Neuroimmune interactions in guinea pig stomach and small intestine

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Liu, Sumei, Hong-Zhen Hu, Na Gao, Chuan Yun Gao, Guodu Wang, Xiyu Wang, Owen C. Peck, Gordon Kim, Xiang Gao, Yun Xia, and Jackie D. Wood. Neuroimmune interactions in guinea pig stomach and small intestine. Am J Physiol Gastrointest Liver Physiol 284: G154–G164, 2003.—Enteric neuroimmune interactions in gastrointestinal hypersensitivity responses involve antigen detection by mast cells, mast cell degranulation, release of chemical mediators, and modulatory actions of the mediators on the enteric nervous system (ENS). Electrophysiological methods were used to investigate electrical and synaptic behavior of neurons in the stomach and small intestine during exposure to β-lactoglobulin in guinea pigs sensitized to cow’s milk. Application of β-lactoglobulin to sensitized preparations depolarized the membrane potential and increased neuronal excitability in small intestinal neurons but not in gastric neurons. Effects on membrane potential and excitability in the small intestine were suppressed by the mast cell stabilizing drug ketotifen, the histamine H2 receptor antagonist cimetidine, the cyclooxygenase inhibitor piroxicam, and the 5-lipoxygenase inhibitor caffeic acid. Unlike small intestinal ganglion cells, gastric myenteric neurons did not respond to histamine applied exogenously. Antigenic exposure suppressed noradrenergic inhibitory neurotransmission in the small intestinal submucosal plexus. The histamine H2 receptor antagonist thioramide and piroxicam, but not caffeic acid, prevented the allergic suppression of noradrenergic inhibitory neurotransmission. Antigenic stimulation of neuronal excitability and suppression of synaptic transmission occurred only in milk-sensitized animals. Results suggest that signaling between mast cells and the ENS underlies intestinal, but not gastric, anaphylactic responses associated with food allergies. Histamine, prostaglandins, and leukotrienes are paracrine signals in the communication pathway from mast cells to the small intestinal ENS.

INGESTION OF FOREIGN ANTIGENS in the form of food or pathogens sensitizes the enteric immune/inflammatory system. Subsequent exposures to the sensitizing antigen are detected by enteric mast cells stimulated to degranulate and release a variety of biologically active chemical substances. Antigen-evoked mast cell degranulation in the small and large intestine starts an immediate (type I) hypersensitivity reaction characterized by mucosal hypersecretion (1, 17, 34) and strong contractions of the musculature (3). Application of tetrodotoxin or atropine in vitro suppresses the secretory and motor responses. Blockade by tetrodotoxin or atropine implicates participation of the enteric nervous system (ENS) in the hypersensitivity responses (2, 3, 17).

Results of earlier electrophysiological studies in colonic submucosal neurons in antigen-sensitized guinea pigs demonstrated paracrine signaling between mast cells and the ENS (10, 12). Characteristics of this signaling supported the hypothesis that enteric immunoneural communication is a meaningful defensive event that results in adaptive behavior of the bowel in response to circumstances within the lumen that are threatening to the functional integrity of the whole animal (35–37). The immunoneural defensive system integrates specialized memory and sensory functions of enteric mast cells with capacity of the ENS for interpretation of paracrine signals and programming of intestinal secretory and motor behavior that is adaptive for the well being of the animal.

Immune detection of a sensitizing antigen by enteric mast cells starts a cascade of signaling events beginning with transfer of paracrine information to neural networks in the ENS. The neural networks interpret the paracrine signal as a threat to the integrity of the bowel and respond by “calling-up” from their program library a specific program that coordinates stimulated mucosal secretion and powerful propulsive motility to quickly clear the antigenic threat from the intestinal lumen (35–37). Symptomatic side effects of operation of the immunoneural defense program are watery diarrhea, fecal urgency, and abdominal pain (35). Several kinds of immune/inflammatory cells are potential sources of paracrine signals to the ENS. Among these are lymphocytes, macrophages, polymorphonuclear leukocytes, and mast cells. Most is known about signaling between mast cells and the elements of the
local neural networks. Paracrine communication between mucosal mast cells and the colonic submucosal plexus was identified as a contributing factor in earlier studies of type I hypersensitivity reactions associated with sensitization to milk in the guinea pig model (6, 7, 17, 36, 37). Antigen cross-linking of immunoglobulin IgE or IgG antibodies attached at FcεRI receptors on the surfaces of mast cells activates degranulation and the release of a variety of mediators (6, 21). Paracrine-mediated excitation of submucosal secretomotor neurons by mast cell products accounts for stimulation of secretion on exposure of the sensitized bowel to the sensitizing antigen (6, 17, 26).

Histamine is implicated as a significant messenger in communication between mast cells and the enteric neural networks. Electrophysiological studies found that when the guinea pig colon was sensitized to the parasite Trichinella spiralis or to cow’s milk, reexposure to T. spiralis somatic antigen or β-lactoglobulin evoked a dramatic increase in neuronal firing that mimicked slow synaptic excitation (10, 12). Excitatory response to the antigen was significantly reduced but not abolished by the histamine H₂ receptor antagonist cimetidine. Antagonism by cimetidine suggested that the increased neuronal excitability was due, in part, to release of histamine. Experimental application of exogenous histamine to enteric neurons simulates the effects of degranulation of mast cells seen in the colon of milk-sensitized guinea pigs (9, 23, 39). Signaling functions of histamine appear to be related exclusively to mast cells and immunoneural communication, because histamine is not found in enteric neurons and is unlikely to function as a neurotransmitter in the ENS (25).

