Endogenous cholecystokinin reduces food intake and increases Fos-like immunoreactivity in the dorsal vagal complex but not in the myenteric plexus by CCK₁ receptor in the adult rat

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Abstract:

We hypothesized that endogenous cholecystokinin reduces food intake by activating the dorsal vagal complex (DVC) and the myenteric neurons of the gut. To test this hypothesis, adult rats were given camostat mesilate; a non-nutrient releaser of endogenous cholecystokinin (CCK), by orogastric gavage, and Fos-like immunoreactivity (Fos-LI) was quantified in the DVC and the myenteric plexus. The results for endogenous cholecystokinin were compared to those for exogenous CCK-8. Exogenous CCK-8 reduced food intake and stimulated Fos-LI in the DVC, and in myenteric neurons of the duodenum and jejunum. In comparison, endogenous cholecystokinin reduced food intake and increased DVC Fos-LI, but did not increase Fos-LI in the myenteric plexus. Similar to CCK-8, devazepide, a specific CCK₁ receptor antagonist, and not L365,260, a specific CCK₂ receptor antagonist, attenuated the reduction of food intake by camostat. In addition, Fos-LI in the DVC in response to both exogenous CCK-8 and camostat administration was significantly attenuated by vagotomy, as well as by blocking CCK₁ receptors. These results demonstrate for the first time that reduction of food intake in adult rats by endogenous cholecystokinin released by a non-nutrient mechanism requires CCK₁ receptors, the vagus nerve, and activation of the DVC, but not the myenteric plexus.
Key words:

- Endogenous cholecystokinin
- Camostat
- CCK
- Fos
- Myenteric
- Dorsal vagal complex
- Vagotomy
- CCK₁ receptor
- Devazepide
Introduction:

In 1994, Robert C. Ritter and colleagues put forth a hypothesis (39) stating that endogenous cholecystokinin secreted by the I cells of the small intestine may decrease food intake by activating first order neurons in the enteric nervous system (ENS) of the gastrointestinal (GI) tract. The ENS consists of two ganglionated plexuses, myenteric and submucosal. The myenteric plexus is located between the outer longitudinal and the inner circular muscle layers of the gut wall, while the submucosal plexus resides under the submucosa. These neurons, and through their connections (mainly via the vagus nerve) with central satiety locations in the dorsal vagal complex (DVC) of the brainstem, e.g., the area postrema (AP), nucleus tractus solitarii (NTS) and the dorsal motor nucleus of the vagus (DMV), may participate in the reduction of food intake by this hormone.

The goal of this work is to examine the role of the myenteric neurons and the dorsal vagal complex (DVC) in the reduction of food intake by endogenous cholecystokinin (CCK), by quantifying Fos-like immunoreactivity (Fos-LI), a marker for neuronal activation, in these peripheral and central neurons.

The above and other hypotheses were previously tested using exogenous CCK-8, with the following results. Exogenous CCK-8 decreased food intake through CCK₁ receptors acting through an intact vagus nerve (14, 46). Exogenous CCK-8 increased Fos-like immunoreactivity (Fos-LI), a marker for neuronal activation, in the DVC (12) and the myenteric plexus of the duodenum and jejunum through CCK₁ receptors (22, 41, 49). Furthermore, bilateral subdiaphragmatic vagotomy attenuated Fos expression only in the DVC, but not in the myenteric plexus (42).
Similar to exogenous CCK-8, Yox et al., demonstrated that intraduodenal infusion of oleate, a long-chained fatty acid and a stimulant of endogenous cholecystokinin release, reduces food intake via CCK₁ receptors and the vagus nerve (53-55). In addition, intraduodenal infusion of fat also increased Fos-LI in the DVC, via CCK₁ receptors and the vagus nerve (24). Finally, we recently have shown that oleate infusions increased Fos-LI in the myenteric neurons via CCK₁ receptors (19). However, the latter studies do not answer the question whether activation of the myenteric neurons via CCK₁ receptors is obligatory for endogenous cholecystokinin to inhibit food intake.

Although fatty acids, e.g., oleate, release endogenous cholecystokinin, as nutrients they have multiple effects in addition to releasing cholecystokinin. For example, infusions of oleic acid stimulate secretion of PYY and GLP-1 (40), which have also been shown to reduce food intake and increase central Fos-LI (3, 4). The actions of PYY on food intake (1), as with CCK-8, require an intact vagus. Furthermore, we have shown that duodenal infusions of oleate increased myenteric Fos-LI (19) to approximately twice the increase caused by a supraphysiological dose of exogenous CCK-8 (50), suggesting that factors other than cholecystokinin are involved in these peripheral Fos-LI increases. In addition, although devazepide, a specific CCK₁ receptor antagonist, significantly attenuated myenteric and DVC Fos-LI in response to oleate, it did not abolish it (19). Therefore, it is clear that oleate has effects that are unrelated to release of cholecystokinin.

