Regulation of excitatory neural input to longitudinal intestinal muscle by myenteric interneurons

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Grider, J. R. Regulation of excitatory neural input to longitudinal intestinal muscle by myenteric interneurons. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G973–G978, 1998.—The circuit of myenteric interneurons that regulate excitatory input to longitudinal colonic muscle was identified using dispersed ganglia and longitudinal muscle strips with adherent myenteric plexus from rat distal colon. The preparations enabled measurement of neurotransmitter release from interneurons and/or excitatory motoneurons innervating longitudinal muscle. 1,1-Dimethyl-4-phenylpiperazinium (DMPP) and somatostatin were used to activate myenteric neurons in dispersed ganglia and muscle strips, respectively. DMPP-stimulated vasoactive intestinal peptide (VIP) release in dispersed ganglia was inhibited by [Met]enkephalin and bicuculline and augmented by naloxone and GABA, implying that inhibitory opioid and stimulatory GABA neurons regulate the activity of VIP interneurons. In muscle strips, VIP stimulated basal and augmented somatostatin-induced substance P (SP) release; the somatostatin-induced increase in SP release was inhibited by VIP-(10–28) and N\(^{\text{N}}\)-nitro-L-arginine, implying that excitatory VIP neurons regulate tachykinin motoneurons innervating longitudinal muscle. Somatostatin inhibited [Met]enkephalin and stimulated VIP release; basal and somatostatin-stimulated VIP release were inhibited by [Met]enkephalin and bicuculline and augmented by naloxone and GABA, implying that inhibitory pathways linking somatostatin, opioid, and GABA neurons regulate VIP interneurons, which in turn regulate tachykinin and probably cholinergic motoneurons.

consistent with the topography of motoneurons, contraction or relaxation of tone in longitudinal muscle is regulated by the level of activity of excitatory motoneurons, which in turn is regulated by a variety of interneurons. Previous studies have shown that VIP, PACAP, and NO, which can cause direct relaxation of longitudinal smooth muscle cells, cause contraction when applied to innervated longitudinal muscle strips; the contraction is blocked additively by muscarinic and tachykinin antagonists and abolished by tetrodotoxin (1, 2, 6, 20–23). Furthermore, both VIP and PACAP stimulate the release of acetylcholine and tachykinins (6, 22, 34, 35). The pattern suggests that excitatory VIP/PACAP/NOS interneurons in the myenteric plexus regulate the activity of cholinergic/tachykinin neurons innervating intestinal longitudinal muscle.

The VIP/PACAP/NOS myenteric interneurons are distinct from VIP/PACAP/NOS motoneurons innervating circular muscle. Previous studies on the colonic peristaltic reflex have shown that the latter are regulated by an integrated circuit of somatostatin, opioid, and GABA neurons (11). During peristaltic activity an increase in the activity of somatostatin and GABA neurons leads to a decrease in the activity of opioid neurons. The resultant decrease in [Met]enkephalin release relieves the restraint exerted by opioid neurons on VIP/PACAP/NOS motoneurons (3, 16), thereby increasing VIP, PACAP, and NO release and inducing circular muscle relaxation (10, 14, 15).

We postulated that the same circuit of somatostatin, GABA, and opioid interneurons may also regulate VIP/PACAP/NOS interneurons, coupled to excitatory motoneurons innervating longitudinal muscle. The activity of interneurons in this circuit should lead to reciprocal contraction of longitudinal muscle during peristaltic activity.

The operation of this circuit, depicted in Fig. 1, was tested in the present study by direct measurement of neurotransmitter release from longitudinal muscle strips with adherent myenteric plexus. The preparation excludes release of neurotransmitters from motoneurons innervating circular muscle. According to the model circuit, somatostatin, opioid, and VIP/PACAP/NOS interneurons are coupled in series to each other and to cholinergic/tachykinin motoneurons innervating longitudinal muscle. An increase in somatostatin release would induce a decrease in [Met]enkephalin release, which in turn would induce an increase in VIP, PACAP, and NO release. The increase in VIP, PACAP, and NO release activates motoneurons leading to release of acetylcholine and tachykinins and inducing longitudinal muscle contraction.

MOTOR INNERVATION of intestinal longitudinal muscle consists largely of excitatory neurons, particularly in smaller mammals (4, 5, 7, 8, 24, 27, 30). In the intestine and colon virtually all motoneurons innervating longitudinal muscle contain acetylcholine; about 50% of these contain also the tachykinins, substance P (SP), and neurokinin A (4, 5, 7). Inhibitory neurotransmitters such as vasoactive intestinal peptide (VIP) are present in <3% of motoneurons (4, 5, 8, 24). In contrast, intestinal circular muscle is densely innervated with excitatory cholinergic/tachykinin neurons and inhibitory motoneurons expressing VIP, pituitary adenylate cyclase-activating polypeptide (PACAP), and nitric oxide synthase (NOS) (7, 8, 18, 24, 26, 27, 32).

