Inhibitory effects on intake of cholecystokinin-8 and cholecystokinin-33 in rats with hepatic proper or common hepatic branch vagal innervation


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abdominal vagal innervation. Specifically, we administered both peptides intraperitoneally to two groups of vagotomized rats. One group (HGD rats) had the common hepatic branch (3, 24, 28, 30) intact and all other abdominal vagal branches sectioned. This has been referred to previously as a hepatic-spared vagotomy (16, 38). This surgical group had vagal innervation of the liver through the hepatic branch proper and vagal innervation of the proximal duodenum, distal ileum, and proximal stomach through the fibers of the gastroduodenal branch that traverse the liver and descend to the small intestine. Previous experiments in HGD rats showed that the common hepatic branch of the vagus was sufficient to mediate the inhibition of intake by CCK-8 in real feeding and sham feeding in some (15, 16) but not all (39) conditions. The inhibitory effect of CCK-8 in HGD rats is presumably mediated by afferent fibers of the common hepatic branch. Because CCK₁ receptors are on the afferent fibers (6, 21), CCK-8 produces electrophysiological stimulation of the afferent fibers of the common hepatic branch (8, 35) and total, abdominal, afferent vagotomy abolished the inhibitory effect of CCK-8 (20, 40).

To our knowledge, CCK-33 has not been tested in HGD rats.

The other group of vagotomized rats (H rats) had vagal innervation of the liver by the fibers of the hepatic branch proper, but had no other abdominal vagal innervation because the gastroduodenal branch of the common hepatic branch and all other abdominal vagal branches were sectioned. Neither CCK-8 nor CCK-33 has been tested in rats with this type of vagal innervation.

On the basis of previous results with intraportal administration of the peptides in vagally intact rats (11, 36), we predicted that CCK-33 would be significantly more potent than CCK-8 in H rats. We also predicted that CCK-33 and CCK-8 would be equipotent in HGD rats as they are after intraperitoneal administration in vagally intact rats (19). The results confirmed the first prediction, but not the second.

**MATERIALS AND METHODS**

Male Sprague-Dawley rats weighing between 200–225 g (Charles River Laboratories, Wilmington, MA) were housed individually in polycarbonate, solid-bottomed cages (24 x 20 x 29 cm) with cob bedding. Room temperature was ~23°C, and there was a 12:12-h light/dark cycle (lights on 0700). The rats were maintained on ground chow (Purina no. 5001; St. Louis, MO) and tap water ad libitum for 1 wk, following which they were also offered a milk diet consisting of equal parts of sweetened condensed milk (Eagle Brand; Borden, Columbus, OH) and tap water supplemented with Poly-Vi-Sol vitamin drops, (2.0 ml/0.6 liter of diet; Mead Johnson, Evansville, IN). Rats were vagotomized after at least 2 wk of access to the milk diet. All procedures and protocols were approved by the Institutional Animal Care and Use Committees of Weill Medical College of Cornell University and Purdue University.

**Surgery.** Rats (250–325 g body wt at surgery) were anesthetized by administration of 3 ml/kg ip of a mixture containing pentobarbital (8.9 mg/ml) and chloral hydrate (42.5 mg/ml). Rats were laparotomized and the right (anterior or ventral) and left (posterior or dorsal) vagal trunks were visualized microscopically (magnification, ×10) as they emerged along the esophagus below the diaphragm. This was facilitated by gentle traction on the ends of a 3-0-vicryl (Ethicon; Johnson & Johnson, Sommerville, NJ) stay suture placed in the middle of the greater curvature of the stomach. The left trunk and the gastric and accessory celiac branches of the right trunk were ligated individually with two 3-0-vicryl ties placed about 5 mm apart and then sectioned between the ligatures. This left the common hepatic branch intact.

Thus HGD rats had the hepatic branch proper and the gastroduodenal branch intact.

