of CGRP 8–37, CP-99,994 and SR 142801 (right). The arteriole was preconstricted before stimulation, as described in Fig. 1. Resting outside diameter was 78 µm. C: summary of the mean dilation area in the presence and absence of the antagonists.
balloon distension studies given that mucosal-activated reflexes were significantly altered by CGRP 8–37 in the same preparation.

Several lines of evidence support the contention that CGRP and tachykinins were released from sensory neurons following mucosal stroking in our experiments. Mucosal stroking in the intestine has been shown in a number of species (10, 11, 13), including the guinea pig, to release CGRP from enteric neurons. This release was dependent on 5-HT (3, 11, 12, 14, 22), which is released from enterochromaffin cells following mucosal stimulation and, in turn, activates CGRP sensory neurons (11, 22). We have also shown in previous studies that the vasodilator reflex activated by mucosal stroking is dependent on 5-HT. This is consistent with the notion that stroking releases 5-HT, which, in turn, activates CGRP containing sensory neurons and initiates the vasodilator reflex. Electrophysiological studies in the guinea pig submucosal plexus also demonstrate that mucosal stimulation and application of 5-HT releases CGRP in the submucosal plexus activating second-order neurons (22). These second-order neurons could activate submucosal VIPergic neurons that project to the myenteric plexus (25) and/or CGRP containing sensory neurons in the submucosal plexus that project to the myenteric plexus (10, 11). In these electrophysiological studies (22), however, they failed to find evidence that mucosal stimulation evoked tachykinin release in the submucosal plexus. In contrast, there is compelling evidence that tachykinin release from sensory neurons in the myenteric plexus activates NK3 receptors on adjacent sensory neurons, initiating reinforcing circuits, as well as on ascending and descending myenteric interneurons (5, 6, 15). We also found that a component of our mucosal-activated vasodilator reflex was blocked by NK3 antagonist (see Fig. 2). When these studies are taken together, they suggest that mucosal stroking causes release of CGRP and tachykinin sensory neurotransmitters and that these activate vasodilator reflexes projecting for significant distances in the myenteric plexus. On the basis of this data, we speculate that the CGRP neurons may predominate in the submucosal plexus and tachykinin responses in the myenteric plexus. We did observe a small dilation that was not blocked by combined CGRP, tachykinin, and hexamethonium antagonists (Fig. 3), and this may suggest a role for other sensory neurotransmitter(s), such as acetylcholine acting at muscarinic receptors.

Although our data are consistent with the release of CGRP from submucosal IPANs, not all studies (5) suggest CGRP originates from these neurons. In the guinea pig, several functional studies concluded that sensory neurons release CGRP (10, 22), but other studies that correlated electrophysiological and immunohistochemistry properties of single neurons only found evidence for tachykinins in these neurons (5). However, studies applying these latter approaches in other species, such as the mouse, do suggest CGRP is found in IPANs (7), and these findings correlate well with functional studies in the mouse (11). These findings highlight species differences as well as potential differences in interpretation resulting from different technical approaches. In the guinea pig, one possibility to explain the discrepancy is that CGRP-containing neurons are second-order neurons (4) that are activated by IPANs.

Our studies also suggested that distension-evoked vasodilator reflexes were mediated by a different profile of noncholinergic sensory neurotransmitters compared with the mucosal-activated reflexes. We found that distension-evoked responses were mediated predominantly by tachykinins and not CGRP, whereas CGRP played a major role in the mucosal reflexes. Our previous studies with this model (23) have shown that the distension-evoked reflexes were not dependent on mucosal sensory mechanisms and appear to originate from activation of sensory mechanisms in the myenteric plexus. This role for tachykinins is also consistent with the previous electrophysiological and immunohistochemical studies demonstrating that IPANs in the myenteric plexus release tachykinins, which in turn activate NK3 receptors on other IPANs and on ascending and descending myenteric interneurons. A significant component of the distension-evoked response was not blocked by high concentrations of the tachykinin antagonists, suggesting that other mechanisms may also be involved. Although cholinergic responses may have accounted for this difference, we have previously shown that >30% of the response is not blocked by hexamethonium (23) and muscarinic responses appear to play little if any role in neurotransmission (30), at least in interneuronal myenteric pathways mediating vasodilation. Given the findings of recent studies, one possible explanation is that these sensory stimuli also directly activate myenteric interneurons (26, 27), which in turn initiate these vasodilator pathways. Regardless of the exact nature of this latter mechanism, this finding coupled with the absence of evidence to support a major role for CGRP following balloon distension provide further evidence to support the contention that distinct populations of afferent neurons mediate the mucosal- and distension-activated reflexes.

In summary, our studies suggest that noncholinergic sensory neurotransmission involving the release of both tachykinins and CGRP is important in initiating vasodilator reflexes mediated by long pathways projecting through the myenteric plexus. These pathways are poised to coordinate mucosal blood flow with complex patterns of neurally mediated secretion and peristalsis such as the mixing behavior that occurs during digestion. These studies also suggest that the relative importance of these neurotransmitters may vary depending on the nature of specific stimuli, e.g., CGRP and mucosal stimulation and tachykinins with both mucosal- and muscle-activated reflexes.

ACKNOWLEDGMENTS
S. Vanner was supported by a grant from the Canadian Institute of Health.

REFERENCES
G790 SENSORY NEUROTRANSMITTERS MEDIATING VASODILATION