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Radioimmunoassay of [Met]enkephalin, VIP, and SP. [Met]-enkephalin was measured as described previously (11) using antibody 18100 at a final dilution of 1:5,000. The limit of detection of the assay was 68 fmol/ml of original sample, and the IC50 was 272 fmol/ml. The concentration of [Met]enkephalin in the samples ranged from 112 to 234 fmol/ml. The antibody reacts fully with [Met]enkephalin and minimally (<3%) with [Leu]enkephalin but does not cross-react with dynorphin-13, SP, VIP, or VIP-(10 – 28).

Substance P was measured as described previously (15) using antibody RAS-7451 at a final concentration of 1:13,000. The limit of detection of the assay was 3 fmol/ml of original sample, and the IC50 was 9 ± 1 fmol/ml. The concentration of SP in the samples ranged from 6 to 30 fmol/ml. The antibody reacts fully with SP but does not cross-react with neurokinin A, somatostatin, [Met]enkephalin, VIP, or VIP-(10 – 28).

VIP was measured as described previously (11, 15) using antibody RAS-7161 at a final concentration of 1:13,000. The limit of detection of the assay was 3 fmol/ml, and the IC50 was 18 ± 3 fmol/ml of original sample. The concentration of VIP in the samples ranged from 9 to 38 fmol/ml. The antibody reacts fully with VIP but not with VIP-(10 – 28), somatostatin, SP, or [Met]enkephalin.

Data analysis. Neurotransmitter release was expressed as femtomoles per 100 mg tissue per minute. Values are means ± SE of n experiments; each experiment was done in tissue obtained from a different animal. Statistical significance was evaluated using Student’s t-test for paired or unpaired values.

Materials. Somatostatin and VIP-(10 – 28) were obtained from Bachem (Torrance, CA); somatostatin, [Met]enkephalin, SP, VIP, 125I-S-P, 125I-VIP, SP antibody RAS-7451, and VIP antibody RAS-7161 were from Peninsula Labs (Belmont, CA); 125I-labeled [Met]enkephalin and [Met]enkephalin antibody 18100 were from Incstar (Stillwater, MN). Naloxone, bicuculline, l-NNA, GABA, DMPP, and all other chemicals were obtained from Sigma Chemical (St. Louis, MO). Nitric oxide was prepared from NO gas (MG Industries, Valley Forge, PA).

RESULTS

Effect of opioid and GABA agonists and antagonists on basal and somatostatin-stimulated VIP release from myenteric plexus-longitudinal muscle strips. Somatostatin was used to stimulate or inhibit the release of various neurotransmitters (11). Somatostatin (1 µM) increased basal VIP release (45.6 ± 4.4 fmol·100 mg·min⁻¹) by 74.5 ± 4.8% (P < 0.001) but decreased basal [Met]enkephalin release (116.5 ± 12.6 fmol·100 mg·min⁻¹) by 52.3 ± 6.9% (P < 0.01; Fig. 2 and Table 1).

[Met]enkephalin (1 µM) decreased basal VIP release by 26 ± 2.6% (P < 0.01) and somatostatin-stimulated VIP release from 74.5 ± 4.8 to 39.3 ± 9.8% above basal level (P < 0.01; Table 1 and Fig. 2). In contrast, naloxone (10 µM) increased basal VIP release by 14.1 ± 2.1% (P < 0.005) and somatostatin-stimulated VIP release to 119.0 ± 18.7% above basal level (P < 0.005; Table 1 and Fig. 2). GABA (1 mM) also increased basal VIP release by 30.9 ± 3.7% (P < 0.005) and somatostatin-stimulated VIP release to 108.9 ± 16.5% above basal level (P < 0.02; Table 1 and Fig. 2). In contrast, bicuculline (20 µM) decreased basal VIP release by 15 ± 5% (P < 0.02) and somatostatin-stimulated VIP release by 26 ± 2.6% (P < 0.01).
somatostatin-stimulated VIP release implied that endogenous release of GABA exerts a sustained stimulatory influence on VIP release from VIP interneurons.

