Vagally mediated, nonparacrine effects of cholecystokinin-8s on rat pancreatic exocrine secretion

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Viard E, Zheng Z, Wan S, Travagli RA. Vagally mediated, nonparacrine effects of cholecystokinin-8s on rat pancreatic exocrine secretion. Am J Physiol Gastrointest Liver Physiol 293: G493–G500, 2007. First published June 14, 2007; doi:10.1152/ajpgi.00118.2007.—Cholecystokinin (CCK) has been proposed to act in a vagally dependent manner to increase pancreatic exocrine secretion via actions exclusively at peripheral vagal afferent fibers. Recent evidence, however, suggests the CCK-8s may also affect brain stem structures directly. We used an in vivo preparation with the aims of 1) investigating whether the actions of intraduodenal casein perfusion to increase pancreatic protein secretion also involved direct actions of CCK at the level of the brain stem and, if so, 2) determining whether, in the absence of vagal afferent inputs, CCK-8s applied to the dorsal vagal complex (DVC) can also modulate pancreatic exocrine secretion (PES). Sprague-Dawley rats (250–400 g) were anesthetized and the common bile-pancreatic duct was cannulated to collect PES. Both vagal deafferentation and pretreatment with the CCK-A antagonist lorglumide was placed on the fourth ventricle before endogenous CCK release was stimulated via intraduodenal casein infusion. Behavioral data by Reidelberger’s group (8, 39) has shown that the effects of systemic administration of CCK-8s are prevented by pretreatment with CCK-A receptor-selective antagonists that cross the blood-brain barrier (BBB) but not by polarized antagonists that do not. These data strongly indicate that the effects of CCK are not limited to activation of the peripheral terminal of vagal afferent (sensory) C-type fibers, but effects at other areas, including vagal brain stem circuits, must be considered. Indeed, in vitro studies have suggested that the actions of CCK in vagovagal circuits also include effects on brain stem neurons and fibers, including vagal efferent fibers, and even involves neuronal areas that do not receive sensory inputs from the periphery (17, 20, 42).

The aim of the present study was thus to investigate the effects on pancreatic protein output of activation of brain stem vagal circuits by CCK in rats that underwent different types of vagal sensory denervation (i.e., surgical or chemical deafferentation) or in rats in which the CCK-A-selective antagonist lorglumide was placed on the fourth ventricle before endogenous CCK release was stimulated via intraduodenal casein infusion.

MATERIALS AND METHODS

Research reported in the present manuscript fully conforms to National Institutes of Health guidelines and was approved by the Pennington Biomedical Research Center-Louisiana State University System Institutional Animal Care and Use Committee.

Pancreatic duct cannulation. All experiments were conducted on Sprague-Dawley rats of either sex (200–400 g; n = 77). Rats were anesthetized with Inactin (130–140 mg/kg ip), the abdominal area was shaved and cleaned with Novalan and alcohol before performing a midline laparotomy. The duodenum was retracted to expose the gut content, and pancreatic exocrine secretion was initiated by microinjection of proteinase (PES). Sprague-Dawley rats (250–400 g) were anesthetized and the common bile-pancreatic duct was cannulated to collect PES. Both vagal deafferentation and pretreatment with the CCK-A antagonist lorglumide was placed on the fourth ventricle before endogenous CCK release was stimulated via intraduodenal casein infusion.
pancreas and the common bile-pancreatic duct, which was cannulated (PE-10 tubing) at a level medial to the sphincter of Oddi. After an initial 30-min stabilization period, pancreatic exocrine secretions (PES) were collected over 10-min periods.

In one series of experiments, a cannula (PE-190 tubing) was inserted through a small incision (2–3 mm) into the duodenum ~1 cm distal to the pylorus to allow infusion of a 10% solution of casein (pH 7.4) at a rate of 4 ml/h for 75 min. The abdominal laparotomy was closed with 6-0 suture and the rat placed on a heated pad to maintain body temperature at 37 ± 1°C.