The vagus is a complex nerve that can be stimulated by cholecystokinin via inputs from several regions of the intestine. There are three known vagal afferents, all of which may be activated by cholecystokinin, intramuscular arrays (IMA), intraganglionic
laminar endings (IGLE) and intravillous arbors (7, 17, 18). The IMAs synapse mainly on the various muscle layers of the gut, and function as mechanoreceptors and tension receptors. The IGLEs are basket-like structures that surround the myenteric ganglia, and function as mechanosensory receptors (muscle stretch or contraction). Beside having mechanoreceptors and tension receptors functions, there is evidence to suggest that both IMAs and IGLEs can be activated by CCK-8, and may have a role in the reduction of food intake by the same peptide (9, 10). The IVAs reach to the enteroendocrine cells and function as chemoreceptors.

To stimulate endogenous cholecystokinin release without the confounding effects of intraluminal nutrients, we carried out studies using camostat, a non-nutrient, non-proteinaceous synthetic trypsin inhibitor that causes release of endogenous cholecystokinin (15, 27, 30, 36, 48). To the best of our knowledge, this is the first report that examines reduction of food intake and Fos-LI in the DVC and the myenteric neurons in response to a non-nutrient, strong releaser of endogenous cholecystokinin. In addition, this is the first work that investigates if the myenteric neurons play an essential role in the reduction of food intake by endogenous cholecystokinin in the same way as exogenous CCK-8. An additional reason for comparing the effects of endogenous cholecystokinin to the previously-described effects of CCK-8 is because the only detectible endocrine form of cholecystokinin released by camostat is CCK-58 (36), which has several physiological effects that differ markedly from CCK-8 (52).
Materials and Methods:

Experimental Procedures

The Tuskegee University Institutional Animal Care and Use Committee approved all the animal protocols. Sprague Dawley rats (250-350g) were housed individually in wire-mesh cages, in a controlled environment (lights were on from 0600 to 1800 h and temperature was maintained at 21.5° C), and given free access to water and pelleted rodent chow (Teklad, WI).

In order to adapt the rats to our experimental protocol and laboratory environment, each rat was handled daily for ten minutes, and orogavaged with 3.5 ml of double distilled water (ddH₂O). All rats were fasted beginning at 1800 h on the day prior to all of the experiments. All injections were made in a volume of 0.5 ml of vehicle (saline or DMSO), and all gavages were made in a volume of 3.5 ml of ddH₂O.

Effect of camostat on myenteric and DVC Fos-LI:

For this experiment we used a total of forty-eight adult, male Sprague Dawley rats divided to twelve treatment groups (n=4 rats per group). Two groups received CCK-8 (Bachem CA, 40µg/kg) or vehicle intraperitoneally (IP), and ten groups received camostat (Camostat mesilate, [FOIPAN: N, N – dimethylcarbamoylmethyl-4-(4-guanidinobenzoyloxy) phenylacetate monomethanesulfonate], Ono Pharmaceuticals Japan, 200 mg/kg) or ddH₂O by orogastric gavage. Based on our previous experience (19-22, 33-35, 49, 50), we sacrificed the two groups of rats that received CCK-8 or vehicle at ninety minutes post injection. This is the time point for producing maximum Fos expression in response to exogenous CCK-8. On the other hand, to evaluate the time
point for maximum Fos-expression in response to camostat, the remaining ten groups that received this treatment or ddH₂O were sacrificed at five different time points post gavage: 90, 105, 120, 135, and 150 minutes. Following the injection or the gavage, all rats were euthanized with an overdose of sodium pentobarbital (10mg/kg, IP).

**Effect of devazepide, a CCK₁ receptor antagonist, on DVC Fos-LI induced by camostat:**

Thirty-two adult, male Sprague Dawley rats were assigned to eight treatment groups (n=4 rats/group). Four groups received the CCK₁ receptor antagonist, devazepide (ML laboratories, Leicester, England, 1000 µg/kg) or dimethyl sulfoxide (DMSO) IP, followed 15 minutes later by exogenous CCK-8 (40µg/kg) or saline IP. The remaining four groups received devazepide (1000 µg/kg) or DMSO IP, followed 15 minutes later by an orogastric gavage of camostat (200mg/kg) or ddH₂O. As we stated previously with regards to the time point of maximum Fos expression in response to CCK-8 or camostat, ninety minutes after the CCK-8 or vehicle injection, and – based on the results of the previous experiment – 105 minutes following the gavage of camostat or ddH₂O all rats were euthanized with an overdose of sodium pentobarbital (10mg/kg, IP).

