Myenteric neurons activate submucosal vasodilator neurons in guinea pig ileum

S. VANNER
Gastrointestinal Diseases Research Unit, Departments of Biology, Medicine, and Physiology, Queen’s University, Kingston, Ontario, Canada K7L 5G2
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Vanner, S. Myenteric neurons activate submucosal vasodilator neurons in guinea pig ileum. Am J Physiol Gastrointest Liver Physiol 279: G380–G387, 2000.—This study examined whether myenteric neurons activate submucosal vasodilator pathways in in vitro combined submucosal-myenteric plexus preparations from guinea pig ileum. Exposed myenteric ganglia were electrically stimulated, and changes in the outside diameter of submucosal arterioles were monitored in adjoining tissue by videomicroscopy. Stimulation up to 18 mm from the recording site evoked large TTX-sensitive vasodilations in both orad and aborad directions. In double-chamber baths, which isolated the stimulating myenteric chamber from the recording submucosal chamber, hexamethonium or the muscarinic antagonist 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine hydrochloride (4-DAMP) almost completely blocked dilations when superfused in the submucosal chamber. When hexamethonium was placed in the myenteric chamber ~50% of responses were hexamethonium sensitive in both orad and aborad orientations. The addition of 4-DAMP or substitution of Ca\(^{2+}\)-free, 12 mM Mg\(^{2+}\) solution did not cause further inhibition. These results demonstrate that polysynaptic pathways in the myenteric plexus projecting orad and aborad can activate submucosal vasodilator neurons. These pathways could coordinate intestinal blood flow and motility.

vasodilator reflexes; intestinal blood flow; enteric reflexes; submucosal arterioles

THE DEMANDS FOR MUCOSAL BLOOD flow in the gastrointestinal microcirculation are significantly greater than in other peripheral vascular beds (14, 17). Mucosal oxygen and transcapillary fluid and solute transport needs vary significantly, depending on the physiological state of the intestine. For example, vascular perfusion to intestinal mucosa increases by up to 100% during digestion (9, 15). The rate of vascular perfusion to the mucosa is directly controlled by the contractile state of submucosal arterioles, the major resistance vessels in the gastrointestinal tract (14). Although multiple regulatory systems are important in modulating this tone (14, 17, 32), neural regulatory mechanisms are poised to rapidly respond to the widely fluctuating demands as stimuli are constantly changing within the lumen of the intestine.

Although extrinsic neural reflexes have been implicated in the regulation of intestinal blood flow, functional studies (4, 5, 14, 17, 19) have demonstrated that important neural vasodilator reflexes exist within the intestine. These studies suggest that enteric neural reflexes can respond to increased mucosal requirements for blood flow as chyme moves through the lumen. For example, in in vivo studies (14, 17) both local mechanical and chemical stimulation of the mucosa have been shown to initiate neurally mediated reflex vasodilation. This action is mediated by enteric nerves because these responses were not blocked by extrinsic denervation (3, 19). The neuronal pathways mediating these enteric neural reflexes could not, however, be deduced from these in vivo studies. More recently, in vitro studies (24) of guinea pig ileum have demonstrated that submucosal cholinergic vasodilator neurons innervating submucosal arterioles appear to be the major “final common pathway” mediating enteric vasodilator reflexes. In vitro studies (32) have also shown that mechanical stimulation of the mucosa can activate these neurons. This reflex, however, was mediated entirely by submucosal neurons because the myenteric plexus had been dissected from this preparation. Nonetheless, there is evidence that implies that vasodilator reflexes are also mediated by the myenteric plexus. For example, in vivo studies show a close relationship between intestinal motility and blood flow (10); such actions may well be coordinated by neural connections between the myenteric and submucosal plexus. The existence of multiple enteric neural pathways is consistent with the parallel and overlapping nature of other effector systems within the intestine that regulate blood flow (14, 17, 32). Which of these systems predominates appears to depend on the physiological and pathophysiological state of the intestine.