**Fourth ventricular drug application.** Following pancreatic duct and duodenal cannulation, a group of rats were mounted on a stereotaxic frame (Kopf Instruments, Tujunga, CA) and the lower medulla was exposed by removal of superficial muscles between the occipital bone and the first cervical vertebra. The dura and pia mater were removed to expose the floor of the medulla, a small (~2 mm²) piece of filter paper was apposed gently onto the surface of the fourth ventricle, and the selective CCK-A receptor antagonist lorglumide (12.6 nmol in 2 μl) was added to the paper pellet 1–2 min before the start of intraduodenal casein perfusion. Although unlikely, to prevent leakage from the fourth ventricle and a potential peripheral effect, the excess liquid was collected via a cotton pellet placed at the junction between the brain stem and the spinal cord.

**Microinjection in the DVC.** Following pancreatic duct cannulation, rats were mounted on a stereotaxic frame and the lower medulla was exposed as above. A glass micropipette (30–40 μm tip diameter) was directed into the dorsal vagal complex (DVC) under microscopic guidance (0.1–0.3 mm mediolateral, 0.1–0.3 mm rostral to calamus scriptorius, and 0.5–0.7 mm dorsoventral to the surface of the medulla) for drug delivery. The volume of the ejectate was measured via a calibrated monocular microscope mounted on the stereotaxic frame and directed at the meniscus inside the micropipette. Vehicle (PBS in mM: 115 NaCl, 75 Na₂HPO₄, 7.5 KH₂PO₄ or CCK-8s (450 pmol in 60 nl PBS; the solution was inspected visually to confirm that CCK-8s with 5-0 suture and the rats recovered for at least 4 days before experimentation.

In an additional series of experiments, rats that underwent afferent rhizotomy also underwent contralateral subdiaphragmatic vagotomy (i.e., complete vagal sensory denervation), performed by removing 3–5 mm of either the anterior branch of the vagus at a level rostral to the hepatic branch bifurcation or 3–5 mm of the posterior vagal branch at midesophageal level. The incision was closed with 5-0 suture and the rats were allowed to recover for at least 4 days before experimentation.

**Measurement of pancreatic protein secretion.** Pancreatic secretion was collected in 10-min aliquots and the volume was measured. Baseline volume of PES was 205 ± 15.7 μl/10 min, and none of the pharmacological procedures increased it significantly. The total protein content in the PES was measured per 5 μl by a BCA protein assay (Pierce, Rockford, IL) and the protein content was expressed as micrograms of total protein per 10 min. Previous studies showed that the protein measured is of pancreatic, not biliary, origin (30). The baseline data are expressed as mean ± SEM of the protein content collected in the 30-min equilibration period before drug treatment. The average protein content following drug administration is expressed as the average ± SEM of the protein content in the 30 min following microinjection or in the 60 min following the beginning of intraduodenal perfusion of casein.

**Verification of injection site.** At the end of the experiment, the rat was perfused transcardially with chilled saline followed by 4% formaldehyde in PBS. The brain stem was removed and stored in fixative at 4°C for at least 48 h. The fixative was removed by washing (60 min in 10% sucrose in PBS) and coronal slices (40-μm thickness) were cut with a freezing microtome. Every third slice was mounted on a gelatin-coated coverglass and air-dried. A Nikon E400 equipped with TRITC filters was used to visualize the microinjection site. Photographs of the microinjection sites were taken with a SPOT camera and software and used to create a map of the microinjection locations.

**Statistical analysis.** Data are expressed as means ± SE. We used Student’s paired (to compare baseline values vs. response after treatment) or grouped (to compare the effects of drug treatments in the different experimental procedures) t-test or χ² test. Significance was defined as P < 0.05.

**Drugs and chemicals.** Capsaicin was purchased from Tocris (Ellisville, MO), and Fluoresbrite carboxy NYO (0.5 μm) microspheres were purchased from Polysciences (Warrington, PA). All other chemicals were purchased from Sigma (St. Louis, MO). Stock solutions in frozen aliquots were prepared in advance.