**Effect of vagotomy on DVC Fos-LI induced by camostat:**

Eight adult, male Sprague Dawley rats underwent bilateral surgical sectioning of the vagus nerve (2 groups, n=4 rats per group). Another group of eight rats underwent sham vagotomy (2 groups, n=4 rats per group) surgery as previously described (42). Briefly, we exposed the right and left vagi along the sides of the esophagus through a
ventral midline celiotomy incision, and severed them under the guidance of a surgical microscope. Sham surgeries were performed by manipulating the nerves without severing them. All rats were allowed two weeks recovery time from the surgery during which they were observed clinically for any abnormal behavior or pain. Vagotony was verified twice, first by measuring reduction of 10% sucrose intake in response to CCK-8 10ug/kg IP for 30 min, and second, by inspection during postmortem examination. During sacrifice all vagotomized rats showed severely distended stomachs, which is an accepted verification of vagotomy.

The vagotomy groups received camostat (200 mg/kg) or ddH₂O and the sham groups received the same treatments, all through orogastric gavage. Again, based on the results of the first experiment all rats were euthanized 105 minutes post gavage of camostat or ddH₂O with an overdose of sodium pentobarbital (10mg/kg, IP).

Immediately following the euthanasia, all rats were perfused transcardially in two stages. First, with 500 ml of Krebs solution (Krebs saline formula in mM: 119 NaCl, 4.7 KCl, 1.2 NaH₂PO₄·H₂O, 25 NaHCO₃, 1.2 MgSO₄, 11.1 glucose, and 1M CaCl₂.) to collect the intestine, and second, with 500ml of 0.4% formaldehyde solution in 0.1 M phosphate buffer saline (PBS) to fix the brains in situ.

The small intestine was exposed through a ventral midline celiotomy incision and the duodenum and jejunum were collected. On the basis of our previous experiments (20-22, 33, 49, 50), the duodenal sample was 5-10 cm aborad from the pylorus and the jejunal sample was 20-25 cm aborad from the pylorus. The lumens of the removed segments were rinsed with Krebs solution, and the segments were placed in Sylgard-coated Petri dishes (World Precision Instruments, FL), opened along the mesenteric
attachment, stretched and pinned with the mucosal side up, and stored overnight in Zamboni’s fixative at 4°C. On the next day, the tissues were unpinned and cleared three times in 100% dimethyl sulfoxide (DMSO), 10 minutes each time, followed by three 10-minute rinses with 0.1 M PBS, pH 7.4. Whole mounts (approximately 1 cm²) of longitudinal smooth muscle with adhering myenteric plexus from the duodenum and jejunum were prepared using a dissecting microscope and processed for immunohistochemical detection of Fos-LI.

After collection of the intestinal tissues, the brainstems were collected and postfixed with 0.4% formaldehyde for two hours at 4°C, followed by overnight placement in a 25% sucrose solution at room temperature. The next day, they were sectioned at 40 microns on a cryostat at -20°C, and blocked with 50% ethyl alcohol for 30 minutes, to reduce the production of free radicals and improve background staining. Consistent with our previous work (19, 20, 33-35, 49, 50), the areas cut included the following levels of the DVC according to the rat brain atlas of Paxinos and Watson (32): the area postrema (AP, -4.5 mm caudal to interaural plane), nucleus of the solitary tract (NTS, -4.5 and -4.8 mm caudal to interaural plane), and the dorsal motor nucleus of the vagus (DMV, -4.5 and -4.8 mm caudal to interaural plane) (please see Figure 1). Sections were taken at multiple levels of the NTS to insure sampling from sites receiving both gastric and intestinal vagal afferent innervation.

Immunohistochemistry

The intestinal whole mounts and the brain sections were incubated for 24 h at room temperature in a primary antiserum raised in rabbit against a peptide consisting of
amino acids 4-17 of human Fos (Oncogene, Ab-5, San Diego, CA, [1:12,000 dilution]). The next day the tissues were rinsed with 0.01M TPBS and incubated overnight in biotintylated donkey anti-rabbit serum (Jackson ImmunoResearch Laboratories, PA [1:500 dilution]). Then, the tissues were incubated for 3 h in avidin conjugated to horseradish peroxidase (HRP), washed with 0.01M TPBS, and reacted with glucose oxidase and diaminobenzidine (DAB, Sigma, MO) intensified with nickel (5, 11).