Multiple neural projections from neurons in the myenteric plexus with axons terminating in the submucosal plexus have been described in immunohistochemical studies (20, 26). Our (22) recent in vitro electrophysiological studies have demonstrated that most submucosal neurons receive synaptic inputs after stimulation of the myenteric plexus, but the functional

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role of these neurons was not known. In the current study, we examined whether these inputs activated submucosal vasodilator pathways and if so, what was the organization of these myenteric reflexes.

**METHODS**

Experimental protocols were approved by the Queen’s University Animal Care Committee. Guinea pigs (150–225 g) were rendered unconscious by isoflurane inhalation and immediately killed by cervical transection. Segments of small intestine were removed from the distal ileum, and dissection of conventional submucosal in vitro preparations (33) was modified to expose the submucosal plexus on one half and the myenteric plexus on the other (Fig. 1). Briefly, the intestine was opened along the mesenteric border and pinned out flat with the mucosa facing upward. One half of the mucosa was stripped off, exposing the submucosa, and the mucosa, submucosa, and circular muscle were removed from the other half, exposing the myenteric plexus. The exposed myenteric plexus was intact with the myenteric plexus lying beneath the exposed submucosa on the other half. The orientation of the preparation was carefully recorded by placing a small hole in the orad end. Preparations (~7.5 mm wide and 15–30 mm long) were pinned in a small organ bath, and a double chamber was created by placing a small Plexiglas divider over the tissue at the junction of the exposed submucosa and myenteric plexus. Silicone gel was placed between the divider and tissue to prevent communication of solutions between the chambers. Each chamber was superfused separately with a physiological saline solution containing (in mM) 126 NaCl, 10.220.33.1 on April 12, 2017 http://ajpgi.physiology.org/ Downloaded from

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**Fig. 1.** A: schematic drawing of the combined submucosal-myenteric preparation. The myenteric ganglia were exposed on one side of the preparation (elliptical shapes joined by fiber tracts), and a bipolar tungsten stimulating electrode was placed so that it just touched the preparation over the ganglia. The submucosal plexus (small irregular elliptical shapes joined by fiber tracts) was exposed on the opposite side of the preparation and remained intact with the underlying myenteric plexus. Changes in the outside diameter of the submucosal arterioles were monitored at a single site (parallel dark lines following arterioles) using a simple videomicroscopy system. A Plexiglas divider with a silicone seal was placed at the junction of exposed submucosal and myenteric plexi, creating two chemically isolated chambers: the myenteric chamber and the submucosal chamber. Antagonists could then be added separately to each compartment. B: a representative trace of the outside diameter of a submucosal arteriole demonstrating the effects of increasing frequency of stimulation of myenteric ganglia as shown in A. The vessel was preconstricted with 9,11-dideoxy-11a,9a-epoxy-methanoprosta
glandin F2α (400 nM) from a resting outside diameter of 70 µm to enable examination of dilator responses. Maximal responses appeared between 10 and 20 Hz. C: a representative trace of the changes in outside diameter of a single submucosal arteriole in response to increasing durations of stimulus trains (20 Hz) in the myenteric ganglia. Large dilations were obtained with durations of 6–9 s. All subsequent experiments used stimulus parameters of 20 Hz for 6 s. D: representative trace showing that TTX (1 µM) blocked nerve-evoked responses in the myenteric plexus. Resting outside diameter, 71 µm.
25 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 5 KCl, 25 NaHCO₃, and 11 glucose gassed with 95% O₂:5% CO₂ at 35–36°C (pH 7.4). Nifedipine (1 μM) was added to prevent muscle movement. Drugs were added to the bath by superfusion. In a few experiments, CaCl₂ was omitted and MgCl₂ was increased to 12 mM. This modified saline solution has been shown previously to block synaptic transmission (18, 30). The integrity of the “seal” between the chambers was checked after each experiment by determining whether there was leakage of trypan blue dye between the chambers.