**RESULTS**

**Duodenal infusion of casein increases pancreatic exocrine secretion.** Following a 30-min collection of baseline PES, intraduodenal infusion of a 10% solution of casein increased the total protein secretion from 125 ± 5.4 to an average of 565 ± 13.6 μg/10 min (P < 0.05; n = 6). Peak secretion (843 ± 55 μg/10 min) was observed 40 min from the start of casein perfusion, and protein secretion returned to baseline within 50 min from cessation of casein infusion (Fig. 1). In rats that underwent surgical vagal sensory deafferentation (n = 5), the increased PES induced by intraduodenal infusion of casein was reduced, but not abolished (from 121 ± 2.4 to an average of 236 ± 21.3 μg/10 min; P < 0.05 vs. baseline; P < 0.05 vs. PES induced in vagally intact rats. Figure 1). Peak secretion was also reduced to 317 ± 72.3 μg/10 min (P < 0.05 vs. vagally intact rats). These data suggest that intraduodenal casein also acts at sites other than peripheral vagal afferent fibers to increase pancreatic protein secretion.

**Casein increases pancreatic exocrine secretion via actions at CCK-A receptors in the DVC.** To investigate whether the effects of intraduodenal casein to increase PES were due to
activation of CCK-A receptors in the DVC, the CCK-A antagonist lorglumide was applied to the floor of the fourth ventricle 1–2 min before casein infusion (n = 4). In the presence of lorglumide, intraduodenal casein infusion increased total PES from 203 ± 23 to an average of 317 ± 34 μg/10 min (P < 0.05 vs. baseline; P < 0.05 vs. PES in the absence of lorglumide; Fig. 1). Peak secretion was also reduced to 387 ± 124 μg/10 min (P < 0.05 vs. PES in the absence of lorglumide). These data suggest that the casein-induced increase in protein PES involves effects at CCK-A receptors in the DVC.

Microinjection of CCK-8s in the DVC increases PES via a vagally dependent pathway. Microinjection of 450 pmol of CCK-8s in the DVC increased PES to 257 ± 76% of baseline secretion (199 ± 21 to an average of 428 ± 50 μg/10 min; P < 0.05), with a peak secretion of 540 ± 104 μg/10 min (P < 0.05; n = 10; Figs. 2 and 3). Interestingly, the increase in protein PES was larger when CCK-8s was microinjected in the left DVC compared with the right DVC. In fact, microinjection of CCK-8s in the left DVC increased protein PES to 378 ± 139% of baseline (163 ± 25 to 587 ± 90 μg/10 min; P < 0.05; n = 5), with a peak secretion of 646 ± 203 μg/10 min (P < 0.05), whereas microinjection of CCK-8s in the right DVC increased protein PES to 140 ± 9% of baseline (220 ± 21 to 300 ± 37 μg/10 min; P < 0.05, n = 5), with a peak secretion of 433 ± 48 μg/10 min (n = 5; P < 0.05; Fig. 2).

Microinjection of PBS in the DVC did not affect protein PES (205 ± 16 and 198 ± 16 μg/10 min, in control and following PBS, respectively, n = 8, P > 0.05; Fig. 2). These data demonstrate that functional CCK-A receptors are present in the DVC and that activation of these receptors increases pancreatic protein secretion.

In five rats that underwent acute (1 h before the experiment) surgical cervical vagotomy, ipsilateral microinjection of CCK-8s in the DVC did not increase PES (197 ± 23 and 177 ± 18 μg/10 min in control and CCK-8s, respectively, P > 0.05; Fig. 2). Vagotomy itself did not affect basal PES (245 ± 67 μg/10 min after 40 min; 241 ± 41 μg/10 min after 120 min; P > 0.05; n = 3; Fig. 2). These data demonstrate that activation of CCK-A receptors in the DVC increases protein PES via a vagally dependent mechanism.

