Organization of intrinsic cholinergic neurons projecting within submucosal plexus of guinea pig ileum

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Moore, B. A., and S. Vanner. Organization of intrinsic cholinergic neurons projecting within submucosal plexus of guinea pig ileum. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G490–G497, 1998.—Electrophysiological techniques were employed to examine the organization of the projections of submucosal neurons in the submucosal plexus of guinea pig ileum. These neurons were activated by focal pressure-pulse application of 5-hydroxytryptamine (5-HT) to single ganglia in submucosal preparations in vitro, and resulting fast excitatory postsynaptic potentials (EPSPs) were recorded intracellularly in S-type neurons. 5-HT-evoked fast EPSPs were blocked by TTX, hexamethonium, and ICS-205-930 (tropisetron). 5-HT was applied either directly to the ganglion containing the neuron recorded intracellularly or to adjacent ganglia positioned at increasing distances on either side of the impaled cell in circumferential or longitudinal orientations. All S-type neurons recorded in this study (n = 103) received nicotinic fast EPSPs from cholinergic neurons when 5-HT was applied directly to the ganglion containing the impaled neuron. Stimulation of adjacent ganglia also evoked nicotinic fast EPSPs, but the number of neurons that received this input decreased as the distance between the stimulus and the impaled cell increased. Maximal projections were 3 mm in the circumferential and orad-to-aboral orientations. There were no significant projections in the aborad-to-orad direction. These findings suggest that S-type neurons in the submucosal plexus are innervated by intrinsic cholinergic neurons that project over relatively short distances and have a distinct orad-to-aboral polarity.

5-hydroxytryptamine; enteric reflexes; submucosal neurons

Almost 100 years have passed since the pioneering studies of Bayliss and Starling (1) ascertained that neural reflexes confined to the intestine were important in the regulation of gastrointestinal function. Since their seminal observations of the peristaltic reflex (1), many of the cellular components of the neural circuits in the myenteric plexus that mediate this reflex have been elucidated (15, 27). These circuits can function independently of connections with the central nervous system (CNS) but contain the basic components of CNS reflexes, i.e., sensory neurons, interneurons, and motoneurons. The interneuron plays a key role in the integration of both CNS and peristaltic reflexes by coordinating the spread of synaptic activity within selected circuits. The neural pathways that underlie the peristaltic reflex involve motoneuronal interneurons, which convey ascending and descending excitatory or inhibitory synaptic inputs to motoneurons controlling smooth muscle contractility (8, 10). Individual interneurons may project up to 10 mm orad and 100 mm aborad (21). In addition, both ascending interneurons and specific populations of descending interneurons form polysynaptic chains (11, 20, 28) and thus have the capacity to coordinate orad and aborad synaptic activity over considerable distances. Evidence suggests (9, 26) that intrinsic neural reflexes confined to the submucosal plexus may regulate mucosal function in the gastrointestinal tract by controlling secretion and blood flow. There is, however, little known about the role played by interneurons in coordinating these responses.

The possibility that interneurons also exist within the submucosal plexus was first suggested by combined selective lesioning and immunohistochemical studies (6). In these studies (6), submucosal neurons from control animals were found to receive innervation from multiple cholinergic fibers, but it was unclear whether these fibers originated from neurons in the submucosal plexus or myenteric plexus. Therefore, studies were repeated on animals in which the axons of myenteric neurons had been selectively ablated after surgical myectomy. Although the number of inputs were significantly reduced after the surgery, cholinergic synaptic inputs could still be found. Because vagal fibers do not appear to innervate the submucosal plexus (15), it was concluded that these inputs must originate from submucosal interneurons projecting to other submucosal neurons. Functional studies have also shown that neural elements that mediate reflex vasodilation of submucosal arterioles after mucosal stimulation exist within the submucosal plexus (26). Many of these responses were blocked by nicotinic antagonists, suggesting that cholinergic interneurons were involved. Together, these studies strongly suggest that interneurons exist in the submucosal plexus but provide little information about how they are organized to integrate neural inputs.

