Neural pathways regulating Brunner’s gland secretion in guinea pig duodenum in vitro

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Received 9 November 1999; accepted in final form 25 April 2000

Neural pathways regulating Brunner’s gland secretion in guinea pig duodenum in vitro. Am J Physiol Gastrointest Liver Physiol 279: G910–G917, 2000.—This study examined the neural pathways innervating Brunner’s glands using a novel in vitro model of acinar secretion from Brunner’s glands in submucosal preparations from the guinea pig duodenum. Neural pathways were activated by focal electrical stimulation and excitatory agonists, and videomicroscopy was used to monitor dilation of acinar lumen. Electrical stimulation of perivascular nerves evoked large dilations that were blocked by TTX (1 μM) or the muscarinic receptor antagonist 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine hydrochloride (1 μM). The nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium iodide (100 μM) had no effect, and the nerve-evoked responses were not inhibited by hexamethonium (200 μM). Dilations were abolished in preparations from chronically vagotomized animals. Activation of submucosal ganglia significantly dilated submucosal arterioles but not Brunner’s glands. Effects of electrical stimulation of perivascular and submucosal nerves were not altered by guanethidine. Capsaicin and substance P also dilated arterioles but had no effect on Brunner’s glands. Cholinergic (choline acetyltransferase-immunoreactive) nerve fibers were found in Brunner’s glands. These findings demonstrate that Brunner’s glands are innervated by cholinergic vagal fibers but not by capsaicin-sensitive or intrinsic enteric nerves.

cholinergic innervation; submucosal plexus; vagus; capsaicin-sensitive nerves

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THE SECRETION OF MUCIN and bicarbonate from the epithelium and Brunner’s glands into the duodenal lumen is thought to play an important role in mucosal defense against gastric acid and proteolytic enzymes (8). The concept that neural reflexes were important in mediating these responses was recognized in the 1940s when it was observed that electrical stimulation of the vagus caused the release of sticky, alkaline fluid into the proximal duodenum of cats in vivo (35) and in the 1960s when the activation of cephalic reflexes by sham feeding caused a similar secretion in dogs (26). It was not clear, however, whether these secretions were derived from the epithelium, Brunner’s glands, or both.

Since these early studies (26, 35), considerably more research has focused on duodenal epithelial secretions because, compared with Brunner’s glands, the epithelium is much more accessible. Studies that examined regions of the duodenum devoid of Brunner’s glands have demonstrated that bicarbonate secretion is regulated by both extrinsic and intrinsic pathways. These include vago-vagal reflexes (2, 7, 15, 16, 23), reflexes within the submucosal plexus of the enteric nervous system (12), C fiber efferent mechanisms (11, 29), and inputs from the sympathetic nervous system (5, 6, 22). The interactions between these neural pathways have also been studied, and the findings suggest that secretomotor reflexes within the submucosal plexus are modified by vagal and sympathetic efferents that provide excitatory and inhibitory inputs, respectively, to submucosal neurons (6,22). These studies demonstrate that neural regulation of epithelial bicarbonate and mucin secretion from intestinal epithelium involves a complex interplay between extrinsic autonomic nerves and intrinsic secretomotor neurons in the submucosal plexus.

There is considerably less known about the neural pathways mediating Brunner’s gland secretion. Early studies (35) used the histological identification of mucin glycoproteins to examine the secretory status of the glands after experimentation. It was observed (35) that prolonged stimulation of the vagus leads to depletion of mucin glycoprotein from Brunner’s glands in some animals. It was not clear whether secretion was mediated solely by extrinsic neurons or whether neural pathways located within the enteric nervous system were also involved. This lack of understanding persists because it is not possible to separate the neural elements controlling glandular secretion from those of the epithelium in vivo.

We (18) recently established an in vitro model of Brunner’s gland secretion and demonstrated that the cholinomimetic carbachol acts directly on acinar cells in guinea pig duodenum to stimulate mucin secretion, suggesting these glands receive cholinergic neural inputs. The current study employs this model and uses focal electrical stimulation and excitatory agonists to
selectively activate neural pathways. The aim of the study was to determine whether Brunner’s glands receive functional innervation and, if so, whether the neural pathways regulating secretion are mediated by extrinsic and/or enteric nerves.