Microinjection of CCK-8s in the DVC increases PES in vagally deafferented rats. To investigate the role of brain stem vagal pathways in PES, we microinjected CCK-8s ipsilaterally in the DVC of deafferented rats. In four rats that underwent a left vagal deafferentation, the increase in PES induced by DVC microinjection of CCK-8s was reduced to 157 ± 18% (P < 0.05 vs. baseline, P < 0.05 vs. vagally intact rats; Table 1). In contrast, right vagal deafferentation did not affect the CCK-8s-induced increase in PES (170 ± 16%; P < 0.05 vs. baseline; P > 0.05 vs. vagally intact rats; n = 4; Table 1).

To eliminate the possibility of spread of CCK-8s to the contralateral DVC (thus potentially activating contralateral vagal efferent neurons), a series of experiments was carried out in rats that had undergone a complete sensory vagal denervation, i.e., unilateral deafferentation and contralateral complete subdiaphragmatic vagotomy; CCK-8s was microinjected ipsilaterally to the deafferentation since the motor output was preserved in this hemisphere. In four rats that underwent left DVC microinjection, the CCK-8s-induced increase in PES was reduced to 133 ± 32% (P < 0.05 vs. baseline; P < 0.05 vs. vagally intact rats; P < 0.05; Table 1). In contrast, four rats that underwent microinjection in the right DVC, the CCK-8s-induced increase in PES was unaffected (172 ± 24%; P < 0.05 vs. baseline; P > 0.05 vs. vagally intact rats; Table 1).

These data indicate that the effects of CCK-8s in the left DVC are determined by activation of CCK-A receptors on both vagal motoneurons and vagal afferent fiber terminals in the nucleus tractus solitarius (NTS). These data additionally suggest that the effects of CCK-8s in the right DVC to increase PES are mediated entirely by the efferent vagus nerve, with little or no effect on the afferent nerve fibers.

Microinjection of CCK-8s in the DVC increases PES in capsaicin-treated rats. The effects of CCK-8s to increase PES were examined in rats that underwent chemical deafferentation. In six rats treated with capsaicin on the left vagus nerve 7–10 days before experimentation, the CCK-8s-induced increase in PES following left DVC microinjection was reduced to 125 ± 6% (P < 0.05 vs. baseline, P < 0.05 vs. vagally intact rats; P < 0.05 vs. unilaterally deafferentation; Table 1). In contrast, in six rats treated with capsaicin on the right vagus nerve before experimentation, the CCK-8s-induced increase in PES following right DVC microinjection was unaffected (179 ± 21%; P < 0.05 vs. baseline, P > 0.05 vs. vagally intact rats; Table 1).
These data indicate that perivagal capsaicin reduces, but does not abolish, the effects of CCK-8s microinjection in the left DVC. These data also put forward the intriguing possibility that capsaicin treatment may have damaged the vagal efferent nerve fibers either as a consequence of remodeling the neurons secondary to loss or damage of the afferent innervation, or, albeit unlikely, via a direct toxic action.

**DISCUSSION**

In this study, we have shown that 1) intraduodenal infusion of casein increases pancreatic exocrine secretion via a CCK-A-mediated effect in the DVC and 2) microinjection of CCK-8s in the DVC increases pancreatic protein output in control as well as in deafferented rats, although the CCK-8s-induced increase is significantly lower than that observed in control rats.

The data from this study suggest that endogenous CCK also increases pancreatic protein secretion in a nonparacrine manner, i.e., via direct activation of CCK-A receptors present on neurons of brain stem vagal circuits. We should like to reinforce once more that we do not dispute the powerful and well-documented paracrine effects of CCK on peripheral vagal afferent fibers. Our data suggest, though, that CCK also modulates pancreatic protein secretion via actions at other sites, including the brain stem. These conclusions are based on the following evidence.