The outside diameter of individual submucosal arterioles was monitored using a computer-assisted videomicroscopy system (Diamtrak), as previously described (21). Briefly, this system uses an Imaging Technology PCVision frame-grabber board in an IBM PC/AT computer to digitize television images of the arteriole. This is converted to an analog signal and stored on a chart recorder. The resolution of the system is <1 μm, and the sampling rate was 15 Hz. Vasodilator responses were monitored by first preconstricting arterioles 80–95% (maximum) with 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin F₂₀ (PGF₂₀). The magnitude of the dilations was quantified by measuring the amplitude and duration at one-half the amplitude of the response and expressing the product of the two in micrometers per second.

Myenteric ganglia were stimulated (at 20 Hz for 6 s) using a Grass S88 stimulus and stimulus isolation unit with a bipolar tungsten electrode modified to stimulate several myenteric ganglia simultaneously (22). Electrodes were fashioned from insulated tungsten wire (75 μm) by bending a 3-mm distal portion parallel with the preparation and removing the insulation from this segment.

Drugs. The following drugs were used: 5-hydroxytryptamine (5-HT), TTX, hexamethonium, nifedipine, and PGF₂₀ were from Sigma (Oakville, ON, Canada); and 4-diphenylacetoxy-N-(2-chloroethyl)-piperdine hydrochloride (4-DAMP) was from Research Biochemicals (Oakville, ON, Canada).

RESULTS

General observations. The outside diameter of vasodilator responses examined in this study ranged from 62 to 87 μm (n = 97). Cursors employed by the videomicroscopy system to monitor changes in the outside diameter of the blood vessels were typically positioned proximally on a first- or second-order branch (see Fig. 1). Preliminary experiments demonstrated that when the preparation was oriented with stimulating electrode placed orad to the recording site, vasodilator responses could be consistently elicited at distances of 5–10 mm. Optimum electrical stimuli were determined by varying the frequency and duration of stimuli in control experiments (Fig. 1). Vasodilator responses were evoked by 2–30 Hz, and maximal responses were evoked at 10–20 Hz (n = 3). Train durations were studied at 20 Hz, and large, reproducible responses were evoked with durations of 6–9 s (n = 3). Based on these findings, all subsequent electrical stimuli were delivered at 20 Hz for 6 s.

Several experiments were conducted to confirm that nerve-evoked vasodilator responses resulted from activation of myenteric neurons. First, to establish that responses evoked by electrical stimulation were neurally mediated, the effects of TTX (1 μM) were examined. TTX blocked all vasodilator responses evoked by electrical stimulation (Fig. 1, n = 12). The possibility that current spread from the stimulating electrode could have activated neurons in the submucosal ganglia in the adjacent bath (see Fig. 1) was examined by cutting the preparation between the exposed myenteric plexus and submucosal plexus borders and then repining the edges together. Nerve stimulation in these preparations had no effect (n = 3).

Effects of 4-DAMP and hexamethonium in the recording chamber. The effect of cholinergic blockade was examined by stimulating myenteric neurons orad to the recording site and superfusion antagonists into the recording “submucosal chamber” (see Fig. 1). When the muscarinic antagonist 4-DAMP (1 μM) was applied (n = 5), the nerve-evoked vasodilator responses in the submucosa were completely blocked in three preparations and only small residual vasodilator responses remained in the other two (10.8 and 13.2 mm · s; Fig. 2). The ganglionic nicotinic receptor antagonist hexamethonium (500 μM) blocked nerve-evoked vasodilator responses by >80% in all preparations (n = 5, Fig. 2).

Orientation and distances of myenteric synaptic inputs. The vasodilator responses elicited by stimulating at sites 5–20 mm from the recording site in the orad and aborad direction were examined. The proportion of preparations responding were grouped into three distances: 5–10, >10–15, and >15–20 mm. Nerve-evoked vasodilator responses were elicited up to 18 mm from the recording site in both directions (Fig. 3) but not at 20 mm. At each of the three distances, there was no significant difference in the proportion of preparations that responded, regardless of whether the preparation was oriented in the orad-aborad or aborad-orad orientation (Fig. 3).