This study examined the organization of submucosal neurons projecting within the submucosal plexus by using focal pressure-pulse application of 5-hydroxytryptamine (5-HT) to activate the cell bodies of neurons within single ganglia. Previous studies (23, 25) have shown that 5-HT is a robust stimulus that readily activates submucosal neurons with discharge of action potentials and ensuing release of neurotransmitter(s). Using this stimulation technique, we identified neurons projecting from within the submucosal plexus when intracellular recordings from adjacent submucosal neurons displayed synaptic potentials in response to 5-HT activation of one or more neurons in a stimulated ganglion. The orientations of the projections of the neurons that elicited these synaptic inputs and the maximum distances over which they could be recorded were determined by positioning the stimulus at increasing distances medial-lateral and orad-aboral to the cell that was recorded intracellularly.
METHODS

Adult Hartley guinea pigs (125–250 g) of either sex were obtained from Charles River Laboratories (Montreal, QC, Canada). Experiments were performed according to the guidelines of the Canadian Council of Animal Care. Animals were rendered immediately unconscious by a blow to the head and killed by carotid and cervical transection. The abdomen was opened, and segments of ileum were excised ~10 cm proximal to the ileocecal junction. The aborad end of each segment was marked with a suture. Ileal segments were opened on the mesenteric border, the tissue was pinned flat, and the mucosa was removed. Submucosal preparations were dissected from the underlying muscle layers as described previously (24). Each preparation was oriented by cutting a small hole in the aborad end. Preparations were pinned in a small organ bath (0.5–1.0 ml) and superfused with a physiological saline solution containing (in mM) 126 NaCl, 2.5 NaH2PO4, 1.2 MgCl2, 2.5 CaCl2, 5 KCl, 25 NaHCO3, and 11 glucose, which was continuously gassed with 95% O2-5% CO2 at 35–36°C.

Intracellular impalements of submucosal neurons lasting 30 min to 3 h were obtained using glass microelectrodes filled with 2 M KCl (70–120 MΩ). Synaptic inputs to these neurons were evoked by electrically stimulating adjacent ganglia using bipolar platinum electrodes (20 Hz, 400-ms pulse duration). Changes in membrane potential were recorded with an Axoclamp 2A amplifier and displayed on a Gould TA240 chart recorder or digitized at 5–10 kHz using a Digidata 1200A acquisitions board and Axoscope software (Axon Instruments). Neurons were classified as S-type neurons based on the absence of a prolonged action potential afterhyperpolarization (<4 s) and the presence of fast nicotinic synaptic inputs (16, 19). Intrinsic submucosal neurons that innervated S-type neurons from which the intracellular recordings were made were selectively sought out using pressure-pulse application of 5-HT (100 µM) from a glass puffer pipette (5- to 10-ms pulse duration) using a variable pressure Picopressor (General Valve). 5-HT was applied to the same ganglia from which the intracellular recording was obtained or to adjacent ganglia. Distances between the ganglia stimulated by 5-HT and the one containing the impaled neuron were measured using a calibrated eyepiece graticule that was accurate to within 0.02 mm. Distances were recorded as 0 (same ganglia) or ≤1–4 mm. Careful attention was made to choose ganglia that very closely followed the orientation in question. Distances recorded for movement of the puffer pipette out of the field of high-power view (×32 objective) were determined by recording the change on the vernier scale on the micromanipulator and, after completion of the experiment, using the eyepiece graticule on lower power (×5 objective) to convert the vernier scale to distance in micrometers.

Chemicals

5-HT, TTX, hexamethonium (Sigma Chemical), and ICS-205-930 (tropisetron; Sandoz) were dissolved in deionized water. 5-HT was diluted from a 10 mM stock solution to 100 µM with Krebs buffer.