To prepare H rats with only the hepatic branch proper intact, the same procedure was performed as in HGD rats, and then the gastroduodenal branch of the common hepatic branch was sectioned using the technique described by MacIsaac and Geary (17). The hepatic artery was exposed, isolated, and then sectioned between two 4-0-vicryl ligatures placed about 2–5 mm apart. The proximal ligature was distal to the bifurcation of the celiac artery into the hepatic and gastroduodenal arteries, so blood flow in the gastroduodenal artery was not compromised. After the hepatic artery was sectioned, forceps were used to strip the bile duct and portal vein with cotton-tipped applicators. A piece of parafilm cut to size was placed behind the bile duct and portal vein to protect the underlying and surrounding tissue from the solution. After application of the solution, the area was flushed copiously with 0.15 M saline and the piece of parafilm was removed. Sham rats underwent laparotomy, but the vagal nerves were not touched. At the end of all surgeries, the abdominal cavity was closed with interrupted 3-0-vicryl sutures, and the skin was closed with wound clips (Clay Adams; Becton Dickinson, Sparks, MD).

**Test procedure.** After recovering from surgery, the rats were slowly weaned from the milk diet and returned to their maintenance diet of ground chow. During this time, the rats were adapted to intraperitoneal injections and the test diet of 10% sucrose.

On test days, rats were food deprived at 0900 and water deprived at 1245. At 1255 rats received an intraperitoneal injection of CCK-8 or CCK-33 in 1 ml of 0.9% bacteriostatic saline (Abbott Laboratories, North Chicago, IL) or 1 ml of 0.9% bacteriostatic saline alone. The doses of CCK-8 (Peninsula Laboratory, San Carlos, CA) were 4, 8, and 16 μg/kg; molar equivalent doses of porcine CCK-33 (Peptide, Osaka, Japan) were 13.2, 26.4, and 52.8 μg/kg. Each test with CCK-8 or CCK-33 was preceded by a control test in which saline alone was injected. At 1300, the rats were given access to a 10% sucrose solution in a graduated burette fitted with a sipper tube. Intake was measured at 1330. Under these conditions, the 30-min intake was the size of a single meal. At the end of a test, rats were returned to their home cages, water was replaced at 1345, and chow was replaced at 1400.

At the end of behavioral testing, rats were shipped to Purdue University for verification of the vagal afferent denervation.

**Verification of vagal afferent denervation.** After arrival at Purdue University, rats were housed individually at 23°C and had ad libitum access to tap water and pelleted rat chow before verification surgery.

Vagotomies were verified with a wheat germ agglutinin-horseradish peroxidase (WGA-HRP) tracer strategy (24, 27). Briefly, each rat was anesthetized with pentobarbital sodium (60 mg/kg ip) and the left nodose ganglion was exposed and pressure injected (PicoSpritzer II; General Valve, NJ) with 4% WGA-HRP (3.0 μl; Vector Laboratories) through a glass micropipette (25 μm ID). The incision was closed and treated with nitrofurazone. Then the rat was returned to its cage.

Three days after receiving WGA-HRP, each rat was deeply anesthetized with pentobarbital (180 mg/kg ip) and injected in the left cardiac ventricle with 0.1 ml heparin (1,000 U/ml; Elkins-Sinn, Cherry Hill, NJ) to prevent coagulation and 0.1 ml propranolol (Ayerst Laboratories, Philadelphia, PA) to produce vasodilatation. The rat was perfused transcardially with 500 ml of 0.9% saline at 40°C followed by 3% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 at 4°C, and finally by 10% sucrose in the same buffer at 4°C.

The hilus of the liver (including the portal vein, arteries, and adventitia) and first 8 cm of the duodenum were prepared as whole mounts (29). The slides were then processed with tetramethyl benzidine (5), mounted, air dried, cleared in xylene, and coverslipped with 1,3-Diethyl-8-phenyloxanithine (DPX; Aldrich, Milwaukee, WI).