Microelectrode recording from single neurons in the enteric microcircuits revealed that suppression of fast nicotinic synaptic transmission occurred when the sensitized intestine was exposed to the sensitizing antigen (10, 12). Pretreatment with the histamine H₃ receptor antagonist burimamide reduced but did not abolish this effect (10, 12). Like the excitatory responses in the cell somas of enteric neurons, suppression of fast nicotinic synaptic transmission seemed also to be mediated partly by mast cell degranulation and release of histamine.

Mucosal mast cells release substances apart from histamine that could potentially influence enteric neurophysiology. Included among these are serotonin, prostaglandins, leukotrienes, cytokines, and possibly others (7, 21). Each of these putative mediators mimics some of the actions of antigenic stimulation when applied to neurons in the submucosal plexus of guinea pig colon (8, 11, 13, 16, 39). Other mast cell mediators are assumed to be coreleased and act on the enteric neurons during antigen exposure. This assumption is on the basis of observations that histamine antagonists often suppress significantly, but do not abolish, the neural effects of antigen exposure in the sensitized guinea pig colon.

Histamine acts at presynaptic inhibitory receptors on sympathetic postganglionic axons to suppress the release of norepinephrine in submucosal ganglia (20). Norepinephrine is an inhibitory neurotransmitter that evokes slow inhibitory PSPs (IPSPs) when released from sympathetic neurons and membrane hyperpolarization when experimentally applied to secretomotor neurons in the submucosal plexus. Release of norepinephrine is a mechanism by which the sympathetic nervous system can exert “braking” action on the excitability of secretomotor neurons and therefore on neurogenic mucosal secretion of H₂O₂, electrolytes and mucus. Release of histamine during exposure to a sensitizing antigen is predicted to stimulate secretion both by excitation of histamine H₂ receptors on secretomotor neurons and removal of the braking action of the sympathetic nervous system. Histamine removes sympathetic braking action by activation of presynaptic H₃ inhibitory receptors at norepinephrine release sites on sympathetic postganglionic axons.

Studies (10, 12) of communication between the mucosal immune system and the ENS in sensitized animals are, so far, limited to the colonic submucosal plexus. An aim of the present study was to extend investigation of immunoneural communication to the myenteric plexus of the gastric corpus and antrum and to the submucosal plexus of the small bowel. An allied aim was to identify specific mediators other than histamine that might be released on antigen challenge and be involved in immunoneural signaling. A third aim was to investigate effects of β-lactoglobulin exposure on noradrenergic inhibitory neurotransmission in small intestinal submucosal neurons of milk-sensitized guinea pigs.

MATERIALS AND METHODS

Sensitization procedure. The general protocol was to induce gastrointestinal hypersensitivity to milk protein by feeding cow’s milk to one group of guinea pigs in parallel with an age-matched, nonsensitized control group. Two-week-old male albino guinea pigs of the Hartley strain were used. The experimental group was sensitized by ingestion of cow’s milk in place of drinking water over a 3-wk period, as described by others (1, 6, 10). Tap water replaced milk for 1–3 days before the animals were killed and set up for electrophysiological recording from the enteric neurons during exposure to β-lactoglobulin in vitro. Nonsensitized animals drank only tap water during the 3 wk and were never exposed to milk protein in the diet. Both groups of animals were fed standard guinea pig chow. All protocols were reviewed and approved by the Ohio State University Laboratory Animal Care and Use Committee.

Tissue preparation. After 3 wk of milk exposure, preparations of the myenteric plexus from the gastric corpus and antrum or the submucosal plexus of the ileum were prepared by microdissection and setup in perfused tissue chambers for neuronal electrophysiological recording. Electrophysiological behavior of the neurons in the milk-sensitized and nonsensitized tissues was recorded, followed by recording of the effects of application of β-lactoglobulin. Specificity of the effects of the milk antigen was tested by application of ovalbumin, which served as an unrelated antigen.

Experimental animals were killed by stunning and exanguination from the cervical vessels according to procedures reviewed and approved by the Ohio State University Labo-
MgCl₂, 1.2 NaH₂PO₄, 14.4 NaHCO₃, 2.5 CaCl₂, and 11.5 mM NaCl, 5.9 KCl, 1.2 glucose. Enteric ganglia were visualized microscopically with differential interference contrast optics and epilumination.

Electrophysiological recording. Our methods for intracellular recording from the myenteric and submucosal plexuses are described in detail elsewhere (27, 28, 31, 32, 40). Transmembrane electrical potentials were recorded with conventional “sharp” microelectrodes filled with 2% biocytin in 2 M KCl containing 0.05 M Tris buffer (pH 7.4). Resistances of the electrodes ranged between 80 and 120 MΩ. The preamplifier (model M767; World Precision Instruments, Sarasota, FL) was equipped with a bridge circuit for intraneuronal injection of electrical current. Constant current rectangular pulses were driven by a stimulator (model S99; Grass Instrument Division, Astro-Med, Warwick, RI). Electrometer output was amplified and observed on an oscilloscope (model 5113; Tektronics, Beaverton, OR). Synaptic potentials were evoked by focal electrical stimulation of interganglionic fiber tracts with electrodes made from 20-μm-diameter Teflon-insulated platinum wire connected through model SIN5 stimulus-isolation units to model S48 stimulators (Grass Instrument Division, Astro-Med). Fast excitatory postsynaptic potentials (EPSPs) were recorded with an expanded time scale and displayed through a real-time digital oscilloscope (model TDS210; Tektronics) and output to a laserjet printer. Chart records were made on Astro-Med thermal recorders. Amplitudes of action potentials on some records were blunted by the low-frequency response of the recorders. All data were recorded on videotape for later analysis.