Duodenal perfusion with casein induces the release of endogenous CCK from intestinal enteroendocrine cells that stimulate PES bypassing the activation of gastric vagovagal reflexes (24, 29). In the present study we demonstrate that the casein-induced increase in PES is attenuated significantly by application of the selective CCK-A receptor antagonist lorglumide to the floor of the fourth ventricle. Furthermore, the casein-induced increase in protein output is still present, although attenuated significantly, following selective sensory vagal deafferentation. These data indicate that the casein-induced increase in protein secretion also includes actions to activate CCK-A receptors in the DVC. In fact, if casein acted to increase PES via exclusively paracrine effects of the released CCK then fourth ventricular pretreatment with lorglumide would not have reduced casein-induced protein secretion and vagal sensory deafferentation would have prevented com-

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**Fig. 2.** Microinjection of CCK-8s in the DVC increases protein output via a vagally mediated action. A: microinjection of CCK-8s (450 pmol) in the DVC increased protein secretion; this CCK-8s-induced increase was prevented by vagotomy. Microinjection of vehicle (PBS) did not affect protein output. B: summary graphic representing the effects of microinjection of PBS or CCK-8s in intact animals and of CCK-8s in surgically vagotomized animals. “Baseline” is the average value of protein output in the 40 min that precede the microinjection, “average” is the mean value of protein output during the 30 min following the microinjection, and “peak effect” represents the peak value of protein output obtained following the microinjection. *P ≤ 0.05 vs. baseline, **P ≤ 0.05 vs. control. C: the increase in protein secretion was larger when CCK-8s was microinjected in the left vs. right DVC. D: summary graphic representing the effects of microinjection of CCK-8s in the right or the left DVC. *P ≤ 0.05 vs. baseline, **P ≤ 0.05 vs. right DVC.
pletely the casein-induced increase in PES. Instead, our results show that the increase in PES persists following either one of these treatments, arguing in favor of both paracrine and direct brain stem sites of action of CCK. Interestingly, our data also show that application of lorglumide to the floor of the fourth ventricle might increase slightly PES; the data, however, are inconclusive and further separate studies are needed to address this observation.

In physiological conditions, or following administration of low doses of exogenous CCK, neural cholinergic pathways play a major role in mediating the increase in PES (22, 23, 31, 35, 46, 50). Cholinergic pathways to the pancreas originate from the preganglionic dorsal motor nucleus of the vagus (DMV) neurons that, in turn, receive modulated sensory inputs from the adjacent NTS. To confirm our hypothesis of direct effects of CCK-8s in the brain stem, we performed microinjections in the dorsal vagal complex (DVC; i.e., the DMV, NTS, and area postrema) while monitoring pancreatic protein output. Our results demonstrate that brain stem microinjection of CCK-8s increased protein PES via activation of vagal pathways, since vagotomy prevented the induced increase in protein output, an effect similar to that observed following systemic administration of CCK-8s (27). Interestingly, microinjection of CCK-8s in the left DVC induced a larger increase in protein secretion than that observed upon administration in the right DVC. This observation suggests a possible functional asymmetry in pancreatic vagal efferent fibers, similar to the asymmetry described previously for both anatomical pathways and insulin-release mechanisms (4, 5, 14, 34, 44; reviewed in Refs. 49). The relevance of this asymmetry should not be underestimated (see below).

The results of the present study demonstrate that microinjection of CCK-8s in the DVC of vagal deafferented rats (see MATERIALS AND METHODS) still increased protein output, albeit significantly less than in intact rats, confirming our hypothesis of a direct brain stem site of action of CCK. Synaptic plasticity and rearrangements of the brain stem circuitry cannot explain

Fig. 3. Localization of the microinjections. A: micrograph depicting the site of injection of CCK-8s (arrow). DMV, dorsal motor nucleus of the vagus; NTS, nucleus tractus solitarius; TS, tractus solitarius; AP, area postrema; XII, nucleus of the hypoglossus. B: schematic representation of the sites of microinjection. Deaff, deafferented.

