Role of CCK<sub>1</sub> and Y<sub>2</sub> receptors in activation of hindbrain neurons induced by intragastric administration of bitter taste receptor ligands

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Hao S, Sternini C, Raybould HE. Role of CCK<sub>1</sub> and Y<sub>2</sub> receptors in activation of hindbrain neurons induced by intragastric administration of bitter taste receptor ligands. Am J Physiol Regul Integr Comp Physiol 294: R33–R38, 2008. First published November 14, 2007; doi:10.1152/ajpregu.00675.2007.—G-protein-coupled receptors signaling bitter taste (T2Rs) in the oral gustatory system and the α-subunit of the taste-specific G-protein gustducin are expressed in the gastrointestinal (GI) tract. α-Subunit of the taste-specific G-protein gustducin colocalizes with markers of enteroendocrine cells in human and mouse GI mucosa, including peptide YY. Activation of T2Rs increases cholecystokinin (CCK) release from the enteroendocrine cell line, STC-1. The aim of this study was to determine whether T2R agonists in the GI tract activate neurons in the nucleus of the solitary tract (NTS) and whether this activation is mediated by CCK and peptide YY acting at CCK<sub>1</sub> and Y<sub>2</sub> receptors. Immunocytochemistry for the protooncogene c-Fos protein, a marker for neuronal activation, was used to determine activation of neurons in the midregion of the NTS, the region where vagal afferents from the GI tract terminate. Intragastric administration of the T2R agonist denatonium benzoate (DB), or phenylthiocarbamide (PTC), or a combination of T2R agonists significantly increased the number of Fos-positive neurons in the mid-NTS; subdiaphragmatic vagotomy abolished the NTS response to the mixture of T2R agonists. Deletion of CCK<sub>1</sub> receptor gene or blockade of CCK<sub>1</sub> receptors with devazepide abolishes the activation of NTS neurons in response to DB, but had no effect on the response to PTC. Administration of the Y<sub>2</sub> receptor antagonist BIIE0246 blocks the activation of NTS neurons to DB, but not PTC. These findings suggest that activation of neurons in the NTS following administration of T2R agonists to the GI tract involves CCK<sub>1</sub> and Y<sub>2</sub> receptors located on vagal afferent terminals in the gut wall. T2Rs may regulate GI function via release of regulatory peptides and activation of the vagal reflex pathway.

bitter taste receptors; vagal afferent; nucleus of the solitary tract; Fos; cholecystokinin 1 receptor; Y<sub>2</sub> receptor

T2R ARE A FAMILY OF G-protein-coupled receptors that are expressed on the tongue and palate epithelium in humans and rodents and are involved in sensing bitter taste (12, 37). The human T2R family is composed of 33 members (38), and both mouse and rat T2R families consist of 36 intact genes (56). T2Rs and the α-subunit of the taste-specific G-protein gustducin (Gogust) are also found in rodent gastrointestinal (GI) tract (25, 50, 55). Gogust is expressed in brush cells of the rat stomach and duodenum (25) and mouse small intestine (50). Colocalization of Gogust with peptide YY (PYY) and glucagon-like peptide-1 in enteroendocrine L cells of human colonic mucosa has been reported (47). In addition, Gogust is colocalized with glucagon-like peptide-1 and 5-hydroxytryptamine in the mouse small intestine (50). T2R mRNA has been identified in the mucosa of stomach and duodenum (55, 56) and in the human colon (47). The presence of transcripts corresponding to the putative denatonium benzoate (DB) and phenylthiocarbamide (PTC) receptors, mT2R108 in mouse (rT2R16 in rat) and mT2R138 (rT2R38), respectively, have been demonstrated in mouse and rat upper GI tract, and in a mouse GI enteroendocrine cell line, STC-1 (56). Application of ligands for the T2Rs, including DB and PTC to STC-1 cells, induces rapid Ca<sup>2+</sup> signaling (14, 56), indicating that T2Rs in these cells are functional. Release of cholecystokinin (CCK) is induced by application of DB to STC-1 cells (14). Thus there is evidence to demonstrate the existence of T2Rs in the GI tract and enteroendocrine cell lines, but little is known of their function.