**Counting Procedures**

Two independent examiners, blinded to the treatments of this study, counted Fos-positive neurons in the myenteric neurons. For each intestinal whole mount 10 non overlapping, 40X microscopic fields were counted and averaged. In the brainstem sections, Fos positive cells were counted by an automated computer software (ImagePro Plus, Media Cybernetics) in the AP, NTS, and DMV at planes -4.5mm and -4.8mm caudal to the interaural plane according to the Rat Brain Atlas (32) (Figure 1).

**Effect of camostat on food intake:**

To establish a baseline for food intake, we measured the intake of 10% sucrose in 12, overnight food-deprived, Sprague Dawley male rats (250-350 gm each), 6 times during a 16-18 day period following orogastric gavage of 3.5 ml ddH₂O or an IP injection of saline. On the experiment day, the rats were deprived of food but not water overnight. On the following day, the rats were divided into four groups (n=5 rats per group), two of which received orogastric gavage of camostat (200mg/kg) or ddH₂O and the remaining two groups of rats received CCK-8 (10µg/kg) or saline IP. The intake of 10% sucrose
was recorded every 5 minutes for 120 minutes. The rats were deprived of regular food for the entire test period.

**Effect of devazepide, a CCK₁ receptor antagonist, and L365,260, a specific CCK₂ receptor antagonist on reduction of food intake by camostat:**

Four groups of rats (250-350gm each, n=5 rats per group) were assigned to this experiment, and deprived from food but not water over night. The following day, each group received two treatments, an IP injection of devazepide (1 mg/kg dissolved in 0.5 ml of 0.01% DMSO) or 0.01% DMSO (0.5ml), followed by orogastric gavage of camostat mesilate (200mg/kg dissolved in 3.5 ml of ddH₂O) or ddH₂O (3.5 ml). Then, the intake of 10% sucrose was recorded every 5 min for 120 min. The rats were deprived of regular food for the entire test period.

**Effect of L365,260, a specific CCK₂ receptor antagonist on reduction of food intake by camostat:**

Four groups of rats (250-350gm each, n=3 rats per group) were deprived from food but not water over night. The following day, each group received two treatments, an IP injection of L365,260 (1 mg/kg dissolved in 0.5 ml of 0.01% DMSO) or 0.01% DMSO (0.5ml), followed by orogastric gavage of camostat mesilate (200mg/kg dissolved in 3.5 ml of ddH₂O) or ddH₂O (3.5 ml). After the gavage, intake of 10% sucrose was recorded every 5 min for 120 min. The rats were deprived of regular food for the entire test period.
Statistical Analysis

Data were analyzed using two-way Analysis of Variance (ANOVA) (treatment and time are the two independent variables) and multiple comparisons were performed using Bonferroni t-test. Results are displayed as mean±SEM. The food intake data was analyzed by a one-way ANOVA and Bonferroni t-test for multiple comparisons. Data were considered statistically significant if P < 0.05.
Results:

**Effect of camostat on DVC and myenteric Fos-LI:**

Figure 1 shows a photomicrographs of three nuclei in the dorsal vagal complex: area postrema (AP), nucleus tractus solitarii (NTS) and the dorsal motor nucleus of the vagus (DMV) captured from a camostat mesilate-treated and control animals. In the AP (Figure 2), orogastric gavage of camostat increased Fos-LI significantly more than ddH₂O only at the 90 min time point. In the DMV/NTS (-4.5mm caudal to the interaural plane) orogastric gavage of camostat increased Fos-LI significantly more than ddH₂O only at the 105 and 135 min time points (Figures 3A and B). In the DMV/NTS (-4.8mm caudal to the interaural plane) orogastric gavage of camostat increased Fos-LI significantly more than ddH₂O only at the 105, 120 and 135 min time points (Figures 4A and B).

Figure 5 depicts Fos-like immunoreactivity in the myenteric plexus of the duodenum in response to camostat, exogenous CCK-8 and saline. Two-way ANOVA revealed no difference in the counts of Fos-positive neurons in the myenteric neurons between camostat and ddH₂O in the duodenum (Figure 6) and jejunum (Figure 7). However, as previously reported the counts of Fos-positive neurons in response to exogenous CCK-8 were significantly more than saline (Figures 6 and 7).

Based on the previous results we decided to sacrifice and perfuse the animals in all of the following experiments after 105 min of the orogastric gavage of camostat because it revealed the maximum Fos expression in the DVC.