Fig. 4. Microinjection of CCK-8s in the dorsal vagal complex also increases protein output in vagally deafferented animals. A: duodenal infusion of 10% casein increased protein secretion; this increase was attenuated either by pretreatment with the CCK-A receptor antagonist lorglumide (120 pmol) placed on the floor of the 4th ventricle or by surgical vagal deafferentation. B: summary graphic representing the effects of microinjection of CCK-8s in intact animals (control), following complete vagal deafferentation 4–6 days before the experiment, or in rats following perivagal capsaicin treatment 7–10 days before the experiment. “Baseline” is the average value of protein output in the 40 min that preceded CCK-8s microinjection, “CCK-8s-average” is the mean value of protein output during the 30 min following CCK-8s microinjection, and “CCK-8s-peak effect” represents the peak value of protein output obtained following CCK-8s microinjection. *P < 0.05 vs. baseline, **P < 0.05 vs. control.
these results, since the modulation of pancreatic function by CCK-8s following vagotomy takes ~20 days to be restored (26), whereas our experiments were conducted within 10 days of deafferentation. Our data, therefore, argue in favor of a direct brain stem site of action of CCK as well as an effect on vagal afferent terminals in the DVC.

The well-accepted idea that CCK-8s acts via exclusively paracrine actions on peripheral, vagal afferent fibers stems mainly from the observations that C-fiber ablation, induced by perivagal capsaicin treatment, greatly attenuates, or even abolishes, the effects of systemic administration of CCK-8s on PES (25, 27, 35). In the present study, however, following perivagal capsaicin treatment, DVC microinjection still increased protein PES, albeit less than in vagally intact rats. Surprisingly, the increase in PES induced by CCK-8s microinjection in capsaicin-treated rats was less than that induced in vagally deafferented rats, but similar to that induced in rats that underwent a complete sensory denervation (unilateral deafferentation plus contralateral subdiaphragmatic vagotomy). These data indicate that perivagal capsaicin-induced degeneration not only of afferent C-fibers, but also of other neuronal elements controlling neuronal pancreatic functions, including, perhaps, vagal efferent motor fibers. It is important to remember that the chemical deafferentation using capsaicin involves localized perineural application of very high concentrations of capsaicin, usually a 1% solution, i.e., 33 mM (7, 16, 25, 27, 38, 47, 53, 55). It is likely that such a high concentration of capsaicin can also induce toxic effects on fibers or neurons other than C-fibers via the massive calcium influx consequent to activation of VR1 receptors. Indeed, administration of elevated doses of capsaicin induces degeneration of central nervous system neurons and fibers, including vagal effferent fibers, that likely express VR1 receptors and even involves neuronal areas that do not receive sensory inputs from the periphery (17, 20, 42). Furthermore, since more than 85% of vagal fibers are afferent, even minor nerve damage induced to vagal efferents or intrapancreatic neuronal cells by perivagal or systemic application of capsaicin not only could be overlooked easily but also would have a potentially major impact on the vagal motor output, leading to an overestimation of the contribution of vagal afferent fibers to the CCK-8s-mediated PES effects (35). Indeed, our results indicate that perivagal capsaicin induces the same effects on CCK-8s microinjection in the DVC as those observed following deafferentation and contralateral vagotomy, suggesting either that there is a nonselective toxic effect of capsaicin or that remodeling of neurons occurred as a consequence of loss or damage of vagal afferent innervation.

In conclusion, the data presented herein suggest strongly that the mechanism of action of CCK to increase pancreatic protein output requires the combined activity of CCK at the level of peripheral vagal sensory fibers (i.e., the well-described paracrine effect) and the direct activation of brain stem vagal circuits. In fact, although the BBB is scarcely permeable to CCK, we have to consider that a portion of both the NTS and the DMV have a leaky BBB, fenestrated capillaries, and enlarged perivascular space that allows the passage of large molecules (11, 15, 43), making it likely that circulating CCK reaches these neuronal circuits. In fact, there is functional evidence that CCK can cross the BBB to activate neuronal elements within the brain stem (8, 21, 54). Our data obtained with endogenous release of CCK induced by perfusion with intraduodenal casein, indeed, appear to support the likelihood of an hormonal effect of CCK to activate NTS-DMV neuronal elements directly or via the adjacent area postrema, which lies entirely outside the BBB.

This possibility is supported by electrophysiology data indicating that CCK-8s excites via a CCK-A mediated effect a subpopulation of pancreas-projecting neurons (50a) as
well as other neurons comprising vagal brain stem circuits (1–3, 9, 37).

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