It is well established that regulatory peptides released from GI endocrine cells in response to luminal stimulation by nutrients result in changes in GI function, including inhibition of gastric emptying, and gastric acid secretion, pancreatic enzyme secretion, intestinal motility, and inhibition of food intake (46). A major pathway by which regulatory peptides induce change in function is via activation of extrinsic neural pathways, predominantly the vagal afferent pathway (21). Vagal afferents play an important role in gut-brain communication, responding to both mechanical and chemical stimuli. Activation of vagal afferents causes excitation of neurons in the nucleus of the solitary tract (NTS), followed by reflex activation of vagal efferent neurons, which results in changes in GI function (22). In addition, information carried in vagal afferents from the GI tract to the central nervous system is involved in the regulation of food intake and feeding behaviors (48) and in nausea and vomiting (5).

CCK is a well-established gut-brain regulatory peptide. It is released from endocrine cells in the upper small intestine in response to the presence of nutrients (17), and it exerts its effect via two receptor subtypes, CCK<sub>1</sub> and CCK<sub>2</sub>. CCK<sub>1</sub> receptors (CCK<sub>1</sub>Rs) are abundant in peripheral organs (24, 35, 49), while the CCK<sub>2</sub> receptors are the predominant form found in the central nervous system (19, 35). CCK acts on CCK<sub>1</sub>Rs on vagal afferent nerve terminals in the intestinal mucosa (9, 45), subsequently resulting in stimulation of neurons in the NTS (13, 15) and activation of vagal reflexes (44). PYY is a 36-amino acid, straight-chain polypeptide, released from the L-type endocrine cells of the GI mucosa.
After release, dipeptidyl peptidase IV cleaves the NH₂ terminal tyrosine-proline residues, forming PYY3–36 (34). PYY1–36 represents ~60%, and PYY3–36 40% of circulating PYY (20). PYY acts through Y-receptor subtypes: Y₁, Y₂, Y₄, and Y₅. PYY1–36 shows high affinity to all four receptors, while PYY3–36 is a specific Y₂ agonist (26). Both PYY1–36 and PYY3–36 cross the blood-brain barrier and participate in appetite and weight control regulation. It has been suggested that PYY3–36 acting through Y₂ receptors in the arcuate nucleus decreases appetite and promotes weight loss (1, 8). However, Y₂ receptors are also expressed by neurons in the nodose ganglion and transported to the vagal afferent nerve terminals (28). The anorectic effect of peripheral administration of PYY3–36 on food intake was abolished following either bilateral subdiaphragmatic total truncal vagotomy or brain stem-hypothalamic pathway transection in rodents (2, 28). These findings indicate that peripheral PYY3–36 may transmit signal to the brain via activating Y₂ receptors on the vagal afferents.

In the present study, we tested the hypothesis that stimulation of T2Rs expressed by the GI mucosa activates the vagal afferent pathway by a mechanism involving CCK₁R and Y₂ receptors. The specific aims were to determine 1) whether activation of T2Rs in the GI tract stimulate neurons in the medial NTS, the region in the brain stem in which vagal afferents from GI tract terminate, and whether this occurs through a vagal pathway, using Fos protein as a marker of neuronal activation and subdiaphragmatic vagotomy; and 2) whether activation of NTS neurons involves the CCK₁R and Y₂ receptors.

**EXPERIMENTAL PROCEDURES**

**Animals.** Experiments were performed using 8-wk-old male C57/B6 mice (JaxWest, University of California, Davis, CA), CCK₁R knockout mice (CCK₁R<sup>−/−</sup>) (29, 52), and their wild-type counterparts (CCK₁R<sup>+/+</sup>; 129S6/SvEi, Taconic, Oxnard, CA). Mice were maintained on regular laboratory chow and fasted overnight on wire-bottom cages, but allowed water ad libitum before all experimental procedures. The studies were performed in accordance with protocols reviewed and approved by UC Davis Institutional Animal Care and Use Committee. The UC Davis institutional guidelines for care and use of laboratory animals were followed throughout the study.