**Effect of devazepide, a CCK₁ receptor antagonist, on DVC Fos-LI (Figures 8A and B):**
In the AP, NTS/DMV at planes -4.5 mm and -4.8 mm caudal to the interaural plane, devazepide significantly attenuated camostat–induced Fos expression.

**Effect of vagotomy on DVC Fos-LI (Figure 9):**

In the AP, NTS/DMV at planes -4.5 mm and -4.8 mm caudal to the interaural plane, vagotomy significantly attenuated Fos expression induced by camostat.

As a positive control we also compared the results of the previous three experiments with exogenous CCK-8. As expected and documented (19-22, 33-35, 49, 50), CCK-8 increased Fos-LI in all of the DVC nuclei, and devazepide and vagotomy significantly attenuated the immunoreactivity.

**Effect of camostat on food intake (Figure 10):**

One-way ANOVA revealed significant reduction of 10% sucrose intake for 120 minutes in response to orogastric gavage of camostat compared to ddH₂O. As expected, exogenous CCK-8 also reduced the intake of 10% sucrose more than saline.

**Effect of CCK₁ and CCK₂ receptor antagonists on camostat reduction of food intake (Figure 11 A and B):**

Devazepide, a CCK₁ receptor antagonist (Figure 11A) but not L365,260, a CCK₂ receptor antagonist (Figure 11B), reversed the reduction of food intake by camostat.
Discussion:

We have shown three important results: first, endogenous cholecystokinin released by camostat increases Fos-LI in the DVC which requires an intact vagus; second, reduces food intake by CCK\textsubscript{1} receptors in adult rats; and third, the pathway by which this reduction is taking place does not appear to involve the myenteric neurons.

In order to explain the pathway by which cholecystokinin decreases food intake, Ritter et al., (39) hypothesized that nutrients which stimulate the release of endogenous cholecystokinin from the I cells of the upper small intestine (duodenum and jejunum) e.g., fat and protein, activate the gut enteric neurons, which in turn activate satiety centers in the DVC, mainly through the vagus nerve, and by activating CCK\textsubscript{1} receptors.

Based on the above hypothesis, we began to test the role for the myenteric neurons in the reduction of food intake by cholecystokinin, supported by the following data. First, there is electrophysiological (29, 43) and immunohistochemical (21, 22, 33, 34, 49, 50) evidence demonstrating activation of myenteric neurons by exogenous CCK-8 and endogenous cholecystokinin released in response to nutrients (19), through CCK\textsubscript{1} receptors (19, 42). Second, myenteric neurons that synapse with vagal afferents that supply the gut contain CCK\textsubscript{1} receptors. (6, 8, 21, 31, 38). Third, this neuronal connection links the gut with important central satiety control nuclei in the DVC e.g., DMV, NTS and AP (2, 44, 45). Furthermore, the three components of this circuit i.e., myenteric neurons, vagus nerve and DVC transmit CCK\textsubscript{1} receptor signals (25, 26), and can be activated by both CCK-8 and endogenous cholecystokinin. In addition, vagal sensory neurons that contain CCK\textsubscript{1} receptors are in close proximity to the cholecystokinin secretting I cells, suggesting that direct activation of the vagus by endogenous
cholecystokinin is also possible. Our current work provides behavioral, immunohistochemical, pharmacological and surgical evidence that camostat, a non-nutrient, synthetic trypsin inhibitor and a strong releaser of endogenous cholecystokinin, reduces food intake and increases Fos-LI, a marker of neuronal activation, by CCK₁ receptors and in the central nervous system, i.e., DVC, but not in the myenteric neurons of the duodenum and jejunum. This reduction and activation was dependent on CCK₁ receptors, and the vagus nerve i.e., the same requirements for reduction of food intake caused by exogenous CCK-8. It should be noted that the enteric nervous system consists of two ganglionated nerve plexuses: myenteric and submucosal. This work tested only the role of the myenteric plexus in the reduction of food intake caused by endogenous cholecystokinin.

Camostat increases circulating endogenous cholecystokinin, in rats and mice (15, 27, 30). In addition, it increases pancreatic endocrine and exocrine secretions and delays gastric emptying, all by CCK₁ receptor (15, 27, 30). In the adult rat, Voigt et al. (48), demonstrated that camostat reduces food intake. However, they failed to reverse this response using 100 µg/kg devazepide compared to the 1000 µg/kg used in the present study. Devazepide at 1000 µg/kg has been reported to not interact with the CCK₂ receptor (30). Furthermore, the lack of influence of the CCK₂ receptor antagonist in the present work suggests that the actions of endogenous cholecystokinin are mediated by the CCK₁ receptor, but not the CCK₂ receptor. Therefore, the current work provides the first evidence in adult rats that cholecystokinin released by a non-nutrient, camostat, causes reduction of food intake, which was mediated by CCK₁ receptors. Only one other study reported reduction of food intake by endogenous cholecystokinin released by a non-
nutrient (soybean trypsin inhibitor) through CCK$_1$ receptors, and this was in neonatal rats (51).