Innervation patterns were assessed using darkfield microscopy in conjunction with a reconstruction strategy standardized for the com-
mon hepatic branch (24, 27). In this procedure, WGA-HRP-labeled axons in the hilus of the liver and proximal 8 cm of the small intestine were analyzed for their characteristic innervation profiles by an observer blind to each animal’s surgical identity and behavioral results. A HGD preparation was considered successful if 1) the afferent innervation of the liver by the hepatic branch proper and of the first 8 cm of the duodenum by the gastroduodenal branch of the common hepatic branch was comparable to the patterns previously reported (24), and 2) the more distal duodenum (innervated by the celiac branch of the vagus) and the pylorus (innervated by the gastric branch) appeared denervated. An H preparation was considered successful if 1) the innervation of the liver by the hepatic branch proper of the common hepatic branch appeared intact, and 2) the proximal duodenum as well as the more distal duodenum and the pylorus were denervated. A vagotomy was considered incomplete or nonselective if either of the celiac or gastric branches were completely or partially spared (27).

Statistical analysis. The intakes after CCK-8 and CCK-33 were expressed as percent inhibitions of the intakes in the preceding control tests. First, the effects of CCK-8 and CCK-33 were analyzed separately by two-way ANOVAs with surgical condition (sham, HGD, and H) as a between-subjects factor and dose as a within-subjects factor. Then, to compare the potencies of the molar equivalent doses of the peptides, separate two-way ANOVAs were done with peptide (CCK-8 and CCK-33) as a between-subjects factor and dose as a within-subjects factor. Tukey’s honestly significant difference test was used to test pairwise differences after significant ANOVAs. Differences were considered significant when \( P < 0.05 \).

RESULTS

Vagal afferent innervation. Seven of eight HGD rats had complete HGD vagotomies and eight of ten H rats had complete H vagotomies. Only these rats were included in the statistical analysis. In both HGD and H rats, the hilus of the liver and the surrounding arterial supply were densely innervated by afferent bundles originating from the hepatic branch proper of the common hepatic branch (Fig. 1). The two groups, however, differed in the pattern of innervation of the first 8 cm of the duodenum. In HGD rats, the initial segment of the duodenum was richly innervated by vagal afferent axons and endings (Fig. 2, A and C), whereas the same region in H rats was completely denervated (Fig. 2B).

CCK-33. Surgical condition affected the potency of CCK-33 significantly \( [F(2, 34) = 7.10, \ P = 0.006, \text{Fig. 3}] \). CCK-33 produced equivalent percent inhibitions in H and sham rats \( (P > 0.05) \), but significantly less inhibition in HGD rats than in sham rats or H rats \( (P < 0.05) \).

There was a significant effect of dose of CCK-33 \( [F(2, 34) = 8.01, \ P = 0.001] \). The largest dose of CCK-33 (52.8 \( \mu g/kg \)) produced a significantly larger inhibition of intake than 13.2 \( \mu g/kg \) \( (P < 0.05) \), but not a larger inhibition than was produced by 26.4 \( \mu g/kg \) \( (P > 0.05) \). There was no significant interaction between dose and surgical condition \( [F(4, 34) = 1.26, \ P = 0.30] \).

CCK-8. Surgical condition also affected the potency of CCK-8 significantly \( [F(2, 34) = 11.63, \ P = 0.001, \text{Fig. 4}] \). In contrast to the effects of CCK-33, however, CCK-8 decreased intake significantly less in H and HGD rats than in sham rats \( (P < 0.05) \). There was no significant difference in the weak inhibitory effects of CCK-8 in H and HGD rats \( (P > 0.05) \).

There was a significant effect of dose of CCK-8 \( [F(2, 34) = 4.59, \ P = 0.017] \). The largest dose of CCK-8 (16 \( \mu g/kg \)) inhibited intake significantly more than did 4 \( \mu g/kg \) \( (P < 0.05) \) but not more than 8 \( \mu g/kg \) \( (P > 0.05) \). There was no significant interaction between surgical condition and dose \( [F(4, 34) = 0.37] \).

Comparison of potencies of CCK-33 and CCK-8. The potencies of the molar equivalent doses of CCK-33 and CCK-8 were not significantly different in sham rats \( (P > 0.05) \) nor in HGD rats \( (P > 0.05) \); compare Figs. 3 and 4). In H rats, however, 13.2 and 26.4 \( \mu g/kg \) of CCK-33 produced significantly larger percent inhibitions of intake than the equimolar doses of CCK-8 (4 and 8 \( \mu g/kg \), respectively; \( P < 0.05) \).

DISCUSSION

CCK-33. The major result of these experiments is that CCK-33 (intraperitoneal) inhibited 30-min intake significantly more than CCK-8 in H rats in which the presence of the afferent innervation of the hepatic branch proper and the absence of the gastroduodenal, gastric, and celiac afferent innervation of the proximal duodenum were anatomically verified. This confirms our prediction and extends previous results obtained with intraperitoneal administration of CCK-33 and CCK-8 in rats in which all of the abdominal vagal innervation was intact (12, 36). Taken together, the results are consistent with the hypothesis that exogenous CCK-33 is a more effective stimulant of vagal afferent terminals in the liver than CCK-8.

It is probable that CCK1 receptors on hepatic vagal afferent terminals mediated the effect of CCK-33 in H rats, because devazepide abolished the inhibitory effect of CCK-33 on intake in rats with intact abdominal vagal innervation (22). These results with devazepide were obtained under different experimental conditions. Devazepide needs to be investigated in H rats in the conditions used in these experiments.

The larger effect of CCK-33 than CCK-8 in H rats after intraperitoneal administration was related to innervation and not to other conditions of these experiments, because CCK-33 and CCK-8 had equivalent potencies in sham rats and equivalently reduced potencies in HGD rats (compare Figs. 3 and 4).
The larger effect of CCK-33 in H rats was unexpected, given the presence of hepatic proper nerves in H and HGD rats. What explains this differential potency? We suggest the following possible answers to that question. First, when CCK-33 is administered intraperitoneally, it stimulates gastroduodenal vagal afferent terminals first, and then after it gains access to the portal blood, it stimulates hepatic vagal afferent terminals. This sequential stimulation could inhibit the effect of CCK-33 on hepatic vagal afferent terminals peripherally via an axon reflex mechanism, if H and HGD are branches of the same fibers of the common hepatic branch, an anatomical relationship that has not been investigated. Alternatively, the inhibitory interaction could occur centrally in the nucleus tractus solitarius or in other regions of the brain involved in processing the vagal afferent stimulation.

In either case, the larger response in H rats than in HGD rats would result from neural disinhibition of the response of afferent H fibers to CCK-33 by the removal of gastroduodenal afferent fibers. Note that intraportal administration of CCK-33 in vagally-intact rats also bypasses the proposed inhibitory interaction produced by sequential stimulation of gastroduodenal and hepatic afferent fibers.

Second, section of the gastroduodenal fibers probably provoked a regenerative response in the hepatic fibers (25, 26). This could increase the response to CCK-33 in the liver by increasing the number of CCK-1 receptors on hepatic terminals or by increasing the efficiency of their transduction mechanisms.

Third, the increased response to CCK-33 in H rats could have been due to a change in the intrahepatic blood flow produced by the surgical procedure used to section the gastroduodenal branch. The procedure eliminated blood flow from the hepatic artery and removed most, if not all, of the postganglionic sympathetic fibers to the liver (1). This could change the distribution of absorbed CCK-33 within the liver so that it had greater access to hepatic vagal terminals.

Fourth, if the postganglionic sympathetic terminals exerted inhibitory control of the vagal afferent terminals’ response to CCK-33, their removal would also disinhibit the vagal terminals.
All of these suggestions are possible, but none of them is supported by experimental evidence. The problem deserves further investigation because inhibitory interactions among afferent terminal fields of the abdominal vagus have not been described or considered to contribute to the integration of afferent vagal stimulation.

CCK-8. There were two important results of the experiments with CCK-8. First, CCK-8 was significantly less potent in HGD rats than in sham rats. Second, the inhibitory potency of CCK-8 in H rats was not significantly different from its potency in HGD rats.