**Reagents.** The CCK₁R antagonist devazepide (kindly donated by Merck, Sharpe and Dohme) was dissolved in 0.1 ml DMSO, followed by 0.1 ml heparinized 0.9% saline (0.1 ml heparin/100 ml saline) followed by 25 ml of 10% paraformaldehyde (Sigma, St. Louis, MO). The brain stem was removed and postfixed in 4% paraformaldehyde for 1 h. The brain stem was cut into 100-μm sections using a vibratome. Sections were incubated for 1 h in goat serum-phosphate-buffered saline (PBS) (Chemicon, Temecula, CA), incubated in primary antibody (1:2,000 rabbit anti-Fos; Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h, followed by incubation with the secondary antibody (1:200 biotinylated goat anti-rabbit; Vector Laboratories, Burlingame, CA) for 2 h. Tissue was incubated for 3 h in avidin-biotin complex solution (Standard Elite Vectastain ABC kit, Vector Laboratories). Diaminobezidine solution (Sigma) was added for a 5-min incubation, and then 50 μl H₂O₂/PBS (0.1 ml 30% H₂O₂/10 ml PBS) was added to catalyze the diaminobezidine reaction; the reaction was stopped with a PBS wash. Tissue was thoroughly washed between each incubation period.

Images were taken on a Provis microscope and analyzed using paint Shop Pro, edition 7. A stereotaxic mouse brain atlas was used to determine the location of the NTS in each section of tissue (39). A region of interest was drawn around the NTS, and all activated neurons in the NTS region of interest were counted.

Neurons were considered immunopositive (above threshold) by their staining and size. Representative sections were chosen, corresponding to different regions of the NTS: caudal NTS (bregma −8.24 to −7.92 mm), mid-NTS (−7.76 to −7.48 mm), and rostral NTS (−7.08 to −6.48 mm). Three sections were chosen for each region for a total of nine sections per mouse. The numbers of labeled neurons per section were summed for each region for each mouse; this value was used in subsequent statistical analyses (53).

**Statistical analysis.** Significant differences in the number of Fos-positive neurons between treatment groups were analyzed by two-way ANOVA followed by Holm-Sidak multiple-comparison test (SigmaStat). Significant level was defined as P < 0.05. Results are presented as the number of Fos-positive neurons ± SE.

**RESULTS**

Effect of intragastric administration of a mixture of bitter taste receptor agonists. Initial experiments were performed with a mixture of bitter taste receptor agonists to activate multiple T2Rs. Intragastric administration of the bitter taste receptor agonist mixture significantly increased the number of Fos-positive neurons in the mid-NTS in both normal and sham-operated mice compared with control mice that received gavage of vehicle (water) (Fig. 1; P < 0.05; n =
The increase in Fos-positive NTS neurons was observed at the level of mid-NTS (bregma -7.32 to -7.76). There was no significant increase in the number of Fos-positive neurons in the NTS at levels caudal or rostral to this area following intragastric gavage of bitter taste receptor agonist mixture (Fig. 2). Therefore, only the number of Fos-positive neurons in the mid-NTS is presented in the subsequent text and figures.

Role of CCK1Rs and Y2 receptors in mediating activation of the NTS in response to DB. Intragastric administration of DB (10 mM) significantly increased the number of Fos-positive neurons in NTS compared with intragastric administration of vehicle in both C57/B6 and CCK1R+/+ mice (Fig. 3, n = 5–6 in each group, P < 0.05). This increase in Fos-positive neurons in NTS in response to intragastric administration of DB was significantly reduced in CCK1R-/- compared with CCK1R+/+ mice (Fig. 3A; P < 0.05, n = 5–6 in each group). In addition, the increase in the number of Fos-positive neurons in the NTS following intragastric administration of DB was completely abolished in mice pretreated with either the CCK1R antagonist devazepide or the Y2 receptor antagonist BIIE0246 (Fig. 3B, P < 0.05, n = 3–5).