Immunohistochemical methods. At the end of each recording session, the marker dye biocytin was injected into the impaled neurons from the recording electrodes by the passage of hyperpolarizing current (0.5 nA for 10–30 min). The anodal end of the preparations was marked, and the tissue was transferred into a disposable chamber filled with fixative containing 4% formaldehyde plus 15% of a saturated solution of picric acid and kept at 4°C overnight. The preparations were cleared in three changes of dimethyl sulfoxide and three 10-min washes with PBS. After they were cleared, the preparations were reacted with fluorescein streptavidin (1:100) for 1 h and examined under a Nikon Eclipse E600 fluorescent microscope with appropriate filters.

Neurochemical coding of the neurons that responded to antigenic stimulation was determined by first reacting the preparations with streptavidin coupled to Texas Red to reveal biocytin fluorescence and assess neuronal morphology. They were then processed for immunohistochemical localization of vasoactive intestinal peptide (VIP) and choline acetyltransferase (ChAT) immunoreactivity. For VIP localization, rabbit anti-VIP (1:250) (code IHC7161, Peninsula, Belmont, CA) was used; for ChAT, goat anti-ChAT (1:100) (code AB144P, Chemicon, Temecula, CA) was used. The preparations were then incubated with secondary antibodies labeled with fluorescein. Fluorescent labeling was examined under a Nikon Eclipse E600 fluorescent microscope equipped with appropriate filters and a SPOT-2 chilled color and black and white digital camera (Diagnostic Instruments, Sterling Heights, MI).

Drug application. Actions of pharmacological agents and β-lactoglobulin antigen were studied by pressure microejection or by application in the superfusion solution. Micropipettes (10-μm-tip-diameter) manipulated with the tip close to the impaled neurons were used to microinject the substances. Pressure pulses of nitrogen with predetermined force and duration were applied to the micropipettes through electronically controlled solenoid valves. The concentrations of antagonists selected for the work were based on pilot studies and the literature as follows: cimetidine (9), BRL 24934 (11), thiorperamide (20), piroxicam (15), MDL 72222 (41).

Chemicals. Chemical agents used and sources were as follows: acetylcholine, anti-guinea pig IgG, biocytin, cimetidine, compound 48/80, ketotifen, β-lactoglobulin, ovalbumin, piroxicam and pyrilamine were obtained from Sigma (St. Louis, MO). PGE₂, leukotriene C₄ (LTC₄) and caffeic acid were from Cayman Chemicals (Ann Arbor, MI). Thiorperamide and MDL 72222 were from Tocris Cookson (Ballwin, MO). BRL 24929 was from SmithKline Beecham (Betchworth, UK). Histamine, R-α-methylhistamine and norepinephrine were from RBI (Matick, MA). Fluorescein and Texas Red streptavidin were from Vector (Burlingame, CA). VIP and ChAT antiserum were from Chemicon (Temecula, CA).

Data analysis. Data are expressed as means ± SE; n-values refer to the number of neurons. Concentration-response relationships were constructed using the following least-squares fitting routine: V = Vₘax/[1 + (EC₅₀/C)]ⁿH, where V is the observed response, EC₅₀ is the concentration that induces the half-maximal response, and nH is apparent Hill coefficient. The graphs were drawn by averaging results from all experiments and fitting to a single concentration-response curve by using Sigma Plot software (SPSS, Chicago, IL). Paired or unpaired Student’s t-test was used to determine statistical significance. P values of <0.05 were considered statistically significant.

RESULTS

Gastric neurons. Results were obtained for 29 gastric myenteric neurons consisting of 16 in the corpus and 13 in the antrum from 10 guinea pigs that were sensitized by ingestion of cow’s milk. Results for nonsensitized H₂O controls were obtained for 24 neurons consisting of 10 neurons in the corpus and 14 in the antrum from 10 animals. Neurons were classified electrophysiologically as gastric I, gastric II, gastric III, or AH-type according to criteria described previously (27, 28, 31, 32). Ratios for the percentages of each of the specific classes of gastric neurons found in milk-sensitized preparations relative to nonsensitized preparations were: 1) 31.33% for gastric I, 2) 48.42% for gastric II, and 3) 21.25% for gastric III. No AH-type neurons were found in either milk-sensitized or nonsensitized preparations. Mean resting membrane potentials for the three types of gastric neurons in milk-sensitized guinea pigs were not significantly different from the...
controls (Table 1). No spontaneous discharge of action potentials was found in either the milk-sensitized preparations or the nonsensitized controls.

In milk-sensitized preparations, application of β-lactoglobulin in concentrations as high as 10 μM did not significantly alter the resting membrane potentials of any of the gastric neurons (Fig. 1A). The mean resting membrane potential for the controls was −45.2 ± 4.1 mV (n = 8), and the mean resting membrane potential during exposure of the same neurons to 10 μM β-lactoglobulin was −44.2 ± 3.9 mV (P > 0.05). The numbers of action potentials evoked in the gastric neurons by injection of depolarizing current pulses did not change in the presence of β-lactoglobulin (Fig. 1B). Absence of any change in the numbers of action potentials evoked by constant-current depolarizing pulses during exposure to β-lactoglobulin suggests that antigen challenge did not affect excitability of the gastric neurons.