METHODS

Guinea pigs (140–225 g) of either sex were obtained from Charles River Laboratories. Experiments were performed according to the guidelines of the Canadian Council of Animal Care. Animals were anesthetized with isoflurane and immediately killed by decapitation. The abdomen was opened, and the entire duodenum was removed from a point 1 cm distal to the pyloric junction to the ligament of Treitz.

Preparation of Tissue

The proximal duodenum was opened along the mesenteric border and pinned flat in Sylgard-lined petri dishes with the mucosal surface facing upward. The tissue was covered with Krebs buffer (in mM: 126 NaCl, 2.5 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 5 KCl, 25 NaHCO₃, and 11 glucose) at room temperature, equilibrated with 95% O₂-5% CO₂. Submucosal preparations containing Brunner’s glands were dissected as previously described (34) for guinea pig submucosal ileal preparations. Briefly, the mucosa was dissected to expose the submucosa, which was then dissected from the underlying muscle. Preparations (0.5–1 cm²) were maintained under continuous oxygenation at room temperature, and all preparations were used for experimental study within 4 h of dissection.

Chronic Subdiaphragmatic Vagotomy

Guinea pigs (200–250 g) were anesthetized, a laparotomy was performed, and the left and right vagal trunks were visualized just beneath the diaphragm. The vagi were completely severed, and animals were allowed to recover for 7 to 10 days so that distal vagal fibers would degenerate.

Experimental Protocol

Submucosal preparations were positioned with the mucosal surface upward in small organ baths (1–2 ml) and continuously superfused at 15 ml/min with oxygenated Krebs buffer warmed to 36 °C (Fig. 1). Videomicroscopy was used to monitor changes in the diameter of the acinus lumen to provide a measure of secretion, as previously described (18). This system was originally developed for in vitro monitoring of changes in blood vessel diameter (20). Briefly, the video camera captures the image seen through the microscope for analysis by computer-driven imaging software (Diamtrak, T.O. Neild). The image is converted to gray scale and displayed on a monitor. Cursors are superimposed on the image and centered across the acinus lumen, which is visualized as parallel dark lines by the software (Fig. 1B, inset) (18). Our previous studies (18) have shown that exogenous application of secretagogues evokes mucin secretion from acinar cells and results in a concentration-dependent dilation of the lumen. Dilation causes the parallel lines to move apart, and the distance between the lines is tracked by the cursors and recorded on a chart recorder. The resolution of this system is ~1 μm, and the sampling rate is 15 Hz. Data were expressed as the percentage of the maximum response to 10 μM carbachol. All drugs were added by superfusion.

Glass focal electrodes (tip diam, 20–50 μm) were used to stimulate submucosal ganglia or perivascular nerves (Fig. 1B). Perivascular nerves were stimulated with electrodes placed on first- or second-order branches of parent arterioles (Fig. 1). Extrinsic nerve fibers enter the submucosa within the perivascular connective tissue of submucosal arterioles (9). In preliminary studies, optimum stimulus parameters were studied by examining frequency (1–20 Hz), pulse duration (1–10 ms), and train durations up to 30 s. Maximal dilations, which recovered to baseline (Fig. 2), were obtained with 20–40 V, 10 Hz, 5-ms pulse, and 30-s train duration, and these parameters were used in all subsequent studies. Our (18) previous studies demonstrated histologically and by electron microscopy that the dilations evoked by exogenous application of the cholinergic agonist carbachol result from mucin secretion from acinar cells into the acinar lumen.