RESULTS

Identification of S-type Neurons and Submucosal Neurons Projecting Within the Submucosal Plexus

S-type neurons. The resting membrane potential of submucosal neurons recorded intracellularly ranged from ~49 to ~65 mV (n = 103). Neurons were classified as S-type neurons based on the presence of fast excitatory postsynaptic potentials (EPSPs) and the absence of prolonged action potential afterhyperpolarizations (16, 19). All cells received fast EPSPs (range 3–10 mV) and slow EPSPs of ≥5 mV (range 5–15 mV) in response to focal electrical stimulation of adjacent ganglia. These synaptic potentials were reversibly blocked by TTX (1 µM; n = 8). Action potential afterhyperpolarizations elicited by intracellular stimulation were typically <500 ms and not >4 s, characteristic of S-type neurons (16, 19). Of the 103 S-type neurons, 95 also received inhibitory postsynaptic potentials (see Fig. 1B). Previous combined electrophysiological and immunohistochemical studies (2, 5, 6, 13) have suggested that these neurons are the vasoactive intestinal polypeptide-containing secretomotor neurons.

Submucosal neurons projecting within submucosal plexus. Submucosal neurons that innervated the intracellularly recorded S-type neurons described above were sought, using 5-HT stimulation applied by pressure-pulse application to the same or adjacent ganglia. These neurons were positively identified when 5-HT stimulation elicited nerve-evoked postsynaptic potentials in the intracellularly recorded neuron. 5-HT pressure pulses (3–10 ms, 100 µM) could be applied repeatedly to the same ganglion at 1- to 2-min intervals without desensitizing the response (Fig. 1). It was concluded that the evoked potentials originated from the neurotransmitter released by a second neuron, because responses were blocked by TTX (1 µM) and hexamethonium (100 µM) (Fig. 1). To ensure that 5-HT released from the pressure-pulse pipette was not diffusing to a ganglia other than the one positioned beneath the puffer pipette, we examined the effect of moving the pipette laterally a short distance off the ganglion (80–120 µm). In each case, the 5-HT-evoked potentials were abolished (Fig. 1). When the 5-HT stimulation failed to elicit fast EPSPs in the recorded ganglia, the stimulation was repeated with pulse durations up to 50 ms. In addition, the S-type neuron was hyperpolarized to ~90 mV, which would increase the amplitude of all nicotinic fast EPSPs (16, 19). Hyperpolarizing the neuron did not expose any fast EPSPs that were not recognized at the resting membrane potential. The viability of the impaled cell was also tested by applying 5-HT directly to the neuron, either by pressure pulse after moving the puffer pipette to the recording site or by adding 5-HT (1 µM) to the superfusate. All of these cells exhibited fast EPSPs and membrane depolarization in response to the direct application of 5-HT.

Submucosal Neurons Projecting Within a Single Ganglion

5-HT (100 µM; 3-ms pulse duration) applied directly to the ganglion containing the impaled neuron (Fig. 1A) evoked fast nicotinic-like excitatory potentials superimposed on biphasic depolarizations in all S-type neurons (Fig. 1C; n = 20). The biphasic depolarization results from the activation of 5-HT receptors located directly on the cell membrane of the neuron recorded intracellularly (12, 23). These depolarizations were not blocked by TTX (Fig. 1). Fast nicotinic-like excitatory potentials
were superimposed on the first phase of the biphasic depolarization (Fig. 1D), and these were blocked by pretreatment for 3 min with TTX (n = 3) and hexamethonium (n = 3). In some cases, fast depolarizations reached threshold for generation of action potentials and somal spikes were recorded (n = 8). Previous studies (12) have shown the first phase of the 5-HT evoked biphasic response is mediated by 5-HT$_3$ receptors on the cell membrane. In the present study, the 5-HT$_3$ receptor antagonist ICS-205-930 (600 nM for 3 min; n = 4) blocked both the first phase response and superimposed fast EPSPs (Fig. 2). ICS-205-930 had no effect on electrically evoked synaptic potentials, as previously described (23, 25).