Individual neurons of the guinea pig stomach were shown to receive single or multiple fast EPSP inputs like those known to be mediated by nicotinic receptors (27, 28, 31, 32). The mean amplitude of the fast nicotinic EPSPs evoked by stimulation of the interganglionic fiber tracts in milk-sensitized preparations was 18.4 ± 1.6 mV (n = 5). The amplitudes of the fast EPSPs were not significantly altered by application of 10 μM β-lactoglobulin in the superfusion solution (Fig. 1C). Mean amplitude of the EPSPs in the same neurons during exposure to β-lactoglobulin was 17.8 ± 1.7 mV (P > 0.05). β-Lactoglobulin did not alter the membrane potential or the fast EPSPs of gastric neurons from nonsensitized animals (data not shown).

**Small intestinal submucosal neurons.** Results were obtained for 54 ileal submucosal neurons from 28 guinea pigs sensitized by cow’s milk and compared with 40 ileal submucosal neurons from 24 milk-free animals. The submucosal neurons were classified electrophysiologically as S- and AH-type according to the criteria of Mihara (22). Ratios of percentages of neuronal types impaled in milk-sensitized relative to nonsensitized preparations were 89:85% for S-type and 11:15% for the AH-type. The mean resting membrane potential for S- and AH-type neurons in the milk-sensitized ileum was not significantly different from the controls (Table 1). Spontaneous discharge of action potentials was found in neurons from both sensitized and nonsensitized preparations. Incidence of spontaneous discharge was highest in the milk-sensitized preparations with 29.6% (16 of 54) of the neurons firing spontaneously compared with the occurrence of spontaneous discharge in 7.5% (3 of 40) of the neurons in nonsensitized preparations. For preparations from milk-sensitized animals, application of β-lactoglobulin (0.1–10 μM) in the superfusion solution evoked a concentration-dependent depolarization of the membrane potential in 41 of 48 S-type and 6 of 6 AH-type neurons (Fig. 2).

Enhanced excitability reflected as neuronal firing and increase in neuronal input resistance were associated with the membrane depolarization. Enhanced excitability in β-lactoglobulin was reflected also by increased numbers of action potentials evoked by intracellular injection of constant-current depolarizing pulses (not shown), the occurrence of spontaneous spike discharge (not shown), and the occurrence of anodal-break excitation at the offset of hyperpolarizing pulses (not shown).

<table>
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<tr>
<th>Animals</th>
<th>Stomach</th>
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<td></td>
<td>Gastric I</td>
<td>Gastric II</td>
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<tr>
<td>Milk-sensitized</td>
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<td>−41.1 ± 2.6(14)</td>
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<tr>
<td>Nonsensitized</td>
<td>−43.5 ± 2.6(8)</td>
<td>−44.9 ± 1.8(10)</td>
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Values are means ± SE in millivolts; number in parentheses is number of neurons. S, S-type neurons; AH, AH-type neurons.

### Table 1. Resting membrane potentials for gastric and small intestinal neurons from milk-sensitized and nonsensitized guinea-pigs

**Fig. 1. Antigen challenge with β-lactoglobulin (β-LG) in stomach preparation from milk-sensitized guinea pigs.** A: application of 10 μM β-LG did not alter the membrane potential or input resistance of a gastric I neuron. Downward deflections are electrotonic potentials evoked by repetitive injection of 100-ms hyperpolarizing current pulses. B: responses to depolarizing current pulses were unaffected by 10 μM β-LG. This gastric I neuron generated a single action potential at the onset of each depolarizing current pulse. Top trace, transmembrane electrical potential; bottom trace, injected current. Action potentials are attenuated by the low frequency response of the chart recorder. C: fast excitatory postsynaptic potentials (EPSP) in a gastric I neuron were unaffected by 10 μM β-LG.
current pulses. These effects of β-LG developed 1.5–5 min after entry of the antigen into the recording chamber and required 10- to 15-min washing with antigen-free Krebs solution for recovery. Changes in membrane potential and excitability were observed with antigen-free Krebs solution for recovery. Changes in membrane potential of submucosal neurons in preparations from nonsensitized preparations during exposure to β-LG. Data points represent means ± SE for 4–8 neurons.

Application of ovalbumin (300 μg/ml) to seven neurons from milk-sensitized animals failed to evoke excitatory responses in any of the 40 neurons (Fig. 2A). This failure of an unrelated antigen to evoke a response was consistent with antigenic stimulus specificity of the neuronal responses evoked by β-lactoglobulin after sensitization to milk.

Application in the superfusion solution of compound 48/80 also evoked membrane depolarization amounting to 8 ± 0.73 mV in five of seven neurons in nonsensitized preparations. Application of anti-guinea pig IgG (1:100) in the superfusion solution depolarized the membrane potential by 7.14 ± 0.94 mV in seven of eight neurons in milk-sensitized preparations (Fig. 3). Application of anti-guinea pig IgG to five neurons in nonsensitized preparations did not alter either the membrane potential or neuronal excitability.