Preliminary studies were also conducted to establish the general distribution of Brunner’s glands, which consistently dilated in response to perivascular stimulation. Dilations of...
Fig. 2. ES of perivascular nerve fibers but not submucosal neurons elicits dilation of the lumen of Brunner’s gland acini. A, top: representative trace of an acinar lumen dilating after ES of perivascular nerve fibers (40 V, 5-ms pulse duration, 10 Hz) (left) and showing maximum measurable dilation after superfusion of carbachol (10 μM) (right). Bottom: the effect of blockade of sodium-dependent neural transmission by TTX in a different preparation. Pretreatment of the preparation for 3 min with TTX completely prevented dilations elicited by ES of perivascular nerve fibers (left). Responses were restored after washout of the drug (right). B: a representative trace showing that ES of submucosal neurons (20 V, 0.7-ms pulse duration, 20 Hz) did not elicit dilation of the Brunner’s gland acinus lumen (left). Stimulation of submucosal vasodilator neurons was studied in the duodenal Brunner’s gland preparations to demonstrate that the stimulus parameters used to test for Brunner’s gland dilation were sufficient to activate submucosal motoneurons and evoke an effector response. The representative trace on right shows submucosal arterioles preconstricted with PGF$_{2\alpha}$ (400 nM) super fused continuously commencing at arrowhead. Stimulation of an adjacent submucosal ganglion (20 V, 0.7-ms pulse duration, 20 Hz for 30 s) evoked a large dilation. Bars above traces indicate duration of treatment.

RESULTS

Electrical Stimulation of Perivascular Nerves and Submucosal Ganglia

Perivascular nerves. Stimulation of perivascular nerve fibers elicited large dilations (Fig. 2A; n = 6) that averaged 47 ± 6% of the maximum response to superfusion of 10 μM carbachol. These dilations typically returned to baseline within 30–60 s after the stimulus train. After a 10-min waiting period, reproducible responses could be obtained two to three times with repeated stimulation. After pretreatment of the preparation for 3 min with TTX (1 μM; n = 4), a blocker of sodium-dependent action potentials, electrical stimulation failed to elicit detectable luminal dilations (Fig. 2B). A representative trace of an acinar lumen dilating after ES of perivascular nerve fibers (40 V, 5-ms pulse duration, 10 Hz) (left) and showing maximum measurable dilation after superfusion of carbachol (10 μM) (right). Bottom: the effect of blockade of sodium-dependent neural transmission by TTX in a different preparation. Pretreatment of the preparation for 3 min with TTX completely prevented dilations elicited by ES of perivascular nerve fibers (left). Responses were restored after washout of the drug (right). B: a representative trace showing that ES of submucosal neurons (20 V, 0.7-ms pulse duration, 20 Hz) did not elicit dilation of the Brunner’s gland acinus lumen (left). Stimulation of submucosal vasodilator neurons was studied in the duodenal Brunner’s gland preparations to demonstrate that the stimulus parameters used to test for Brunner’s gland dilation were sufficient to activate submucosal motoneurons and evoke an effector response. The representative trace on right shows submucosal arterioles preconstricted with PGF$_{2\alpha}$ (400 nM) super fused continuously commencing at arrowhead. Stimulation of an adjacent submucosal ganglion (20 V, 0.7-ms pulse duration, 20 Hz for 30 s) evoked a large dilation. Bars above traces indicate duration of treatment.

Statistical Analysis

Results are expressed as means ± SE. Data were compared using the two-tailed Student’s t-test for paired values or by ANOVA followed by Tukey’s post-test where appropriate. P < 0.05 was considered significant.

Materials

Carbachol was purchased from Aldrich, and TTX, hexamethonium, guanethidine, capsaicin, PGF$_{2\alpha}$, and substance P were from Sigma Chemical. 4-Diphenylacetoxy-N-(2-chloro-ethyl)-piperidine hydrochloride (4-DAMP) and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) were from Research Biochemicals. All other materials were of reagent grade.
2A). After a 10-min washout of TTX, dilations could be evoked with perivascular nerve stimulation.