The results of the current work also provide the first immunohistochemical evidence that demonstrates that camostat increases Fos-LI in the DVC. This increase was due to release of endogenous cholecystokinin, because a specific CCK$_1$ receptor antagonist i.e., devazepide, attenuated this response.

In a majority of measurements, Fos-LI differed substantially between CCK-8 treatments compared to camostat treatment. In some cases, the differences were quantitative, e.g., Fos-LI in the area postrema was 3-fold higher in CCK-8 treated rats compared to camostat, although both increases were statistically significant (Fig. 2). Likewise, CCK-58 increased Fos-LI more than 2-fold higher in the NTS (Fig. 3B). However, although CCK-8 markedly increased Fos-LI in the myenteric plexus of the duodenum (Fig. 6) and jejunum (Fig. 7), camostat had a very small, non-significant effect in these neurons. These differences between responses to CCK-8 vs. endogenous cholecystokinin may be due to differences in dose of CCK-8 received (50), or to differences in biological actions between CCK-8 vs. endogenous cholecystokinin. CCK-8 was injected at 40 µg/kg, which produces much greater than physiological levels of cholecystokinin in the circulation, and such doses consistently produce acute edematous pancreatitis in rats (16, 28). In contrast, gavage of camostat at 100 mg/kg in rats increased circulating cholecystokinin to physiological levels of 17 pM (36), nearly identical to that (15 pM) caused by the dietary protein casein (37). Therefore, the quantitative differences in Fos-LI between CCK-8 and camostat might be due to the much greater amounts of cholecystokinin bioactivity in rats given CCK-8. On the other
hand, the near absence of an increase in Fos-LI, compared to high and significant Fos-LI in the myenteric plexuses of the duodenum and jejunum could also suggest qualitative differences between CCK-8 and endogenous cholecystokinin in inducing Fos-LI. At this time we cannot determine if the differences in Fos-LI results from quantitative, qualitative or both differences between CCK-8 and endogenous cholecystokinin. This is consistent with several studies showing marked qualitative as well as quantitative differences in biological activity between CCK-8 and CCK-58 (13, 23, 47, 52), the endogenous form of cholecystokinin. Because CCK-8 is apparently not a physiological endocrine form of cholecystokinin in the rat, the differential effect of CCK-8 vs. camostat on Fos-LI in the myenteric plexus may provide another example of the marked differences in pattern of bioactivity between CCK-8 and CCK-58.

Figure 12 illustrates how exogenous CCK-8 or endogenous cholecystokinin could activate the vagus. The only route for i.p. CCK-8 to reach vagal afferent fibers is through the circulation. Therefore, all three vagal afferent fibers, IMA, IGLE and IVA (17, 18) should receive similar concentrations of CCK-8 after i.p. injection. However, these three vagal afferent fibers may be exposed to different concentrations of endogenous cholecystokinin. The IVA and the IGLE are close enough to the cholecystokinin secreting I cell for the endogenous peptide to these fibers by a paracrine route. However, the IMA may be exposed only to circulating cholecystokinin and therefore may be at much lower concentrations of cholecystokinin than encountered by fibers activated by the paracrine route. All three afferent fiber types are connected to the central nervous system, at the level of the DVC, through the vagus or the tenth cranial nerve. Alternatively, endogenous cholecystokinin may directly activate the DVC by an
endocrine route, causing reduction of food intake. In conclusion, our data show for the first time that endogenous, peripherally produced cholecystokinin reduces food intake by a central mechanism that involves an intact vagus nerve and functional CCK₁ receptors. Furthermore, these data demonstrate that there is not a clear role for the myenteric plexus in the reduction of food intake caused by endogenous cholecystokinin. Finally, a possible role for the submucosal plexus in mediating CCK-reduction of food intake remains to be determined.
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Figure 1:

Photomicrographs of three nuclei in the dorsal vagal complex (A and A’): -4.5mm caudal to interaural plane: area postrema (AP), nucleus tractus solitarii (NTS) and the dorsal motor nucleus of the vagus (DMV) captured from a camostat mesilate-treated animal (A) and a double distilled water-treated animal (A’). Camostat increased Fos-like immunoreactivity, shown by the black dots and indicated by the arrow in A. Bar=100 microns, and cc=central canal.
Figure 2:

Effect of camostat on Fos-like immunoreactivity in the area postrema. Camostat (200 mg/kg) or double distilled water (ddH₂O) were given by orogastric gavage (3.5 ml) to adult rats (each bar represent n=4 rats). The animals were sacrificed at different time points to determine the time point for maximum Fos expression, and Fos-positive neurons were counted in the area postrema (32) as total counts. Camostat increased Fos expression significantly (* = p < 0.05) more than ddH₂O after 90 min following the gavage. Exogenous CCK-8 also increased Fos expression more than saline after 90 min.
Figure 3A:

Effect of camostat on Fos-like immunoreactivity in the dorsal motor nucleus of the vagus at -4.5mm caudal to the interaural plane.
Figure 3B:

Effect of camostat on Fos-like immunoreactivity in the nucleus tractus solitarii at -4.5mm caudal to the interaural plane.
Camostat (200 mg/kg) or double distilled water (ddH₂O) were given by orogastric gavage (3.5 ml) to adult rats (each bar represent n=4 rats). The animals were sacrificed at different time points to determine the time point for maximum Fos expression, and Fos-positive neurons were counted in dorsal motor nucleus of the vagus (3A) and the nucleus of the solitary tract (3B) at -4.5mm caudal to the interaural plane (32). * denotes significant difference compared to vehicle.
Figure 4A:

Effect of camostat on Fos-like immunoreactivity in the dorsal motor nucleus of the vagus at -4.8mm caudal to the interaural plane.
Figure 4B:

Effect of camostat on Fos-like immunoreactivity in the nucleus tractus solitarii at -4.8mm caudal to the interaural plane.
Camostat (200 mg/kg) or double distilled water (ddH₂O) were given by orogastric gavage (3.5 ml) to adult rats (each bar represent n=4 rats). The animals were sacrificed at different time points to determine the time point for maximum Fos expression, and Fos-positive neurons were counted in the dorsal motor nucleus of the vagus nucleus (DMV, 4A) and the nucleus of the solitary tract (NTS, 4B) at -4.8mm caudal to the interaural plane (32). * denotes significant difference compared to vehicle.
Figure 5:

A photomicrograph depicting Fos-like immunoreactivity in the myenteric plexus of the duodenum in response to camostat (A), double distilled water (A’), exogenous CCK-8 (B), and saline (B’). Only CCK-8 increased Fos expression (indicated by the gray arrow in B) more than saline. The black arrow points to a cell body of a myenteric neuron that did not express Fos. Scale bar=200 µm.
Figure 6:

Effect of camostat on Fos-like immunoreactivity in the myenteric plexus of the duodenum. Orogastric gavage of camostat mesilate (200 mg/kg) or double distilled water (ddH₂O), or intraperitoneal injection (IP) of exogenous CCK-8 (40µg/kg) or saline were given to adult rats (each bar represents n=4 rats), and Fos-like immunoreactivity was quantified in the myenteric neurons of the duodenum. * denotes significant difference compared to vehicle.
Figure 7:

Effect of camostat on Fos-like immunoreactivity in the myenteric plexus of the jejunum. Orogastric gavage of camostat mesilate (200 mg/kg) or double distilled water (ddH2O), or intraperitoneal injection (IP) of exogenous CCK-8 (40μg/kg) or saline were given to adult rats (each bar represents n=4 rats), and Fos-like immunoreactivity was quantified in the myenteric neurons of the jejunum. * denotes significant difference compared to vehicle.
**Figure 8A:**

Effect of devazepide, a CCK₁ receptor antagonist, on camostat-induced Fos expression in the dorsal vagal complex.

Four groups of rats (n=4 rats per group) received two treatments each, 15 min apart. Dimethyl sulfoxide (DMSO, 0.5ml) / camostat (Cam, 200mg/kg in 3.5 ml of double distilled water [ddH₂O]), DMSO / ddH₂O (3.5 ml), Devazepide (Dev, 500µg/kg in 0.5 ml of DMSO) / camostat, and Dev / ddH₂O. Devazepide and DMSO were given intraperitoneally in a volume of 0.5 ml, and camostat and ddH₂O were given by orogastric gavage in a volume of 3.5 ml. Fos-positive neurons were quantified in the area postrema (AP), nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMV) (in planes -4.5 and -4.8 mm caudal to the interaural plane (32). * denotes significant difference compared to vehicle. † significant difference between Dev/Cam and DMSO/Cam.
**Figure 8B:**

![Graph showing effect of devazepide on CCK-8-induced Fos expression](image)

Effect of devazepide, a CCK$_1$ receptor antagonist, on CCK-8-induced Fos expression in the dorsal vagal complex.