**Neurochemical identification.** Immunofluorescence was used to help assess the functional identity of the neurons. Data on immunoreactivity for VIP and ChAT were obtained for 23 submucosal neurons with electrophysiological responses to antigen challenge. Data on immunoreactivity for VIP and CHAT were obtained for 23 submucosal ganglion cells. Ten S-type neurons with Dogiel type I morphology were examined for VIP immunoreactivity. Immunoreactivity for VIP was expressed by 4 of the 10 neurons (Fig. 4, A–C). Thirteen S-type neurons with Dogiel type I morphology were tested for ChAT immunoreactivity. Immunoreactivity for ChAT was expressed in 7 of the 13 neurons (Fig. 4, D–F).

**Mast cell stabilization.** Ketotifen was used as a pharmacological tool to investigate further the involvement of mast cell degranulation in the neuronal responses of sensitized preparations to application of β-lactoglobulin. Ketotifen is a drug with anti-allergic and anti-inflammatory properties. Its mechanism of action is stabilization of mast cell membranes and prevention of release of histamine and other mediators (14, 18).

Ketotifen (10–100 μM) was added to the superfusion solution 30 min before the addition of 5 μM β-lacto-

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**Fig. 2.** Effects of β-LG on submucosal neurons from the small intestine of milk-sensitized guinea pigs. A: application of 5 μM β-LG to an S-type neuron evoked a slowly activating depolarizing response associated with enhanced excitability reflected by action potential discharge. Application of ovalbumin (300 μg/ml) did not evo- catory responses in the same neuron. B: effects of β-LG on the membrane potential of submucosal neurons in preparations from milk-sensitized animals were concentration-dependent. No membrane depolarization was found in neurons from nonsensitized prepa- rations during exposure to β-LG. Data points represent means ± SE for 4–8 neurons.

**Fig. 3.** Mast cell degranulation mimicked responses evoked by β-LG in a submucosal neuron in a milk-sensitized preparation. A: slowly activating depolarizing response evoked by 5 μM β-LG. B: slowly activating depolarizing response to mast cell degranulation induced by 10 μg/ml compound 48/80. C: slowly activating depolarizing re- sponse to mast cell degranulation evoked by anti-guinea pig IgG. All records were from the same neuron.
globulin. Pretreatment with ketotifen resulted in concentration-dependent suppression of the depolarizing responses evoked by β-lactoglobulin in the sensitized preparation (Fig. 5).

**Pharmacology of antigen-evoked responses.** Histaminergic and serotonergic receptor antagonists and drugs that suppress prostaglandin and leukotriene synthesis were used to test the hypothesis that histamine, prostaglandins, and leukotrienes were paracrine mediators of β-lactoglobulin-evoked neuronal responses found in the milk-sensitized preparations. Application of cimetidine, a histamine H₂ receptor antagonist, significantly reduced the depolarizing responses evoked by β-lactoglobulin in sensitized preparations (Fig. 6). The membrane potential in 10 neurons was depolarized by 13.7 ± 2.0 mV after the addition of 5 μM β-lactoglobulin to the superfusion solution. The presence of 10 μM cimetidine reduced the depolarization to 44.2 ± 4.8% of the control values (P < 0.001).

Application of the H₁ receptor antagonist pyrilamine (10 μM) or the H₃ receptor antagonist thioperamide (10 μM) had no effect on the depolarizing responses to 5 μM β-lactoglobulin in six neurons (P > 0.05).

Putative suppression of prostaglandin synthesis by 60 μM piroxicam significantly reduced the depolarization evoked by 5 μM β-lactoglobulin to 60.2 ± 5.1% of control values of 15.1 ± 1.9 mV in 10 neurons (P < 0.001; Fig. 6). Putative suppression of leukotriene synthesis by 30 μM caffeco acid also decreased the β-lactoglobulin-evoked depolarization to 67.3 ± 5.6% of control values of 14.6 ± 1.8 mV in eight neurons (P < 0.01). On the other hand, the 5-HT₁₄ receptor antagonist BRL 24924 (10 μM) and the 5-HT₃ receptor antag-
onist MDL 72222 (10 μM) in combination did not reduce the depolarizing responses to β-LG in seven neurons (P > 0.05; Fig. 6).

The finding that blockade of histaminergic H2 receptors or suppression of prostaglandin or leukotriene synthesis reduced the responses to β-LG suggested that each of the paracrine messages may be released simultaneously and act individually during antigen exposure. We tested this suggestion by applying 10 μM cimetidine, 60 μM piroxicam, and 30 μM caffeic acid in combination before the addition of 5 μM β-LG in the bathing solution. The three drugs acting together were much more effective in suppressing the antigen-evoked responses than either of the drugs acting alone. Together, the three drugs suppressed the β-LG-evoked depolarizing responses to 7.7 ± 3.5% of control responses of 16.5 ± 2.0 mV in six neurons (P < 0.01; Fig. 6). The enhanced action of the drugs in combination is consistent with the hypothesis that the antigen-evoked depolarizing responses in neurons of sensitized preparations reflect the release and additive actions of histamine, prostaglandins, and leukotrienes.