Effect of opioid and GABA agonists and antagonists on basal and somatostatin-stimulated VIP release from dispersed myenteric ganglia. Parallel studies in dispersed myenteric ganglia confirmed the sustained inhibitory influence of [Met]enkephalin and stimulatory influence of GABA on VIP release from interneurons. DMPP (100 µM) increased VIP release by 12.8 ± 1.4 fmol·100 ganglia⁻¹·min⁻¹ or 164 ± 14% above a basal level of 7.7 ± 0.9 fmol·100 ganglia⁻¹·min⁻¹ (n = 4, P < 0.01; Fig. 3). DMPP-stimulated VIP release was abolished by [Met]enkephalin (0.9 ± 0.4 fmol·100 ganglia⁻¹·min⁻¹) but augmented 109 ± 10% by naloxone to 26.2 ± 2.4 fmol·100 ganglia⁻¹·min⁻¹ above basal level (n = 4, P < 0.01; Fig. 3). DMPP-stimulated VIP release was augmented 54.7 ± 8.9% by GABA to 19.8 ± 1.9 fmol·100 ganglia⁻¹·min⁻¹ above basal level (n = 4, P < 0.01) and inhibited 45 ± 10% by bicuculline to 7.4 ± 1.0 fmol·100 ganglia⁻¹·min⁻¹ above basal level (n = 4, P < 0.01; Fig. 3).

Effect of opioid and GABA agonists and antagonists on basal and somatostatin-stimulated SP release from myenteric plexus-longitudinal muscle strips. Somatostatin (1 µM) increased basal SP release (24.9 ± 2.7 fmol·100 mg⁻¹·min⁻¹) by 90.9 ± 15.7% (P < 0.001; Table 1). [Met]enkephalin (1 µM) increased basal SP release by 7.8 ± 0.4% (P < 0.01) and somatostatin-stimulated SP release from 90.9 ± 15.7 to 37.3 ± 7.6% above basal level (P < 0.001; Table 1 and Fig. 4). In contrast, naloxone (10 µM) increased basal SP release by 69.1 ± 14.4% (P < 0.01) and somatostatin-stimulated SP release to 234.1 ± 21.3% above basal level (P < 0.001; Table 1 and Fig. 4). GABA (1 mM) increased basal SP release by 103.9 ± 14.5% (P < 0.005) and somatostatin-stimulated SP release to 185.3 ± 15.7% above basal level (P < 0.005; Table 1 and Fig. 4).

Table 1. VIP and SP release from rat colonic longitudinal muscle strips with adherent myenteric plexus

<table>
<thead>
<tr>
<th>Agents</th>
<th>ΔVIP</th>
<th>ΔSP</th>
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<tbody>
<tr>
<td>Basal release</td>
<td>45.6 ± 4.4</td>
<td>24.9 ± 2.7</td>
</tr>
<tr>
<td>Somatostatin (1 µM)</td>
<td>+34.0 ± 4.1</td>
<td>+19.9 ± 2.3</td>
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<tr>
<td>(P &lt; 0.001)</td>
<td>(P &lt; 0.001)</td>
<td></td>
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<tr>
<td>[Met]enkephalin (1 µM)</td>
<td>-13.5 ± 3.9</td>
<td>-17.7 ± 0.4</td>
</tr>
<tr>
<td>(P &lt; 0.02)</td>
<td>(P &lt; 0.02)</td>
<td></td>
</tr>
<tr>
<td>Naloxone (10 µM)</td>
<td>+5.7 ± 1.0</td>
<td>+19.5 ± 4.6</td>
</tr>
<tr>
<td>(P &lt; 0.001)</td>
<td>(P &lt; 0.005)</td>
<td></td>
</tr>
<tr>
<td>GABA (1 mM)</td>
<td>+10.7 ± 1.1</td>
<td>+25.9 ± 7.3</td>
</tr>
<tr>
<td>(P &lt; 0.005)</td>
<td>(P &lt; 0.02)</td>
<td></td>
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<tr>
<td>Bicuculline (20 µM)</td>
<td>-7.4 ± 2.9</td>
<td>-5.2 ± 1.7</td>
</tr>
<tr>
<td>(P &lt; 0.05)</td>
<td>(P &lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>NO (1 µM)</td>
<td>+27.2 ± 7.2</td>
<td>+15.3 ± 2.0</td>
</tr>
<tr>
<td>(P &lt; 0.02)</td>
<td>(P &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>L-NNA (100 µM)</td>
<td>NA</td>
<td>-2.4 ± 2.1</td>
</tr>
<tr>
<td>VIP (1 µM)</td>
<td>NA</td>
<td>+12.9 ± 1.4</td>
</tr>
<tr>
<td>(P &lt; 0.01)</td>
<td>(P &lt; 0.01)</td>
<td></td>
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<tr>
<td>VIP-(10–28) (10 µM)</td>
<td>NA</td>
<td>-5.6 ± 3.6</td>
</tr>
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<td>(NS)</td>
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Values are means ± SE in fmol·100 mg wt⁻¹·min⁻¹. Vasoactive intestinal peptide (VIP) and substance P (SP) release in response to various agents is expressed as the change (Δ) from basal release. NO, nitric oxide; l-NNA, N⁵-nitro-l-arginine; NA, not applicable; NS, not significant.
contrast, bicuculline (20 µM) decreased basal SP release by 22 ± 2% (P < 0.01) and somatostatin-stimulated SP release to 46.4 ± 5.1% above basal level (P < 0.01; Table 1 and Fig. 4). As for VIP release, the pattern of inhibition of basal and somatostatin-stimulated SP release by [Met]enkephalin and bicuculline and stimulation by GABA and naloxone was consistent with the postulates of the model depicted in Fig. 1. The fact that naloxone augmented basal and somatostatin-stimulated SP release implied that endogenous release of [Met]enkephalin exerts a continuous restraint on SP release either directly or indirectly via inhibition of the release of VIP from VIP interneurons. Similarly, the fact that bicuculline inhibited basal and somatostatin-stimulated SP release implied that endogenous release of GABA exerts a sustained stimulatory influence on SP release either directly or indirectly via stimulation of the release of VIP from VIP interneurons.

Effect of VIP, a VIP antagonist, NO, and a NOS inhibitor on basal and somatostatin-stimulated SP release from myenteric plexus-longitudinal muscle strips. VIP (1 µM) increased basal SP release by 59 ± 11% and augmented somatostatin-stimulated SP release from 90.9 ± 15.7 to 174.8 ± 9.5% above basal level (P < 0.01; Table 1 and Fig. 5). In contrast, VIP-(10—28) had no significant effect on basal SP release but decreased somatostatin-stimulated SP release to 30.0 ± 9.9% above basal level (P < 0.01; Table 1 and Fig. 5). Exogenous NO, like VIP, increased basal SP release by 65 ± 9% (P < 0.01) and augmented somatostatin-stimulated SP release to 248.6 ± 23.1% above basal level (P < 0.01; Table 1 and Fig. 5). The NOS inhibitor, L-NNA (100 µM) had no significant effect on basal SP release but decreased somatostatin-stimulated SP release to 26.3 ± 4.1% above basal level (P < 0.01; Table 1 and Fig. 5).

DISCUSSION

We have previously shown that a modulatory circuit consisting of somatostatin, opioid, and GABA neurons regulates the activity of inhibitory VIP/PACAP/NOS motoneurons innervating the circular muscle layer of the rat colon (11). The present study shows that a similar circuit regulates also the activity of excitatory VIP/PACAP/NOS interneurons, which in turn regulate the activity of excitatory tachykinin neurons innervating the longitudinal muscle layer. The study was made possible by the use of longitudinal muscle strips with adherent myenteric plexus, thereby precluding release of neurotransmitters from neural projections to the circular muscle layer. The results were corroborated by studies in dispersed ganglia isolated from the myenteric plexus of the rat colon.

Evidence for the operation of the neuronal circuit (Fig. 1) regulating longitudinal muscle function is based on the use of selective antagonists to block the effect of neurotransmitters released at specific synapses. The evidence for the interplay of neurons in this circuit may be summarized as follows.

Somatostatin inhibited [Met]enkephalin release but stimulated VIP and SP release; the stimulation of VIP release resulted from elimination of the restraint exerted by opioid neurons on excitatory VIP interneurons. The serial coupling of two inhibitory pathways involving somatostatin and opioid neurons resulted in activation of excitatory VIP interneurons, which in turn activated tachykinin motoneurons.

[Met]enkephalin inhibited basal and somatostatin-stimulated VIP and SP release, whereas naloxone had the opposite effect. The stimulatory influence of naloxone implied that opioid neurons exert a continuous

![Fig. 4. Effect of opioid and GABA agonists and antagonists on somatostatin (SS)-stimulated SP release from excitatory tachykinin motoneurons.](Image)

![Fig. 5. Effect of VIP and NO and the VIP antagonist VIP-(10—28) and NOS inhibitor N⁶-nitro-L-arginine (L-NNA) on somatostatin (SS)-stimulated SP release from excitatory tachykinin motoneurons.](Image)
restraint on VIP interneurons and SP motoneurons (9, 19). The continuous restraint exerted on VIP interneurons was corroborated by studies in dispersed myenteric ganglia, where naloxone was shown to augment DMPP-stimulated VIP release. The results in isolated myenteric ganglia are consistent with immunocytochemical evidence for the existence of VIP interneurons in the myenteric plexus (7, 8, 24, 25) and imply that opioid neurons can project to or influence the activity of VIP interneurons.