Submucosal Neurons in Adjacent Ganglia Projecting Circumferentially

5-HT was applied by puffer pipette to ganglia positioned at increasing distances on either side of the neuron recorded intracellularly in the circumferential orientation (Fig. 3A). Typically, only one to three gan-
stimulation is repeated in the presence of the 5-HT3 antagonist superimposed fast EPSPs and an action potential. Right: first phase response on an expanded time scale, demonstrating neuron from which the intracellular recording was obtained. Middle: the first phase of the biphasic depolarization is completely blocked, as previously shown (23). Middle right: expanded time scale shows that the fast EPSPs were also blocked. Bottom: after washout of ICS-205-930, reaplication of 5-HT evokes the control biphasic depolarization (left) and fast EPSPs (right). Resting membrane potential of the neuron was -51 mV.

glia could be stimulated during each intracellular recording. Fast EPSPs were recorded in submucosal S-type neurons in response to pressure-pulse application of 5-HT (5-10 ms pulse duration) in 26 of 48 cells (Fig. 3B). Fast EPSPs were abolished by pretreatment with hexamethonium (n = 6) or TTX (n = 4) or by moving the puffer pipette off the stimulated ganglion. The percentage of submucosal neurons receiving 5-HT-evoked fast EPSPs decreased as the distance between the recording site and the stimulated ganglia was increased (Fig. 4). No fast EPSPs were recorded when this distance exceeded 3 mm.

Activation of Submucosal Neurons Projecting in Orad-Aborad Orientation

Figure 5 shows results obtained in response to the activation of ganglia containing neurons that project aborad to the neuron recorded intracellularly. The puffer pipette was placed on ganglia positioned at varying distances orad to the recorded cell (Fig. 5A). Fast EPSPs were recorded in response to pressure-pulse application of 5-HT (10 ms pulse duration) in 15 of 32 cells (Fig. 5, B and C). The fast EPSPs were abolished by hexamethonium (n = 4), TTX (n = 3), ICS-205-930 (n = 3), or by moving the puffer pipette off the stimulated ganglion. No synaptic responses could be elicited in cells >3 mm from the site of stimulation (see Fig. 7).

When 5-HT pressure-pulse stimulation was applied to ganglia positioned aborad (0.3-3.0 mm) to the recording site of the intracellularly impaled neuron (Fig. 6A), no synaptic responses could be elicited in cells >0.3 mm from the site of stimulation (Fig. 6B; n = 16). Fast EPSPs were recorded, however, when 5-HT was applied directly to the ganglion containing the neuron recorded intracellularly (Fig. 6C). This response was abolished by moving the puffer pipette off the ganglion (Fig. 6D). In this series of experiments, only one cell received fast EPSPs after 5-HT stimulation of adjacent ganglia, and this ganglion was 300 µm from the ganglion containing the impaled neuron (Fig. 7).

DISCUSSION

This study describes the properties and projections of cholinergic neurons originating within the submucosal plexus that provide synaptic input to S-type neurons. All S-type neurons examined received nicotinic fast EPSPs on activation of these cholinergic neurons by pressure-pulse application of 5-HT. Studies that examined the projections of these neurons indicate that they function in a polarized fashion. These findings suggest that submucosal cholinergic neurons are poised to provide an important mechanism for the integration of neural reflexes in the submucosal plexus.

The interpretation of the results of this study hinges on the conclusion that 5-HT activated the cell bodies of submucosal neurons and evoked the discharge of action potentials with ensuing release of neurotransmitter onto neurons that they innervated. The possibility that the 5-HT-evoked potentials were the result of a direct effect of 5-HT on the membrane of the S-type neuron recorded intracellularly, rather than a second submucosal neuron, was excluded by studies that examined the effects of TTX and hexamethonium. TTX, which selectively blocks sodium channels, completely blocked the 5-HT-evoked fast EPSPs, demonstrating that action potential discharge was a necessary prerequisite. Fast EPSPs were completely blocked by hexamethonium, indicating that 5-HT-evoked responses were mediated by the interaction of ACh with nicotinic receptors. Evidence against the nonselective diffusion of 5-HT from the stimulation site was provided by studies in which the effects of moving the 5-HT pipette off the stimulated ganglia were examined. In each case, this resulted in loss of the 5-HT-evoked fast EPSPs. The possibility that 5-HT acted on pre synaptic terminals rather than directly on the cell membrane of the stimulated neuron was also addressed in this study by demonstrating that all 5-HT-evoked fast EPSPs were blocked by the 5-HT3 receptor antagonist ICS-205-930. Previous studies have shown that 5-HT3 receptors are found on the cell soma but not on pre synaptic terminals (12, 23). Taken together, the data provide strong evidence that 5-HT activates 5-HT3 receptors on one or more submucosal neurons, which depolarizes the cell, eliciting action potential discharge and the ensuing
release of ACh from nerve terminals onto nicotinic receptors on S-type neurons.

The cholinergic neurons described in this study may derive from more than one class of submucosal neuron. Four chemical classes of neurons have been identified within the submucosal plexus, and specific functional roles have been suggested based on combined immunohistochemical and electrophysiological studies (5, 6, 11). Three of these classes are immunoreactive for choline acetyltransferase (ChAT; a marker of cholinergic activity) and thus could potentially provide nicotinic synaptic inputs to other submucosal neurons. These include the substance P (SP)-immunoreactive putative intrinsic sensory neurons, the neuropeptide Y (NPY)-immunoreactive cholinergic secretomotor neurons, and a third group encoded by ChAT only, lacking both SP and NPY. Studies employing tracer and lesioning techniques indicated that ChAT-only neurons projected to other ganglia within the submucosal plexus, whereas SP and NPY neurons did not (5, 6, 11). It was concluded that the ChAT-only neurons were most likely interneurons and thus would be the predominant source from within the submucosal plexus of cholinergic input to submucosal neurons. More recently, this view has been challenged by tracer studies of single submucosal neurons that provide morphological evidence that other classes of neurons could potentially act as interneurons (13). This study (13) demonstrated that projections from NPY- and SP-immunoreactive neurons form axonal “tufts” around other submucosal neurons in the same and adjacent ganglia, suggesting that they make functional connections with these neurons. However, electrophysiological studies (18) suggest that sensory neurons do not provide fast EPSPs to S-type neurons. In these studies, simultaneous intracellular impalements were made from myenteric sensory neurons and S-type neurons. When the sensory neurons were stimulated intracellularly with a pulse train, only slow depolarizations were recorded in the paired S-type neuron. Because myenteric sensory neurons exhibit electrophysiological and immunohistochemical properties similar to those of sensory neurons in the submucosal plexus (7) and since slow EPSPs were not recorded in response to 5-HT application in the current study, it appears unlikely that sensory neurons constitute a significant source of cholinergic input to S-type neurons under the stimulus conditions employed here. When these findings are taken together, the data favor the concept that the cholinergic neurons described in this study represent one or more neuronal subclasses acting as interneurons, but a contribution by submucosal sensory neurons cannot be entirely excluded.

Fig. 3. Submucosal neurons are innervated by adjacent submucosal cholinergic neurons projecting in a circumferential direction. A: schematic drawing shows relative placement of stimulating and recording devices as described in Fig. 1. The distance between the 5-HT-stimulating puffer pipette and the intracellular recording electrode was 0.5 mm. B: representative trace shows that 5-HT pressure-pulse application (100 µM, 10 ms; arrow) evoked a burst of fast EPSPs in control recordings. When 5-HT stimulation was repeated in the presence of hexamethonium (100 µM) and TTX (1 µM), fast EPSPs were blocked. In each case, 5-HT-evoked fast EPSPs returned after a 10-min washout of the antagonist. 5-HT-evoked fast EPSPs were also abolished by moving puffer pipette 100 µm off the ganglion. Resting membrane potential was −60 mV.