Histamine, PGE2, and LTC4. Results obtained with histamine H2 receptor antagonist and drugs expected to suppress prostaglandin or leukotriene synthesis suggested that endogenous histamine, prostaglandins, and leukotrienes may partially mediate the excitatory responses to antigen challenge in small intestinal neurons from milk-sensitized guinea pigs. To qualify as mediators, the actions of histamine, prostaglandins, and leukotrienes should mimic the effects β-lactoglobulin when they are applied to the neurons of sensitized preparations. Application of histamine (10 μM), PGE2 (100 nM), and LTC4 (100 nM) evoked membrane depolarization and enhanced excitability in the same neurons that responded to antigen challenge (Fig. 7). Histamine, PGE2, and LTC4 also evoked membrane depolarization and enhanced excitability in neurons of the nonsensitized small intestine, whereas β-lactoglobulin was without effect (Fig. 7). In contrast to the small intestine, neither histamine in 21 neurons, PGE2 in 15 neurons, nor LTC4 in 12 neurons evoked depolarizing responses when applied to gastric neurons in milk-sensitized or nonsensitized preparations (data not shown).

**Sympathetic neurotransmission.** Short-train electrical stimulation (20 Hz, 0.2 s or less) applied to the interganglionic fiber tracts evoked slow inhibitory postsynaptic potentials (IPSPs) in most of the submucosal neurons from milk-sensitized and nonsensitized guinea pig small intestine. Past experience suggested that the IPSPs were mediated by the release of norepinephrine from sympathetic nerve terminals and its action at α2-adrenergic receptors (20, 24). Application of 5 μM β-lactoglobulin in the superfusion solution reversibly suppressed the IPSPs by 60.7 ± 4.7% of control in 14 of 17 neurons from sensitized preparations.
Histamine H\textsubscript{3} receptors are known to be present on sympathetic nerve terminals, and action of histamine at these presynaptic receptors is known to inhibit the release of norepinephrine and thereby suppress slow IPSPs (9, 20). The histamine H\textsubscript{3} receptor antagonist thioperamide selectively blocks the presynaptic inhibitory action of histamine at slow noradrenergic synapses in the submucosal plexus (20). In the present study, application of thioperamide (10 \mu M) partially reversed the suppression of slow IPSPs during antigenic challenge in seven neurons (Fig. 8A). The histamine H\textsubscript{2} receptor antagonist cimetidine or the histamine H\textsubscript{1} receptor antagonist pyrilamine did not offset the inhibitory action of antigen challenge on stimulus-evoked slow IPSPs in five neurons (data not shown). These results generally support the conclusion that inhibition of slow IPSPs during antigen exposure in the sensitized preparations is mediated, in part, by the release of histamine and its action at presynaptic inhibitory receptors of the histamine H\textsubscript{3} subtype on sympathetic postganglionic axons in the submucosal plexus.

The cyclooxygenase inhibitor piroxicam (60 \mu M) also partially reversed \beta-lactoglobulin-induced suppression of slow IPSPs in seven submucosal neurons (Fig. 8A). A combination of thioperamide and piroxicam abolished the suppression of slow IPSPs that occurred during antigen challenge in six neurons (Fig. 8A). Unlike the actions of piroxicam and thioperamide, the 5-lipoxygenase inhibitor caffeic acid (30 \mu M) did not reverse suppression of noradrenergic IPSPs during antigenic challenge in five neurons (data not shown). Neither the 5-HT\textsubscript{3p} receptor antagonists BRL 24924 (10 \mu M) nor the 5-HT\textsubscript{3} receptor antagonist MDL 72222 (10 \mu M) antagonized suppression of the slow IPSPs during antigen challenge in five neurons (data not shown).

In milk-sensitized animals, application of either 10 \mu M histamine or the selective histamine H\textsubscript{3} receptor agonist R-\alpha-methylhistamine (3 \mu M) suppressed noradrenergic IPSPs in the same neurons in which exposure to \beta-lactoglobulin suppressed the IPSPs (Fig. 8C). Application of 100 nM PGE\textsubscript{2} also mimicked antigen-evoked suppression of the slow IPSPs (Fig. 8C). On the other hand, application of LTC\textsubscript{4} had no effects on the noradrenergic slow IPSPs (Fig. 8C).

**DISCUSSION**

Guinea pigs in the present study developed an allergic reaction to milk protein that was manifest in the ENS of the small intestine without coincident signs of food allergy in the ENS of the stomach. In this respect, the stomach differed from the ENS of the guinea pig colon that, like the small bowel, develops allergic responses to milk protein(10). Absence of antigenic sensitization in the stomach was unexpected, because mast cells occupy the gastric mucosa. No expression of functional receptors for histamine, prostaglandins, or leukotrienes by gastric enteric neurons was the most plausible explanation for failure to find an allergic reaction in the stomach. Absence of expression of re-

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**Fig. 8. Effects of \beta-LG on noradrenergic neurotransmission in submucosal neurons of milk-sensitized guinea pigs.** A: focal electrical stimulation (20 Hz, 0.2 s) evoked a noradrenergic inhibitory postsynaptic potentials (IPSP) in a neuron from an animal sensitized to milk. Amplitude of the IPSP was suppressed during exposure to 5 \mu M \beta-LG in the bathing solution. Application of 10 \mu M thioperamide or 60 \mu M piroxicam in the presence of the antigen partially restored the amplitude of the IPSP. The combined presence of thioperamide and piroxicam abolished suppression of the IPSP during antigen exposure. B: exposure to \beta-LG did not suppress the hyperpolarizing response to norepinephrine (NE) in neurons from milk-sensitized preparations. Amplitudes of the hyperpolarizing responses to “puffs” of NE were the same before, during exposure to the antigen, and after washout of the antigen. C: inhibitory action of \beta-LG on noradrenergic IPSPs was mimicked by histamine, R-\alpha-methylhistamine, and PGE\textsubscript{2}, but not by LTC\textsubscript{4}. No suppression of IPSPs occurred on application of the antigen to preparations from nonsensitized animals.