Effects of hexamethonium, 4-DAMP, and 0 Ca²⁺ and 12 mM Mg²⁺ in the stimulating myenteric chamber. The possibility that nerve-evoked vasodilator responses were elicited by nicotinic receptor-mediated pathways in the myenteric plexus was examined by studying the effects of hexamethonium (500 μM) superfused into the stimulating myenteric chamber (see Fig. 1) (n = 16). Two types of effects were observed. In ~50% of preparations, nerve stimulation evoked vasodilator responses that were completely or partially blocked by hexamethonium. In the other preparations, hexamethonium-insensitive responses were elicited (Fig. 4). No significant difference in the relative proportion of hexamethonium-sensitive to-insensitive responses was observed when stimulating orad or aborad to the recording site (Fig. 4).

Electrophysiological studies have demonstrated that many myenteric neurons receive cholinergic slow excitatory postsynaptic potentials (EPSPs) mediated by muscarinic receptors (25). The possibility that these synaptic inputs in the myenteric plexus may play a significant role in mediating the vasodilator responses was examined by studying the effect of the muscarinic antagonist 4-DAMP (1 μM) in addition to hexamethonium (500 μM), compared with hexamethonium alone. The addition of 4-DAMP did not cause a significant decrease in vasodilator responses elicited by stimulation orad or aborad to the recording site (Fig. 5). The
The possibility that other synaptic responses (i.e., noncholinergic, fast 5-HT$_3$, fast purinergic responses) may also play an important role was examined by studying the effect of 0 Ca$^{2+}$ and 12 mM Mg$^{2+}$. This solution has been shown previously to inhibit synaptic responses (17, 27). Compared with controls, a large residual vasodilator response remained in the presence of the Ca$^{2+}$-free, 12 mM Mg$^{2+}$ solution when stimulating both orad and aborad to the recording site (Fig. 5).

**DISCUSSION**

This study demonstrated that stimulation of myenteric neurons activated submucosal neural pathways,
causing vasodilation of submucosal arterioles. These vessels are the major resistance within the intestine, controlling blood flow to the mucosa (19). Our previous electrophysiological studies (22) and others (6) identified synaptic connections between the myenteric and submucosal plexus, but the functional identity of these neurons was unknown. The present study provides direct functional evidence that the myenteric plexus can play a major role in regulating mucosal function through the modulation of vasodilator reflexes in the submucosal plexus. In addition, there is evidence that some vasodilator neurons may have a dual secretomotor function (16), raising the possibility that these pathways might also coordinate secretion.

A fundamental requirement in these studies was to ensure that the nerve-evoked vasodilator responses could be activated by stimulating myenteric neurons without any direct stimulation of submucosal neural or nonneural pathways. The neural origin of the vasodilator response was confirmed by demonstrating that TTX blocked all electrically evoked vasodilator responses. The possibility that current spread from the stimulating electrode directly activated submucosal vasodilator neurons (24) was excluded by several methods. The most direct method was to demonstrate that responses were completely ablated when the preparation was severed between the edges of the exposed myenteric and submucosal halves (see Fig. 1). In these studies, the stimulating electrode was positioned within 500 μm of the cut edge. The lack of vasodilator responses in the adjacent recording site would suggest that current spread from the electrode tips did not carry for significant distances beyond this point. Additional evidence for the myenteric origin of the vasodilator responses was obtained in the studies examining the effects of antagonists in the myenteric stimulating chamber because the antagonists inhibited the responses. An additional pathway that might have been implicated in these results consisted of capsaicin-sensitive nerves. However, submucosal arterioles with their perivascular nerves were completely excised from the stimulating chamber and therefore very few capsaicin-sensitive nerves projecting from the submucosa were present in the preparation. Furthermore, all responses were blocked by TTX, whereas our previous studies (31) have shown that capsaicin-sensitive vasodilator responses are not TTX-sensitive in this preparation.

A number of conclusions can be drawn about the connections of myenteric neurons in the submucosal plexus based on the findings of this study and those of previous investigators. Several studies (1, 24) have provided strong evidence that intrinsic cholinergic submucosal vasodilator neurons are the major final com-
mon pathway mediating enteric vasodilator reflexes in this preparation. The most direct evidence was obtained in electrophysiological studies (24) in which intracellular recordings were made from single submucosal neurons. These studies (24) showed that activation of single submucosal neurons evoked a nerve-mediated dilation of adjacent submucosal arterioles. In these (24) and subsequent studies (1), it was shown that these neurons released acetylcholine from perivascular nerve terminals that in turn activated M₃ receptors on the blood vessels. The ensuing vasodilation resulted from release of nitric oxide, presumably from endothelial cells. These studies failed to find any evidence that the myenteric plexus directly innervated submucosal arterioles in normal physiological settings (24), although myenteric pathways may emerge under at least some pathophysiological settings (12, 13). In the current study, the responses were almost completely blocked by hexamethonium or 4-DAMP when placed in the submucosal recording chamber (see Fig. 2). Given the findings of the previous studies described above, this suggests that myenteric neurons activate submucosal vasodilator neurons through stimulation of nicotinic receptors within the submucosal plexus.

These findings are also consistent with our recent electrophysiological studies (22) that demonstrate that most submucosal neurons receive fast EPSPs from myenteric neurons. The possibility that nicotinic synapses in the myenteric plexus, located in close proximity to the recording site (i.e., located in the recording chamber directly above the recording site), also play a role cannot, however, be excluded. Taken together, these data suggest that myenteric neurons activate nicotinic receptors on submucosal vasodilator neurons either directly, or through stimulation of interneurons, and that the vasodilator neurons in turn release acetylcholine, which binds to muscarinic receptors on submucosal arterioles.

A somewhat unexpected finding in this study was the lack of polarity to the myenteric-submucosal vasodilator pathway. Our previous electrophysiological studies (22) have shown a clear predominance of aborad-oriented synaptic inputs from myenteric neurons. These findings correlate with a number of immunohistochemical studies (8, 9, 20, 26, 28, 29) that demonstrate several descending pathways [choline acetyltransferase (ChAT)/5-HT, ChAT/somatostatin, and ChAT/nitric oxide synthase/vasoactive intestinal polypeptide (VIP) immunoreactive fibers] from the myenteric to the submucosal plexus. An ascending myenteric interneuron pathway (ChAT/tachykinin/enkephal...
lin immunoreactive fibers) has also been identified (7), making it a likely candidate for the ascending myen-
teric-evoked vasodilator responses described in the current study. There are several possible explanations
that might explain the lack of polarity observed in the present study. First, it is probable that the majority of,
or possibly all, neurons recorded from in our electrophysiological study (22) were nonvasodilator motoneu-
rons, because these neurons appear to comprise only one or two neurons in each ganglia (24). Alternatively,
the few ascending synaptic inputs defined in these electrophysiological studies were those that predomi-
nantly innervated vasodilator submucosal motoneu-
rons. Differences in innervation of submucosal vasodi-
lator and secretomotor neurons would suggest they can be activated under in different settings, reflecting their separate functions in the intestine.

The results of this study demonstrate that acetylcho-
line is the major excitatory neurotransmitter mediat-
ing synaptic connections in myenteric plexus-evoked vasodilator reflexes. Hexamethonium caused signifi-
cant inhibition of >50% of the responses when stimu-
lating either orad or aborad to the recording site (see Fig. 4), but the addition of the muscarinic antagonist
4-DAMP did not cause further inhibition. Conse-
quently, these pathways appear to be mediated by
acetylcholine acting at nicotinic receptors on postsyn-
aptic myenteric neurons. A significant role for a non-
cholinergic component of neurogenic vasodilation by
the peptide VIP has been suggested by a large body of
previous in vivo and in vitro work (4, 5, 15, 27) per-
formed on cat blood vessels. Nonetheless, the in vitro
studies (1, 24) of the innervation of submucosal arte-
rioles in guinea pig ileum did not detect a significant noncholinergic component (however, see Ref. 33).
These differences were not resolved in the current
study as noncholinergic components were not observed
within the pathways mediating the myenteric-evoked
vasodilator responses.