VIP and NO stimulated basal SP release and augmented somatostatin-stimulated SP release. The VIP antagonist VIP-(10—28) and the NOS inhibitor L-NNA inhibited somatostatin-stimulated SP release. The inhibitory influence of VIP-(10—28) and L-NNA implied that VIP/NOS interneurons stimulate the activity of tachykinin motoneurons.

GABA increased basal VIP and SP release and augmented somatostatin-stimulated VIP and SP release, whereas the GABA<sub>A</sub> receptor antagonist bicuculline had the opposite effect. The inhibitory influence of bicuculline implied that GABA neurons exert a sustained stimulatory influence on VIP and SP release. This conclusion was corroborated by studies in dispersed myenteric ganglia, where GABA augmented and bicuculline inhibited DMPP-stimulated VIP release. The results obtained in myenteric ganglia imply that GABA neurons project to and can influence the activity of VIP interneurons. Previous studies (11) in whole segments of rat colon had shown that GABA and opioid neurons are coupled via reciprocal inhibitory pathways, such that a decrease in opioid neuronal activity leads to an increase in GABA neuronal activity.

The excitatory VIP/NOS interneurons also express PACAP in rat intestine (18). It is therefore likely that PACAP is released together with VIP and NO to activate tachykinin motoneurons; the latter also releases acetylcholine and is more suitably viewed as cholinergic/tachykinin motoneurons (4, 5, 7). Studies in myenteric plexus-longitudinal muscle preparations from guinea pig intestine confirm the ability of VIP, PACAP, and NO to stimulate acetylcholine and SP release and induce muscle contraction that can be additively blocked by atropine and tachykinin antagonists (1, 2, 6, 19–23).

The fact that some opioid neurons also project into longitudinal smooth muscle raises the possibility that opioid neurons may also exert a continuous presynaptic restraint on cholinergic/tachykinin motoneurons (8, 9, 19, 24). Elimination of this restraint by somatostatin or presynaptically by naloxone should also lead to activation of cholinergic/tachykinin motoneurons. Thus two pathways involving somatostatin and opioid neurons could lead eventually to activation of cholinergic/tachykinin motoneurons innervating longitudinal muscle: one pathway involves serial coupling of somatostatin, opioid, and VIP/PACAP/NOS interneurons as depicted in Fig. 1; the other pathway represents direct presynaptic regulation of cholinergic/tachykinin motoneurons by opioid nerve terminals in longitudinal muscle.

The stimulatory influence of GABA neurons on VIP/NOS interneurons demonstrated in the present study parallels the stimulatory effect previously demonstrated in whole rat colonic segments (11). As shown in Fig. 1, elimination of the inhibitory restraint exerted by opioid neurons on excitatory GABA neurons leads to an increase in VIP release and thus to an increase in SP release. It is possible, however, that SP release could result also from an excitatory presynaptic effect of GABA on cholinergic/tachykinin motoneurons. Here again, two pathways may mediate the excitatory effects of GABA on cholinergic/tachykinin motoneurons innervating longitudinal muscle: one pathway involves serial coupling of somatostatin, opioid, GABA, and VIP neurons as depicted in Fig. 1, for which corroborative evidence exists based on studies in isolated ganglia; the other pathway represents direct presynaptic regulation of cholinergic/tachykinin motoneurons by GABA acting via bicuculline-sensitive GABA<sub>A</sub> receptors located on cholinergic/tachykinin nerve terminals (28, 31). The operation of the latter pathway depends on whether GABA neurons can be shown to project into the longitudinal muscle layer.

As previously shown (11) the circuit consisting of somatostatin, GABA, and opioid neurons coupled to inhibitory VIP/PACAP/NOS motoneurons innervating circular muscle determines relaxation of circular muscle during the descending phase of the peristaltic reflex. The same circuit appears to regulate also the activity of excitatory VIP/PACAP/NOS interneurons coupled to excitatory cholinergic/tachykinin motoneurons innervating longitudinal muscle. This should result in reciprocal contraction of longitudinal muscle (12, 29, 33). Recent studies (12), in effect, provide strong evidence that relaxation of circular muscle is accompanied by contraction of longitudinal muscle during the descending phase of the peristaltic reflex; the pathway mediating longitudinal muscle contraction consists of an integrated circuit of somatostatin, opioid, GABA, and VIP/PACAP/NOS interneurons serially coupled to cholinergic/tachykinin motoneurons.

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