Micropressure “puffs” of norepinephrine onto the cell somas evoked IPSP-like hyperpolarizing responses in submucosal neurons from both milk-sensitized and nonsensitized preparations. Application of \beta-lactoglobulin to sensitized preparations never suppressed any of the IPSP-like responses to norepinephrine in seven neurons (Fig. 8B). Failure of antigenic exposure to suppress IPSP-like responses to norepinephrine was consistent with a presynaptic inhibitory action of mediators that were released during antigen exposure and that acted to suppress the release of norepinephrine from sympathetic nerve terminals in the submucosal plexus.
ceptors for the mast cell mediators was reflected by lack of any action of exogenously applied histamine, PGE$_2$, or LTC$_4$. This contrasts with the ENS of the small and large intestine where exposure to any one of these three mediators depolarizes the resting membrane potential and augments neuronal excitability. Aside from failure of expression of receptors for the mast cell mediators, the possibility that gastric mast cells do not express Fc$r$ receptors for sensitizing antibodies to $\beta$-lactoglobulin cannot be ruled-out as part of an explanation for failure to find signs of allergic sensitization in the stomach.

Involvement of mast cells. Several immune/inflammatory cell types including polymorphonuclear leukocytes, lymphocytes, macrophages, and dendrocytes in addition to mast cells are present at all levels of the alimentary canal and are often found in close association with neural elements of the ENS (29, 30). Histological and immunohistological observations suggest that cells of the enteric immune system are strategically positioned to establish a first line of defense against foreign invasion at a vulnerable interface between the body and the outside environment. The cell type involved in antigenic responses in antigen-sensitized guinea pigs in the present and in earlier studies (10, 12) appears to be mast cells that synthesize and store histamine and other paracrine mediators. Mast cell involvement is supported by the finding, in the present study that application of a mast cell degranulating agent (i.e., compound 48/80) or anti-$\gamma$G evoked neuronal electrical behavior in milk-sensitized preparations that mimicked the effects of antigenic stimulation. Moreover, ketotifen, which is a mast cell stabilizing drug, attenuated responses to antigen challenge in milk-sensitized preparations.

Mast cell mediators. Microdissected preparations in the present study consisted of myenteric or submucosal plexus, mast cells, and other cellular elements in the connective tissue matrix. Exposure of the sensitized mast cells to $\beta$-lactoglobulin was expected to trigger degranulation and release of a variety of mediators including histamine, prostaglandins, leukotrienes, cytokines, nitric oxide, and proteases, all of which are known either to be stored in cytoplasmic granules or to be newly synthesized on antigenic stimulation. Observations in the present study are consistent with previous work (10) that identified histamine as one of the mediators that stimulate neuronal excitability. Mast cells are most strongly implicated as the source of endogenous histamine released by antigenic stimulation, because histamine is not generally found in enteric neurons (25).

Elevated levels of endogenous histamine available for action at receptors on submucosal neurons account for part of the excitatory responses found in milk-sensitized small intestine after challenge with $\beta$-lactoglobulin. Experimental application of histamine to enteric neurons of both normal and milk-sensitized guinea pigs resulted in membrane depolarization and augmentation of excitability that resembled the effects of antigenic challenge. Most evidence suggests that the excitatory histaminergic receptors on enteric neurons of the guinea pig belong to the H$_2$ receptor subtype (9, 23). Evidence from the present study pointed to the H$_2$ receptor as the involved subtype, because cimetidine, but not pyrilamine or thiopropamide, suppressed antigen-evoked responses in the milk-sensitized preparations.

Our results suggest that, in addition to histamine, prostaglandins and leukotrienes are also paracrine mediators for the excitatory responses to $\beta$-lactoglobulin. This is based on the finding that piroxicam and caffeic acid reduced but did not abolish excitatory responses to $\beta$-lactoglobulin. Piroxicam is a specific inhibitor of cyclooxygenase that does not affect phospholipase, thromboxane, or prostacyclin synthase or lipoxygenase activity (5). Caffeic acid is a specific inhibitor of 5-lipoxygenase and 12-lipoxygenase (19). Attenuation of the $\beta$-lactoglobulin-induced membrane depolarization by piroxicam and caffeic acid was evidence for the involvement of prostaglandins and leukotrienes as supplementary mediators. Application of exogenous PGE$_2$ or LTC$_4$ mimicked the responses to antigen challenge. Excitatory responses to PGE$_2$ or LTC$_4$ were evidence for the expression of excitatory receptors for prostaglandins and leukotrienes by small intestinal submucosal neurons. Unlike the findings for PGE$_2$ and LTC$_4$, results suggest that 5-HT was not involved in antigenically evoked neuronal excitation, because neither 5-HT$_1$ nor 5-HT$_3$ receptor antagonists suppressed the excitatory effects of exposure to $\beta$-lactoglobulin in the sensitized preparations.