**Submucosal ganglia.** Submucosal neurons were stimulated (20 V, 20 Hz, 0.7-ms pulse duration for 30 s) with electrodes placed on ganglia located adjacent to the Brunner’s gland selected for videomicroscopy monitoring. Stimulation failed to elicit any dilation of Brunner’s gland acini (Fig. 2B) in 70% of the submucosal neurons (5 of 7 neurons) and only very small dilations (<10% of maximum) in the other 30% (2 of 7 neurons). Previous studies (21) in the guinea pig ileum have demonstrated that electrical stimulation of submucosal ganglia activates submucosal vasodilator neurons that innervate submucosal arterioles. In the current study, nerve-evoked dilations of submucosal arterioles in the Brunner’s glands preparations in the duodenum (Fig. 1) were examined to directly demonstrate that the stimulus parameters were adequate to activate submucosal motoneurons and evoke an effector response. Stimulation of submucosal ganglia in the duodenum, using the same parameters examined for Brunner’s gland secretion (20 V, 20 Hz, 0.7-ms pulse duration for 30 s) evoked large dilations of adjacent submucosal arterioles (Fig. 2B; n = 3).

**Inhibition of Sympathetic Neurotransmitter Release**

Perivascular nerve stimulation activates sympathetic nerve fibers and has been shown to release neurotransmitters in the submucosal plexus causing pre- and postsynaptic inhibition (28). The possibility that these actions might have masked excitatory actions in other nerves was studied by comparing control responses with those obtained with guanethidine (10 μM) in the bath. Guanethidine blocks neurotransmitter release from sympathetic nerve terminals (28). Control responses elicited by electrical stimulation of perivascular nerves were not significantly different from those obtained with guanethidine (10 μM) superfused for 3 min into the bath (Fig. 3; n = 8). Similarly, no difference was found with 30 μM guanethidine (control, 63 ± 10.5% vs. guanethidine, 59 ± 7.9%; n = 3). The finding that stimulation of submucosal ganglia did not elicit significant dilations (see Submucosal ganglia) was also not altered when guanethidine (10 μM) was in the bath (n = 8). However, guanethidine (10 μM) completely blocked inhibitory postsynaptic potentials (mean amplitude, 17 ± 4 mV) recorded intracellularly in submucosal neurons (n = 3). These synaptic potentials were elicited by electrical fiber tract stimulation (20 Hz, 0.8-ms pulse duration, 400-ms train). In the same preparations in which the effects of submucosal ganglia stimulation on Brunner’s gland dilation were assessed, typical large dilations (Fig. 3; n = 5) were evoked when the electrode was repositioned on the perivascular nerves.

**Effects of Cholinergic Agonists and Antagonists**

Pretreatment of preparations with the nicotinic receptor antagonist hexamethonium (200 μM; n = 7) for 3 min had no effect on dilations of glandular acini elicited by stimulation of perivascular nerves, whereas dilations were almost completely blocked by the muscarinic receptor antagonist 4-DAMP (1 μM; n = 7) (Fig. 4A). The activation of submucosal neurons by superfusion of the nicotinic receptor agonist DMPP (100 μM; n = 4) failed to stimulate Brunner’s gland acinar dilation. The effects of DMPP on dilation of submucosal arterioles were examined to establish that DMPP was an adequate stimulus to activate submucosal motoneurons and elicit an effector response. Superfusion of DMPP (100 μM; n = 4) evoked large dilations of submucosal arterioles (Fig. 4B). These responses were blocked by TTX (1 μM; n = 3), demonstrating that the site of action of DMPP was nicotinic receptors on submucosal vasodilator neurons (21, 32, 33).

**Effects of Subdiaphragmatic Vagotomy**

The possibility that acinar dilation elicited by stimulation of perivascular nerve fibers was mediated by vagal fibers was assessed by studying the effects of subdiaphragmatic vagotomy. In vagotomized animals, stimulus-evoked dilations were almost completely abolished (95%) (Fig. 5; n = 9) compared with controls (n = 5).

**Effects of Capsaicin and Substance P**

Nerve terminals of extrinsic sensory afferent neurons can release peptides and play a local effector role in the intestine (13). A potential role for this sensory efferent process in the regulation of Brunner’s gland secretion was examined by studying the actions of capsaicin, a selective neurochemical probe, and the putative neurotransmitter substance P (13, 30). Our previous studies (31), in the guinea pig ileum, have shown that capsaicin (2 μM) selectively activates capsaicin-sensitive nerves with ensuing release of neurotransmitter. In the current study, superfusion of capsaicin (2 μM; n = 5) or substance P (30 or 100 nM; n =
4 for each concentration) had no effect on the luminal diameter of Brunner’s gland acini (Fig. 6A). In the same preparations, maximal dilations were recorded in response to 10 μM carbachol. The effects of capsaicin on dilation of duodenal submucosal arterioles in the Brunner’s gland preparation were examined to establish that capsaicin is a sufficient stimulus to activate extrinsic sensory nerves and evoke an effector response. Both capsaicin (600 nM; n = 3) and substance P (30 nM; n = 3) evoked large arteriolar dilations, similar to those described in the ileum (30, 31).

**ChAT Immunoreactivity in Brunner’s Glands**

Previous studies (10) have demonstrated dense projections of ChAT-immunoreactive and cholinesterase-positive nerve fibers within the guinea pig duodenal submucosal plexus. In the present study, confocal microscopy was used to examine the distribution of ChAT-immunoreactive nerves in Brunner’s glands. Numerous ChAT-immunoreactive fibers were evident within the periglandular connective tissue of Brunner’s glands and between individual acini (Fig. 7; n = 3 animals). Immunoreactivity was also localized within cell bodies and processes of a subpopulation of submucosal neurons and on nerve fibers within the perivascular connective tissue of submucosal arterioles (Fig. 7), as previously described (10).

**DISCUSSION**

This study employed a novel in vitro model of Brunner’s gland secretion that enabled intrinsic and extrinsic neural pathways to be selectively activated and their role in the regulation of glandular secretion to be examined. We (18) have previously shown that cholinomimetics are potent activators of Brunner’s gland secretion in this model. These agents acted directly on the acinar cell to stimulate concentration-dependent exocytosis of mucin-containing secretory granules, suggesting these glands may receive cholinergic innervation. In the current study, ChAT-immunoreactive nerve fibers were found in close association with Brunner’s glands, and studies of perivascular electrical stimulation provided direct evidence that Brunner’s glands receive functional cholinergic innervation.

Several lines of evidence demonstrated that electrically evoked dilations were mediated by cholinergic neural mechanisms directly activating acinar cells. The neural origin of the responses was confirmed by demonstrating that TTX, the selective blocker of so-
were obtained in response to superfusion of 10 μM capsaicin. Bars indicate duration of superfusions.

**B.** Substance P (30 nM) also produced a large dilatation of the arteriole. Capsaicin (600 nM; right) evoked large dilations of the arteriole. Bars indicate duration of superfusions. **A.** Capsaicin (2 μM left) and Substance P (30 nM right) did not dilate the acinar lumen. *Bottom:* summary of results in A. Bars are means ± SE for >4 trials. Maximal dilations were obtained in response to superfusion of 10 μM carbachol.