In summary, this study has demonstrated that long
neural pathways in the myenteric plexus can activate
submucosal vasodilator neurons. These findings high-
light the importance of myenteric projections modulat-
ing submucosal neural activity and describe pathways
that may be important for coordinating motor and
mucosal blood flow in the intestine. For example, this
pathway could explain the finding that hyperemia ac-
companies every migrating motor complex in conscious
dogs (10). The mechanism by which the pathways
described in the current study may be activated remains
to be determined, but in the in vivo dog studies hyper-
emia was not observed if luminal contents were
drained out in the cephalad direction, suggesting that
reflexes are initiated by luminal stimulation of muco-
sal factors. 5-HT release from enterochromaffin cells
(5, 19), with ensuing activation of sensory submucosal
and/or myenteric neurons projecting within the myen-
teric plexus (2), appears to be a likely candidate for
such a role. Similar pathways may activate the reflexes
described in the current study.

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REFERENCES
1. Andriansitohaina R and Surprenant AM. Acetylcholine re-
leased from guinea-pig submucosal neurons dilates arterioles by
releasing nitric oxide from endothelium. J Physiol (Lond) 453:
2. Bertrand PP, Kunze WA, Bornstein WA, JC, Farness JB,
and Smith ML. Analysis of the responses of myenteric neurons
in the small intestine to chemical stimulation of the mucosa.
3. Biber B, Fara J, and Lundgren O. Intestinal vasodilation in
response to transmural electrical field stimulation. Acta Physiol
4. Biber B, Fara J, and Lundgren O. A pharmacologic study of
intestinal vasodilator mechanisms in the cat. Acta Physiol Scand
5. Biber B, Lundgren O, and Svanvik J. Studies on the intesti-
nal vasodilation observed after mechanical stimulation of the
6. Bornstein JC, Farness JB, and Costa M. Sources of excita-
tory synaptic input to neurochemically identified submucous
neurons of the guinea-pig small intestine. J Auton Nerv Syst 18:
7. Brookes SJH, Meedeniya ACB, Jobling P, and Costa M.
Orally projecting interneurons in the guinea-pig small intesti-
8. Costa M, Brookes SJH, Steele PA, Gibbins I, Burcher E,
and Kandiah CJ. Neurochemical classification of myenteric
neurones in the guinea-pig small intestine. Neuroscience 75:
949–967, 1996.
9. Fara JW. Postprandial mesenteric hyperemia. In: Physiology of
the Intestinal Circulation, edited by Shepherd AP and Granger
10. Fioramonti J and Bueno L. Relationship between intestinal
motility and mesenteric blood flow in conscious dog. Am J
11. Farness JB and Costa M. Neurons with 5-hydroxytryptamine-
ilike immunoreactivity in the enteric nervous system. Their pro-
jections to the guinea-pig small intestine. Neuroscience 7:
12. Galligan JJ, Costa M, and Farness JB. Changes in surviving
erve fibers associated with submucosal arteries following ex-
trinsic denervation of the small intestine. Cell Tissue Res 253:
Substance P mediates neurogenic vasodilation in extrinsically
denervated guinea-pig submucosal arteries. J Physiol (Lond)
14. Granger DN, Kvietsy PR, Korthuis RJ, and Premen AJ.
Microcirculation of the intestinal mucosa. In: Handbook of
Physiology. The Gastrointestinal System. Motility and Circula-
39, p. 1405–1474.
16. Jiang MM, Kirchessner AL, Gershon MD, and Sur-
prenant A. Cholera toxin-sensitive neurons in guinea pig sub-
mucosal plexus. Am J Physiol Gastrointest Liver Physiol 264:
17. Jodal M and Lundgren O. Neurohormonal control of gastro-
intestinal blood flow. In: Handbook of Phsyiology. The Gastroin-
18. Katz B and Maledi R. The timing of calcium action during
neuromuscular transmission. J Physiol (Lond) 189: 553–544,
1967.
19. Lundgren O, Svanvik J, and Jivegard L. Enteric nervous
system. I. physiology and pathophysiology of the intestinal tract.