Fig. 4. Summary of % of neurons innervated by 5-HT-activated submucosal cholinergic neurons projecting circumferentially. Nos. in parentheses represent the no. of cells that received synaptic input over the total no. of cells examined at any given distance from the site of 5-HT stimulation. The % of neurons innervated by submucosal neurons decreases as the distance from the site of stimulation increases.
The projections of the cholinergic neurons were confined to relatively short distances. This observation is consistent with previous Ussing chamber studies (17) that demonstrated the absence of submucosal secretomotor responses >4 mm from the stimulus. These findings contrast with those in the myenteric plexus in which single interneurons have been found to project for up to 100 mm and can form synapses with other interneurons to form polysynaptic chains (11, 20, 21, 28). The short projections observed for the submucosal neurons may suggest that submucosal reflexes, whose neural elements are confined to the submucosal plexus, may serve to precisely control mucosal function (for example, secretion) by providing a very localized response to luminal stimuli.

An unexpected finding in this study was that the submucosal cholinergic neurons described displayed a distinct orad-to-aborad polarity. Neurons projected up to 3 mm in the aborad direction, but no neurons could be found projecting for any significant distance in the orad direction (see Figs. 6 and 7). Previous studies that examined, using retrograde tracing techniques, the projections of submucosal secretomotor neurons to the intestinal mucosa showed that secretomotor neurons did not display polarity in the orad-aborad orientation (22). More recently, however, studies performed in guinea pig colon showed that chloride-dependent fluid secretion elicited in response to electrical field stimulation was found to be greater when the stimulus was applied on the orad side than when it was applied on the aborad side of a longitudinally oriented preparation (14). Thus activation of descending interneurons that innervate secretomotor neurons could serve to coordinate synaptic activity to evoke descending secretory reflexes. This polarity may enable the intestine to respond locally to luminal stimuli and/or to coordinate secretion with motility as a bolus moves through the intestine.

It was not possible to determine whether polysynaptic pathways were involved in mediating the synaptic responses elicited in this study. One approach would have been to determine whether the latency of the evoked synaptic response was variable, suggesting the pathway was polysynaptic, or alternatively very constant, implying a monosynaptic pathway was involved. This technique could not be applied, however, because the stimulation with the 5-HT pressure-pulse applications lacked sufficient time resolution, and electrical stimulation could not be employed in place of 5-HT stimulation, because synaptic responses would be contaminated by the activation of cholinergic fibers traveling “en passe” from the myenteric plexus. It seems
likely that some polysynaptic pathways could be involved, but if so, these must involve neurons projecting over very short distances given the findings of this study.

Electrophysiological studies have suggested that electrical stimulation of submucosal neurons elicits both fast and slow EPSPs in some classes of submucosal S-type neurons (6, 13). This finding originates from experiments performed in submucosal preparations after surgical myectomy, which ablates inputs from the myenteric plexus (6). Although the number of slow synaptic inputs was substantially reduced after myectomy, many cells continued to exhibit slow EPSPs in response to electrical stimulation of submucosal fiber tracts. However, in the current study, no slow EPSPs were recorded after pressure-pulse application of 5-HT. It is possible that, although 5-HT stimulation was sufficient to elicit short bursts of fast EPSPs recorded in the current study, it was not sufficient to evoke release of the neurotransmitter(s) that mediates the slow EPSP. Interestingly, in the above-mentioned electrophysiological study (6), after myectomy many neurons required multiple stimuli (3–6 impulses at 3 Hz) to elicit slow EPSPs, whereas only one pulse would have sufficed for neurons from control preparations. It is possible that submucosal interneurons and myenteric interneurons differ in their intrinsic activation properties that release the neurotransmitter(s) mediating the slow EPSP. Alternatively, it is possible that the electrical stimulation of axons in submucosal fiber tracts activated fibers from intrinsic sensory neurons as well as from interneurons. The sensory neuron has been suggested, at least in the myenteric plexus, to exclusively release neurotransmitters that elicit slow EPSPs (18).

In summary, this study has demonstrated that S-type submucosal neurons are innervated by cholinergic neurons that project over short distances circumferentially and in an orad-to-aborad direction. These findings imply that neural reflexes confined to the submucosal plexus in the ileum are organized to respond in a localized, circumferential and descending fashion to stimuli from the lumen. It is also known that important projections exist between the myenteric plexus and submucosal plexus (2–4, 6). It may be that reflexes that project over longer distances are mediated through the myenteric plexus.

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