Four groups of rats (n=4 rats per group) received two treatments each, 15 min apart. Dimethyl sulfoxide (DMSO, 0.5ml) / cholecystokinin-8 (CCK-8, 10 µg/kg), DMSO / saline, Devazepide (Dev, 500µg/kg in 0.5 ml of DMSO) / CCK-8, and Dev / Saline. All injections were given intraperitoneally in a volume of 0.5 ml. Fos-positive neurons were quantified in the area postrema (AP), nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMV) (in planes -4.5 and -4.8 mm caudal to the interaural plane (32). * denotes significant difference compared to saline. † significant difference between Dev/CCK-8 and DMSO/CCK-8.
Effect of vagotomy on camostat-induced Fos expression in the dorsal vagal complex. Four groups of rats (n=4 rats per group) underwent bilateral subdiaphragmatic vagotomy surgery (VGX) or sham vagotomy (SHAM). Two groups received orogastric gavage of camostat (200 mg/kg in 3.5 ml double distilled water [ddH$_2$O]) and the other two received ddH$_2$O (3.5 ml VEH). Fos-positive neurons were quantified in the area postrema (AP), nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMV) (in planes -4.5 and -4.8mm caudal to the interaural plane (32). * denotes significant difference compared to vehicle. † significant difference between VGX-CM and Sham-CM.
Figure 10:

Intake of 10% sucrose in response to orogastric gavage of camostat or intraperitoneal injection of exogenous CCK-8 in the adult rat. The intake was measured for 120 minutes in rats gavaged with camostat (200mg/kg dissolved in 3.5 ml water) or CCK-8 (10 µg/kg, intraperitoneally). * denotes significant difference between CCK-8 and saline. † denotes significant difference between camostat and ddH2O.
Figure 11A:

Effect of devazepide, a CCK₁ receptor antagonist, on the reduction of food intake by camostat mesilate. Four groups of rats (n=5 per group) were injected intraperitoneally with devazepide (Dev, 1 mg/kg in 0.5 ml of 0.01% DMSO) or 0.01% of dimethyl sulfoxide (DMSO, 0.5 ml) followed, fifteen min later by an orogastric gavage of Camostat mesilate (Cam) or double distilled water (ddH₂O). The intake of 10% sucrose solution was measured for 120 min. * denotes significant difference between DMSO/Cam and DMSO/ddH₂O. † denotes significant difference between Dev/Cam and DMSO/Cam. Camostat reduced food intake significantly (*), and devazepide blocked this attenuation (†).
Effect of L365,260, a CCK2 receptor antagonist, on the reduction of food intake by camostat mesilate. Four groups of rats (n=3 per group) were injected intraperitoneally with L365,260 (1 mg/kg in 0.5 ml of 0.01% DMSO) or 0.01% of dimethyl sulfoxide (DMSO, 0.5 ml) followed, fifteen min later by an orogastric gavage of Camostat mesilate (Cam) or double distilled water (ddH2O). The intake of 10% sucrose solution was measured for 120 min. * denotes significant difference between DMSO/Cam and DMSO/ddH2O. ** denotes significant difference between L365,260/Cam and L365,260/ddH2O and not significantly different from DMSO/Cam. Camostat reduced food intake significantly (*), and L365,260 failed to block this reduction.
Illustration showing routes by which cholecystokinin inhibits food intake. Starting from the lumen of the intestine the layers include: mucosa (contains the endocrine cholecystokinin-secreting I cells), submucosa (SM), muscularis (consisting of an inner circular muscle layer [CM] and an outer longitudinal muscle layer [LM]), and serosa. Within the wall of the gut, two ganglionated nerve plexuses reside, submucosal plexus (SP) and myenteric plexus (MP). These plexuses synapse with a minimum of three vagal afferent fibers, intramuscular arrays (IMA), intraganglionic laminar endings (IGLE), and intravillous arbors (IVA). Fat and protein (or trypsin inhibitors like camostat) stimulate the release of cholecystokinin from the I cells into interstitial fluid. Cholecystokinin
activates vagal afferent fibers directly, or indirectly through the submucosal plexus, which then activates the dorsal vagal complex causing reduction of food intake. Cholecystokinin can also diffuse from the interstitial fluid into the venous circulation to stimulate the dorsal vagal complex. (A&V=artery and vein).
References:


