Leptin and CCK selectively activate vagal afferent neurons innervating the stomach and duodenum


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Results from our laboratory and others suggest that primary vagal afferent neurons may be one site at which this interaction occurs. For example, studies of receptor expression (5, 6, 7, 11) and electrophysiological data (14, 28, 29) support the conclusion that leptin and CCK both are capable of activating vagal afferents. Furthermore, in vitro studies have shown that leptin and CCK acutely depolarize cultured vagal afferent neurons (8, 14, 21), and single-cell calcium measurements demonstrated that this occurred in an overlapping population of neurons (12). Finally, we recently demonstrated that microgram infusions of leptin into the celiac artery, which perfuses the upper gastrointestinal (GI) tract, including the stomach and duodenum, decreases meal size (13). The effect of intraceliac leptin depends on capsaicin-sensitive, subdiaphragmatic vagal afferent neurons (13). These findings suggest that leptin and CCK act on vagal afferent neurons to acutely regulate meal size.

It is well known that gastric mucosal leptin, and CCK from intestinal I cells, are secreted in response to ingestion of a meal (1, 24). The fact that these areas are also heavily innervated by vagal afferents suggests that vagal afferent fibers that innervate these structures may be selectively responsive to leptin and CCK. To investigate this hypothesis, we selectively labeled vagal afferent neurons that innervated either the stomach or duodenum using a fluorescent retrograde neuronal tracer. We then isolated the cell bodies of these neurons from the nodose ganglion and then measured cytosolic calcium responses to leptin and CCK in specifically labeled neurons using single-cell imaging techniques with the fluorescent calcium indicator fura-2 AM. We found that leptin and CCK increased calcium levels in a significantly greater proportion of neurons labeled from the stomach and duodenum than in nonlabeled vagal afferents. Additionally, the vagal afferents activated by leptin and CCK were largely overlapping.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats purchased from Simonsen Laboratories were subjects for all experiments. The animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited quarters under a 12:12-h light-dark cycle and were provided with ad libitum pelleted chow. All procedures performed were approved by the Washington State University, Institutional Animal Care and Use Committee. For all surgical procedures, the rats were anesthetized (ketamine 25 mg/100 g, xylazine 2.5 mg/100 g), and surgeries were performed under aseptic conditions.
Retrograde tracer application. Fluorescent latex microspheres (530 nm excitation, 590 nm emission) (Lumafluor, Naples, FL) were used to retrogradely label vagal afferent neurons innervating the stomach and proximal duodenum. In each tissue, microspheres were injected in 20 sites (0.5 μl/site) using a microsyringe (Hamilton, Reno, NV). The injection sites formed a grid pattern across the dorsal and ventral surfaces of the stomach and circumferentially around the proximal duodenum, beginning 1–2 mm from the pylorus and extending 20 mm aborally. To test for possible nonspecific labeling of vagal afferent neurons, we injected microsphere tracer into the abdominal wall in a pattern similar to our stomach and duodenal injections. These abdominal wall injections should mimic any nonspecific diffusion to the peritoneal cavity equivalent to our selective injections of stomach and duodenum. Any spillage of tracer from the injection site was immediately removed with a cotton-tipped swab. Although some labeling in the nodose ganglia was observed within 1 wk following surgery, a minimum of 2 wk was allowed before nodose ganglia were collected for culture. This period appeared optimal for labeling the maximum number of vagal afferent cell bodies in the nodose ganglia following injection of this tracer to these sites. Isolations were taken from at least nine animals for both the stomach- and duodenum-labeled groups.

Cell culture. Dissociated vagal afferent cell bodies were obtained as previously described (22). Briefly, nodose ganglia, which contain the vagal afferent cell bodies, were isolated from anesthetized (ketamine 25 mg/100 g, xylazine 2.5 mg/100 g) adult male Sprague-Dawley rats (280–320 g) under aseptic conditions. The ganglia were digested for 90 min at 37°C in 3 ml of Ca²⁺- and Mg²⁺-free HBSS containing 1 mg/ml dispase II (Boehringer-Mannheim, Indianapolis, IN) and 1 mg/ml collagenase type Ia (Sigma, St. Louis, MO). Cells were dispersed by gentle trituration through silanized Pasteur pipettes, washed two times with HEPES-buffered DMEM (HDMEM; Life Technologies, Grand Island, NY) containing 10% FCS (Life Technologies) supplemented with antibiotic (penicillin-streptomycin, 100 U/ml and 100 μg/ml, respectively), and then plated onto poly-l-lysine-coated coverslips and maintained in HDMEM with 10% FCS at 37°C in a 5% CO₂ atmosphere. All measurements were performed within 48 h of collecting the nodose ganglia.

Calcium measurements. Ratiometric measurements using the fluorescent calcium indicator fura-2 AM (Molecular Probes, Eugene, OR) were performed using MetaFluor Software (Universal Imaging, West Chester, PA). All manipulations and measurements were made at room temperature (22°C) in a physiological saline solution (in mM: 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 6 glucose, and 10 HEPES, with pH adjusted to 7.4 with NaOH). Cells were loaded with 2 μM fura-2 AM for 30 min followed by a 30-min wash. The coverslips containing the loaded cells were mounted into an open chamber and constantly perfused with physiological saline. Image pairs (340 and 380 nm excitation, 510 emissions) were collected every 6 s. Ratios of fluorescence intensity were converted to calcium concentrations using a standard curve.

Neuron identification. Labeled neurons were identified by the intracellular presence of intensely fluorescent microspheres that emitted in the red spectral range. Fluorescence of the microspheres was visualized using a band-pass filter (575–650 nm; Omega Optics, Brattleboro, VT), and typical fluorescence of labeled neurons was 2–10 times brighter compared with unlabeled neurons. Although nonviable (necrotic or apoptotic) neurons sometimes exhibited high levels of autofluorescence in this range, they were easily excluded on the basis of their deteriorating morphology, inability to maintain constant basal calcium levels, or inability to respond to a KCl challenge.

Experimental protocol. Murine leptin (Pepro Tech, Rocky Hill, NJ) and the sulfated form of CCK-8 (Peptides International, Louisville, KY) were used. In some measurements, an additional challenge to capsaicin (Sigma) was made at the end of the experiment to identify whether the neuron belonged to A-type or C-type populations (21).

Leptin and CCK were dissolved directly in physiological saline, while capsaicin was first dissolved into 0.1% DMSO and further diluted in physiological saline. Solutions containing hormones and capsaicin were applied by switching solutions flowing through a common manifold upstream of the recording chamber (~15 to 30 s required for new solution to reach neurons). Concentrations of hormones and capsaicin used are indicated in RESULTS. On all coverslips, neuronal viability was affirmed following the hormonal challenges by a brief challenge to 55 mM KCl solution (reciprocal reduction in bath NaCl). Only cells with at least a 40-nM rise in cytosolic Ca²⁺ in response to the high K⁺ challenge were used in the analysis.

Statistics. The χ² analysis was used to determine whether responses to CCK or leptin occurred selectively in labeled neurons vs. nonlabeled neurons in the same cultures and whether responses to leptin and CCK selectively colocalized to the same population of neurons. P values <0.05 were considered statistically significant.

RESULTS

Neurons innervating the stomach (Fig. 1A) and duodenum (Fig. 1B) were identified by epifluorescent illumination of microspheres that had been injected into the specific endorgan as outlined in MATERIALS AND METHODS. No labeling was observed when the microsphere tracer was applied to the abdominal wall (Fig. 1C). To determine the responsiveness of neurons, we sequentially challenged labeled neurons with concentrations of leptin (40 ng/ml) and CCK (10 nM) that we have found to produce reliable activation in sensitive cells. Among neurons that innervated the stomach, we observed three types of responses: neurons nonresponsive to both CCK and leptin (Fig. 2A), neurons responsive only to CCK (Fig. 2B), and neurons responsive to both leptin and CCK (Fig. 2C). We did not observe neurons responsive to leptin only. Distribution of responses are summarized in Fig. 2D. Specifically, 17 of 53 neurons responded to CCK only (32%), 22 of 53 neurons responded to both leptin and CCK (42%), and the remaining neurons (14 of 53, 26%) did not respond to either leptin or CCK. Among neurons sensitive to CCK, 56% (22 of 39) were also responsive to leptin, whereas all of the neurons responsive to leptin were also reactive to CCK (22 of 22, 100%). There was a significant overlap in responsiveness to leptin and CCK among neurons labeled from the stomach (F = 11.27, df = 1, P < 0.001).

Among neurons that innervated the duodenum, we observed four types of responses: neurons responsive only to CCK (Fig. 3A), neurons responsive only to leptin (Fig. 3B), neurons responsive to both leptin and CCK (Fig. 3C), and neurons nonresponsive to either leptin or CCK (example not shown). Distribution of responses are summarized in Fig. 3D.
that 8 of 29 (28%) responded only to CCK; 1 of 29 (3%) responded only to leptin; and 13 of 29 responded to both leptin and CCK (45%). The remaining neurons (7 of 29, 24%) did not respond to leptin or CCK. Among neurons sensitive to CCK, 13 of 21 (62%) were also responsive to leptin, whereas all except one of the neurons responsive to leptin were also reactive to CCK (13 of 14, 93%). There was a significant overlap in responsiveness to leptin and CCK among neurons labeled from the stomach ($F = 3.85, df = 1, P < 0.05$).

Summarized in Fig. 4 is the distribution of all neurons responsive to leptin and all neurons responsive to CCK from both the stomach and duodenum. We observed that leptin activated a greater percentage of vagal afferent neurons that innervated the stomach ($F = 3.94, df = 1, P < 0.05$) and duodenum ($F = 4.03, df = 1, P < 0.05$) compared with nonlabeled neurons that were examined simultaneously in the same culture (Fig. 4A). CCK also activated a significantly greater percentage of the neurons labeled from both the stomach ($F = 19.7, df = 1, P < 0.001$) and the duodenum ($F = 10.95, df = 1, P < 0.001$) than nonlabeled neurons (Fig. 4B).

Vagal afferent neurons can be classified into either A- or C-type neurons, according to their sensitivity to the VR1 agonist, capsaicin (21). C-type neurons, but not A-type neurons, exhibit depolarization and increased intracellular calcium in response to capsaicin exposure. To determine whether CCK or leptin selectively activated A- or C-type nodose ganglion neurons, we applied capsaicin (10 nM) to a subgroup of stomach-labeled cells after assessing their sensitivity to leptin and CCK. We found that there were both capsaicin-sensitive (21 of 41, 51%; Fig. 5A) and capsaicin-insensitive (20 of 41, 49%; Fig. 5B) neurons labeled from the stomach. Sixty percent (18 of 30) of the CCK-responsive neurons that innervated the stomach were capsaicin-sensitive C-type neurons (Fig. 5A), whereas 50% (11 of 22) of the leptin-responsive neurons...
innervated by the stomach were capsaicin sensitive (data not shown). Finally, we found that neurons that innervated the duodenum consisted of both capsaicin-sensitive (12 of 17, 71%; (Fig. 5C) and capsaicin-insensitive (5 of 17, 29%) sub-populations (Fig. 5D) cells. Eighty percent (12 of 15) of the CCK-responsive neurons that innervated the duodenum were capsaicin sensitive, whereas 89% (8 of 9) of the leptin-responsive neurons were activated by capsaicin.

DISCUSSION

The results of experiments reported here indicate that the proportion of CCK-sensitive and leptin-sensitive vagal afferents retrogradely labeled by tracer injections into the stomach and duodenum is markedly and significantly greater than that of the unlabeled afferents collected from the same nodose ganglia. The proportion of these unlabeled neurons responding to CCK was very close to the proportion of the total vagal afferent population that we previously reported as sensitive to CCK (22) and are in close agreement with estimates of the number of vagal afferents that express mRNA coding for the CCK1 receptor (5), which we have shown mediates virtually all vagal afferent responses to this peptide (22). Thus it appears that the unlabeled population was fairly representative of the overall prevalence of CCK-responsive afferents in the vagus. One might argue that subtraction of labeled vagal afferents from the unlabeled population should increase the proportion of unlabeled CCK-nonresponsive neurons. However, it is important to point out that the fluorescent microspheres we used for retrograde labeling in this study are nondiffusible. Therefore, the area of innervation exposed to our injections, and hence the proportion of the total gastric and duodenal vagal afferent population labeled must be exceedingly small, such that the actual number of labeled afferents is insignificant relative to the total number of gastric and duodenal afferents in the nodose ganglia. Therefore, we conclude that vagal afferents innervating the upper GI tract, specifically the stomach and duodenum, are highly sensitive to CCK; with other organs likely receiving little or no CCK-responsive afferent innervation. Targeting of the CCK-responsive vagal afferent popula-

Fig. 4. Percentage of neurons in which leptin (40 ng/ml) (A) and CCK (10 nM) (B) induced an increase in cytosolic calcium in neurons labeled from the stomach (stom) (n = 54), duodenum (duo) (n = 29) or among nonlabeled neurons (non) (n = 97). *Statistically significant difference from nonlabeled neurons by χ² analysis (P < 0.05 for leptin and P < 0.001 for CCK).

Fig. 5. Characterization of responsiveness to CCK, leptin, and capsaicin in neurons labeled from the stomach and duodenum. The bars over the traces indicate when either leptin (40 ng/ml), CCK (10 nM), or capsaicin (10 nM) was applied. CCK activated both capsaicin-sensitive (A) and capsaicin-insensitive (B) neurons labeled from the stomach. In neurons labeled from the duodenum, CCK and leptin activated both capsaicin-sensitive (C) and capsaicin-insensitive (D) cells. Overall, 51% (21 of 41) of cultured nodose neurons labeled from the stomach and 71% (12 of 17) of the neurons labeled from the duodenum were activated by capsaicin. Specifically, 60% (18 of 30) of the CCK-responsive neurons and 50% (11 of 22) of the leptin-responsive (example not shown) neurons labeled from the stomach were activated by capsaicin. Within nodose neurons innervating the duodenum, 80% (12 of 15) of the CCK-responsive neurons and 89% (8 of 9) of the leptin-responsive neurons were sensitive to capsaicin.
tion to the upper GI tract is not entirely surprising, as CCK participates in the initiation of many upper GI reflexes, and its principle functions seem to be coordination of upper GI digestive processes and satiation for food.

The proportion of vagal afferent neurons that were responsive to leptin also was higher in neurons labeled by gastric and duodenal microsphere injections than it was in the unlabeled population. In the case of leptin, however, the differences between the unlabeled population and the labeled populations were not as great as for CCK, suggesting that leptin-responsiveness is more widely distributed among vagal afferent innervations than CCK responsiveness. It is possible that leptin responsiveness simply is distributed more widely in GI vagal afferents. However, it also seems reasonable that sensitivity to a hormone that reflects adipose mass might exert modulatory control of cardiovascular or pulmonary afferents as well. Additional experiments will be required to assess these possibilities.

Our current results are consistent with results of electrical recordings from vagal afferent fibers in vivo. For example, Wang et al. (28) reported that leptin and CCK activate afferent fibers in the gastric branch of the vagus. Our current study adds a number of salient points to prior observations. First, as we have pointed out in the past, the fact that our measures occurred in vitro in neurons deprived of their end organ connections obviates the possibility that the afferent responses we recorded were secondary to mechanical or chemical responses of the end organs. Second, in our experiments, we were able to quantify the proportion of responses attributable to specific vagal afferent innervations, and to compare these proportions to estimates of vagal afferent responsiveness made by nonelectrophysiological means (5). Like the first point, this latter point is not obviated by in vivo recording from individual vagal branches, such as the gastric branch, because the gastric branch innervates nongastric sites as well as the stomach (3), just as fibers in the hepatic branch also innervate nonhepatic sites in addition to the liver. Through in vitro measurements from prelabeled cultured afferents, we can now be more confident that vagal afferent sensitivities to CCK and leptin are direct effects on vagal afferents themselves, and we have a compelling indication of which organ systems contain the terminals that are CCK and leptin responsive.

Nearly all of the CCK- and leptin-responsive neurons innervating the duodenum were capsaicin sensitive. This observation is consistent with anatomical reports that most of the duodenal vagal afferent innervation is destroyed by capsaicin (4). However, we found that leptin and CCK activated both capsaicin-sensitive C-type afferents and capsaicin-insensitive A-type afferents from the stomach. This observation is consistent with our previously reported electrophysiological finding that CCK depolarizes both C- and A-type afferents (21). Electrophysiological (18) and anatomical data (4) indicate that the stomach receives a significant capsaicin-insensitive vagal afferent innervation. Furthermore, it appears that this capsaicin-resistant innervation is distension responsive (18). Gastric distension and CCK injection have been shown to interact to reduce food intake (26). Although some of the responses to CCK are mediated by capsaicin-sensitive vagal afferents (15–17, 19, 25), recently published data demonstrate that the response of capsaicin-resistant gastric vagal afferents to distension are enhanced by CCK (27), suggesting that the action of CCK at A-type afferents may be to enhance responses to other stimuli like distension. The role of leptin responsiveness of gastric vagal afferents is not known. However, because all leptin-responsive gastric vagal afferents also were CCK responsive, it seems reasonable that leptin may enhance the responses of gastric afferents to distension and/or CCK.

The concentration of leptin- and CCK-responsive afferents within the vagal afferent populations innervating stomach and duodenum suggest that these innervations should exhibit a high level of functional sensitivity to leptin and CCK. Indeed, we and others (13) have demonstrated that rats are more sensitive to the satiation effects of CCK when it is infused into the arterial supply of the upper GI tract than when it is administered intravenously. Recently, we reported that the upper GI innervation does appear to be selectively sensitive to reduction of food intake by leptin and CCK. We have found that infusion of low doses of leptin into the celiac artery reduces short-term food intake via a vagally dependent mechanism. Furthermore, the effect of leptin was synergistically enhanced in the presence of CCK (13). The celiac artery perfuses the upper GI organs, including the stomach, proximal small intestine, liver, and pancreas. Hence, we were not able to determine the exact site(s) where the leptin and CCK were acting. Nevertheless, our finding that cultured nodose ganglion neurons labeled from the stomach or duodenum were highly sensitive to leptin and CCK suggests that the vagal afferent terminal fields within these organs are a potential site for endogenous leptin and CCK action and interaction and may mediate all or part of the vagally dependent effects of leptin and CCK on food intake.

Our celiac arterial leptin infusions did not elevate systemic leptin concentrations beyond the physiological range (13). However, it is likely that leptin and CCK concentrations near the gastric and duodenal vagal afferent terminals were higher than those in the systemic circulation and higher than would be generated in this area by adipose-derived leptin. Bado et al. (1) have demonstrated that leptin is synthesized and stored within exocrine and endocrine cells of the gastric mucosa. Furthermore, this source of leptin is depleted postprandially and is probably secreted in response to food intake (1). Although the total amount of leptin released from the stomach may be relatively small and likely does not significantly increase systemic circulating concentrations, it is possible that leptin secreted from the gastric mucosa may act locally within the extracellular space of the upper GI tract in a paracrine fashion. Such a mode of action has previously been proposed for CCK-induced vagal afferent activation. Furthermore, the fact that leptin and CCK exhibit pronounced synergy in activation of vagal afferents in vitro and the induction of satiation in vivo raises the possibility that endocrine concentrations of one peptide may enhance paracrine concentrations of the other in vivo. Such a paracrine/endocrine mode of cooperation would be consistent with our finding that leptin and CCK activate vagal afferents that innervate the stomach, which secretes leptin, but not CCK, and also activate afferents that innervate the duodenum, which secretes CCK, but presumably not leptin.

In summary, we report that vagal afferent neurons that innervate the stomach and duodenum are highly responsive to both leptin and CCK. The demonstration that leptin and CCK activated an overlapping population of gastric and duodenal vagal afferents suggests that these two agents may influence physiological parameters referable to these specific innerv-
tions, including the control of food intake. Finally, the observation that leptin- and CCK-responsive neurons consisted of both capsaicin-sensitive and capsaicin-insensitive populations suggests that they may modify signaling of multiple modalities of vagal afferent information. The extensive distribution of vagal afferent neurons innervating the stomach and duodenum, which are responsive to both leptin and CCK, may provide a substrate for their synergistic activation of vagal afferent neurons and may explain part of their endogenous interactions on food intake and energy expenditure. These findings, taken together, are consistent with the hypothesis that gastric leptin and CCK act locally within the upper GI tract to activate vagal afferent neurons and contribute to the control of food intake.

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