The decreased potency of CCK-8 in HGD rats compared with sham rats demonstrates that the vagal afferent innervation of the small intestine by the gastroduodenal branch is not sufficient to mediate the normal inhibitory effect of CCK-8 on food intake under these conditions. This confirms our previous result (39) but is different from that of Le Sauter and Geary (16). The reason for the different results is not clear.

The weak potency of CCK-8 in HGD rats suggests that the celiac or gastric afferent innervation of the small intestine that is not present in HGD rats is necessary for the normal potency of CCK-8, at least under these conditions. The celiac afferent innervation may be more important than the gastric because the celiac nerves innervate much more of the small intestine than does the gastric branch (2). Furthermore, celiac afferent vagotony decreased or abolished the inhibitory effect on food intake of nutrients infused into the small intestine that release endogenous CCK (44). The relative importance of gastric and celiac innervation could be decided by measuring the potency of CCK-8 in HGD rats in which the celiac or gastric vagal innervation was preserved. This experiment has not been reported.

The second important result was that CCK-8 was not more potent in H rats than in HGD rats. Thus when the same intraperitoneal route of administration was used, CCK-8 was still significantly less potent than CCK-33 on hepatic vagal afferent terminals. Our working hypothesis is that this difference in potency occurs because CCK-8 is more vulnerable than CCK-33 to intrahepatic uptake and metabolism. This hypothesis needs more testing.

Vagal afferent innervation. The differential duodenal vagal afferent innervation in H and HGD rats (Figs. 1 and 2) demonstrates that surgical section of the gastroduodenal branch produces a novel preparation for the investigation of the anatomy and function of the fibers of the common hepatic branch of the abdominal vagus. Bellinger and Williams (1) provided evidence that their surgical method of sectioning the gastroduodenal branch eliminated visible nerve fibers to the liver along the bile duct and portal vein. We extend that observation by demonstrating that the surgical method also eliminates the afferent innervation of the proximal duodenum by these fibers.
Combining the H or HGD procedures with section of the gastric, accessory celiac, and celiac vagal branches optimizes the analysis of the peripheral and central effects of the hepatic proper and gastroduodenal branch fibers because these fibers are the only abdominal vagal innervation in such rats. A limitation of the technique, however, is that it permits the analysis of the functions of the hepatic proper fibers in the absence of the gastroduodenal fibers, but not the reverse.

Although the anatomical verification demonstrated that the procedure for cutting the gastroduodenal branch was effective, it did not evaluate the loss of other neural fibers.

The procedure lesioned vagal efferent fibers traveling in the gastroduodenal branch and nonvagal fibers, such as spinal visceral afferent fibers and postganglionic sympathetic efferent fibers that ascend into the liver along the hepatic artery and portal vein as previously shown by Bellinger and Williams (1). The extent of this nonvagal damage needs to be assessed in future experiments, because it may be involved in the increased potency of CCK-33 observed in H rats compared with its potency in HGD rats.

In summary, we validated a new surgical procedure to determine the response of hepatic proper and gastroduodenal fibers of the common hepatic branch of the abdominal vagus nerve to intraperitoneal administration of CCK-8 and CCK-33. When the hepatic and gastroduodenal vagal fibers were the only abdominal vagal fibers intact, CCK-8 and CCK-33 decreased intake significantly less than in rats that had all of the abdominal vagal innervation intact. In contrast, when only the hepatic vagal fibers were intact, CCK-33 was as potent as it was in rats with all of the abdominal vagal innervation intact and CCK-33 was significantly more potent than CCK-8.

The relevance of our results with exogenous CCK-33 to the physiological role of endogenous CCK in the inhibition of meal size is uncertain, because a new method for the detection of CCK peptides in the peripheral circulation of rats found that CCK-58 was the only circulating form (32). It is possible that the inhibitory effect of endogenous CCK-58 on intake will be similar to the inhibitory effect of exogenous CCK-33 when an appropriate experiment is done. The preliminary report (23) that equimolar doses of exogenous CCK-58 and CCK-8 decreased meal size equivalently in vagally-intact rats is encouraging, but a decisive answer can only come from further experiments with CCK-58.

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