Two cholinergic pathways, involving extrinsic autonomic nerves and/or intrinsic enteric nerves, were likely candidates for the cholinergic-mediated dilations observed in this study. Vagal efferents have been implicated in previous in vivo studies that have shown vagal stimulation causes depletion of Brunner's gland mucin (35), although it was unclear whether this action resulted from direct innervation of the glands or was mediated through activation of submucosal secretomotor neurons. A role for submucosal secretomotor neurons has also been suggested (10), because submucosal ganglia are closely associated with Brunner's glands (Fig. 1), there are dense projections of cholinergic nerves around the glands (Fig. 7), and submucosal secretomotor neurons stimulate epithelial mucin and electrolyte secretion (3,19). In the current study, we provided direct evidence that the cholinergic perivascular fibers were vagal in origin, because these responses were abolished in animals that had been surgically vagotomized (see Fig. 5). The possibility that these vagally evoked responses were mediated through the activation of submucosal secretomotor neurons or, alternatively, that there was a separate secretomotor pathway mediated by submucosal secretomotor neurons, was examined by selective activation of these neurons both electrically and with exogenous application of the nicotinic receptor agonist DMPP. Neither of these stimuli had a significant effect on Brunner's gland secretion. In a small number of preparations, very small dilations (<10% maximum) were elicited by electrical stimulation. Given the lack of effect of DMPP and the small size of the dilations, these responses most likely represent activation of vagal fibers passing "en passe" or in close proximity to the ganglia. However, both DMPP stimulation and electrical stimulation appear to be adequate to activate submucosal motoneurons (see Figs. 2 and 4), because we were able to demonstrate in the same preparation that they evoke large dilations of submucosal arterioles. In addition, the vagally evoked Brunner's gland dilations were also hexamethonium insensitive, providing further evidence that submucosal neurons did not participate in these actions. These data suggest that vagal fibers directly innervate duodenal Brunner's glands and that submucosal secretomotor neurons do not play a significant role in the innervation of these glands.

Sympathetic nerve fibers also enter the submucosa via the perivascular connective tissue where they project to multiple effector systems, including enteric neurons and the submucosal arterioles (9). There is evidence, at least in some species, that they may also project to Brunner's glands (27) and that activation of the splanchnic nerves can inhibit Brunner's gland secretion (24). In the current study, activation of perivascular sympathetic nerves could potentially inhibit mucin secretion by release of inhibitory neurotransmitter onto enteric secretomotor neurons (28) and/or postsynaptic inhibition at the acinar cell, potentially masking simultaneous activation of additional secretomotor pathways. However, we found no evidence for such actions, because the responses to perivascular...
stimulation and submucosal ganglia stimulation were not altered when guanethidine, which blocks release of sympathetic neurotransmitter(s) (4), was added to the bath.

Several noncholinergic neural pathways have also been proposed to activate Brunner’s glands. Previous studies (14), including our (18) own, have shown that exogenous application of vasoactive intestinal polypeptide (VIP) stimulates mucin secretion from Brunner’s glands in this model and in the rat and that VIP-immunoreactive fibers form a dense network around the acini of Brunner’s glands in the rat. VIP immunoreactivity has also been found in vagal nerve fibers (17) and in enteric secretomotor neurons (10), implying both of these neural pathways could be a source of this putative neurotransmitter. Despite these findings, we found no evidence for a significant noncholinergic component to the electrically evoked Brunner’s gland dilations observed in this study. Another noncholinergic effector system that has been suggested to innervate Brunner’s glands was capsaicin-sensitive sensory nerves. These nerves release neuropeptides, such as substance P and calcitonin gene-related peptide (30), from nerve terminals within the intestine, which evokes multiple effector responses (13), including duodenal bicarbonate secretion (29) and mucosal blood flow (1). Capsaicin is a selective neurochemical probe for activation of these nerves but failed to stimulate Brunner’s gland secretion in our study. Similarly, the putative neurotransmitter substance P had no effect. Both capsaicin and substance P appear to be adequate stimuli to evoke effector responses in this model, because we were able to demonstrate that they can dilate submucosal arterioles in the same preparation. (see Fig. 6).

In summary, this study provides direct evidence that Brunner’s glands in the guinea pig duodenum are functionally innervated by cholinergic nerves but not by noncholinergic and capsaicin-sensitive nerves. This cholinergic neural pathway is mediated by vagal fibers.
within the duodenum that directly innervate the Brunner’s gland. Unlike other secretory glands (25), in which vagal fibers activate intrinsic secretomotor neurons in periglandular ganglia, secretomotor neurons in periglandular duodenal submucosal ganglia played no role in stimulating adjacent Brunner’s glands. This study was supported by a grant from the Medical Research Council of Canada (MRC) (S. Vanner) and the MRC and Pharmaceutical Manufacturer’s Association of Canada Health Program Fellowship Award with the Canadian Association of Gastroenterology (CAG)/Astra (B. A. Moore). David Kim was supported by a summer studentship from the CAG.

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