Submucosal plexus. Neural networks in the submucosal plexus are an important part of the neurophysiological control of intestinal secretion. These networks are synthetically “wired” with neurons that can be differentiated according to their electrical and synaptic behavior, morphology, and neurochemistry. Our immunohistochemical results revealed that the submucosal neurons with elevated excitability during antigen exposure also expressed immunoreactivity for VIP or ChAT. Most evidence suggests that neurons with VIP immunoreactivity and slow IPSPs in the guinea pig submucosal plexus are secretomotor neurons (4). This population of secretomotor neurons innervates the intestinal crypts of Lieberkühn and releases VIP as a neurotransmitter, which stimulates the secretion of H$_2$O, electrolytes, and mucus. Submucosal neurons with ChAT immunoreactivity are identified as either a second population of secretomotor neurons or as interneurons (4). Results of the present study indicate that secretomotor neurons were among those neurons with elevated excitability during antigen exposure in the sensitized preparations. Elevation of secretomotor neuronal excitability would be expected to enhance mucosal secretion. Excitation of the secretomotor neurons may be the neural correlate of the enhanced mucosal secretion reported to occur in guinea pigs sensitized to cow’s milk and later challenged with $\beta$-lactoglobulin (17, 34). Augmented stimulation of secretomotor neurons can be implicated in the secretory diarrhea associated with food allergy (35, 37).
**Sympathetic neurotransmission.** Electrical stimulation of sympathetic postganglionic axons in the submucosal plexus evokes slow IPSPs in the cell bodies of secretomotor neurons (20, 38). The slow IPSPs are mediated by release of norepinephrine and its action at \( \alpha_2 \)-adrenoceptors on the secretomotor neurons (24, 38). Aside from augmented neuronal excitability, antigenic exposure in milk-sensitized preparations resulted in inhibition of sympathetic neurotransmission as revealed by suppression of stimulus-evoked slow IPSPs. On the other hand, we found that antigen exposure did not suppress slow IPSP-like responses evoked by micropressure “puffs” of norepinephrine. Failure of antigen exposure to suppress the IPSP-like responses to norepinephrine, while suppressing slow noradrenergic IPSPs in the same neuron, satisfies criteria for presynaptic inhibition of norepinephrine release from the sympathetic nerve terminals. Presynaptic inhibition at slow noradrenergic synapses is a common occurrence in the enteric microcircuits during exposure to inflammatory mediators, such as bradykinin (15), interleukin-1\( \beta \), interleukin-6 (39), and enterotoxins (38). Presynaptic inhibition is also one of the actions of histamine on enteric neurons. The presynaptic histaminergic receptor behaves pharmacologically like the histamine H\( _3 \) subtype and is selectively blocked by the H\( _3 \) receptor antagonist thioperamide (20). PGE\( _2 \), but not LTC\( _4 \), also acts at presynaptic inhibitory receptors to suppress noradrenergic IPSPs (16). Our finding that the 5-lipoxygenase inhibitor, caffeic acid, did not block \( \beta \)-lactoglobulin-evoked suppression of the slow IPSPs suggests that unlike histamine or prostaglandins, release of leukotrienes does not affect noradrenergic neurotransmission.

In conclusion, this study adds to existing evidence for direct communication between the mucosal immune/inflammatory system and the ENS in guinea pig small intestine but not in the stomach. The physiological significance of immunoneural communication is illustrated in Fig. 9. Mast cells degranulate in response to specific antigenic stimulation and release histamine, prostaglandins, leukotrienes, and undoubtedly other inflammatory mediators. Once released, these mediators become paracrine signals to the ENS. Secretomotor neurons located in the submucosal plexus release VIP and/or acetylcholine to stimulate the secretion of H\( _2 \)O, electrolytes, and mucus from the intestinal crypts of Lieberkuhn. Histamine, prostaglandins, and leukotrienes excite the secretomotor neurons in the submucosal plexus, and this stimulates mucosal secretion. Secretomotor neurons receive inhibitory noradrenergic synaptic input from postganglionic sympathetic axons. Sympathetic neuronal firing inhibits secretomotor neuronal activity, which in turn reduces mucosal secretion. The “sympathetic brake” is an important determinant of the balance between absorption and secretory function. Histamine and prostaglandins, but not leukotrienes, act presynaptically to suppress the noradrenergic inhibitory input to the secretomotor neurons. Shutting off the “sympathetic brake” coincides with enhanced excitability of the secretomotor neurons leads to maximal secretomotor neuron firing and hyperstimulation of mucosal secretion. Elevated secretomotor activity is postulated to be a significant pathophysiological factor in the secretory diarrhea associated with food allergies.

Fig. 9. Heuristic model for enteric immunoneural communication in food allergy. Intestinal mucosal mast cells learn to detect specific antigens in food and signal their presence to the enteric nervous system. Cross-linking of a sensitizing antigen with antibodies attached to the surfaces of mast cells degranulates the mast cells and releases a melange of signal substances that include histamine, prostaglandins, and leukotrienes. Once released, the signal substances spread by diffusion to act on neural elements of the enteric nervous system. The present study found one action of the signal substances to be increased excitability of neurons in the submucosal plexus. Two of the mediators, histamine and prostaglandins, also acted to suppress release of NE from sympathetic nerve terminals that form inhibitory synapses with submucosal neurons. A subpopulation of neurons in the submucosal plexus consists of secretomotor neurons that stimulate secretion from the crypts of Lieberkuhn. Elevation of excitability in secretomotor neurons increases the volume of mucosal secretion. Two mechanisms elevate excitability. One mechanism directly increases excitability through the direct action of a mediator at excitatory receptors on the neurons. The second mechanism increases excitability indirectly by removing sympathetic braking action from the neurons. Removal of sympathetic braking action is achieved by action of mast cell mediators at presynaptic inhibitory receptors at NE release sites on postganglionic sympathetic nerve fibers.
REFERENCES