Role of CCK1Rs and Y2 receptors in mediating activation of the NTS in response to PTC. Intragastric administration of PTC (10 mM) to C57/B6 or CCK1R+/+ mice significantly increased the number of Fos-positive neurons in the NTS (Fig. 3; n = 4–5, P < 0.05). However, there was no significant difference in the number of Fos-positive neurons in response to PTC in the CCK1R+/+ compared with CCK1R-/- mice (n = 4–5, P > 0.05). Blockade of Y2 receptors in C57/B6 mice had no effect on the activation of the NTS in response to PTC (Fig. 3B, n = 4 in each group, P > 0.05).

DISCUSSION

The present study shows that activation of multiple T2Rs, presumably expressed by endocrine cells in the mucosa of the GI tract, induces c-Fos expression in neurons in the midregion of the NTS. The increase in the number of Fos-positive neurons in response to intragastric gavage of bitter taste receptor agonists was completely abolished in mice in which the subdiaphragmatic vagus nerves had been sectioned (Fig. 1; P < 0.05, n = 5). In mice following sham operation or in which the subdiaphragmatic vagus nerve was sectioned and were gavaged with water, the numbers of Fos-positive neurons were not significantly different from those of control mice (data not shown).

Fig. 1. Photomicrographs of the brain stem of mice showing immunoreactivity for Fos protein following intragastric administration of a mixture of bitter taste receptor (T2R) agonists. Vehicle (water) or a mixture of bitter taste receptor agonists was administered intragastrically by gavage in control, sham-operated, or vagotomized mice, and the number of Fos-positive neurons in the nucleus of the solitary tract (NTS) were detected and counted using immunocytochemistry and image analysis. Top: section of the brain stem at mid-NTS illustrating the area of NTS where Fos-positive neurons were counted. AP, area postrema; DMN, dorsal motor nucleus. Scale bar = 200 μm. Bottom: photomicrographs of mouse brain stem showing Fos expression in neurons in the mid-NTS (one side) in control mice (water or T2R agonists gavage), and sham-operated or vagotomized mice following taste mixture gavage. Note the abundance of Fos immunoreactive neurons following T2R agonist administration in nonoperated (T2R agonists) and sham-operated (sham+T2R agonists) animals compared with control (water) and vagotomized animals that received taste agonist mixture. Scale bar = 200 μm.

Fig. 2. Number of Fos-positive neurons (mean ± SE) in three regions of the NTS (caudal, mid, and rostral), in control (water or T2R agonists gavage), sham-operated, and vagotomized mice following taste mixture gavage. *Significant difference vs. administration of vehicle and vs. vagotomized animals that received gavage of bitter taste receptor agonists (P < 0.05).
of the NTS, where the majority of afferent fibers from the proximal GI tract terminate (3, 4), with little activation of rostral and caudal regions. The increase in the number of Fos-positive NTS neurons was prevented by subdiaphragmatic vagotomy, suggesting that the response of NTS neurons was induced by an action of bitter taste receptor agonists at the level of the subdiaphragmatic viscera. However, we cannot discern the exact site of action, which could be in the stomach, or the proximal small intestine, or both. Taken together, these data suggest that bitter taste receptor agonists in the gut lumen act on receptors localized to endocrine cells in the mucosa of the GI tract, resulting in activation of the vagal afferent pathway.

CCK1Rs and Y2 receptors are expressed by nodose neurons and localized to vagal afferent nerve terminals in the GI mucosa (9, 28). We investigated whether the response of NTS neurons to intraluminal administration of bitter taste receptor agonists involved either CCK1Rs or Y2 receptors. Intragastric administration of DB alone, a putative selective ligand for mT2R108, elicited a significant activation of NTS neurons that was not observed in mice lacking CCK1Rs (CCK1R−/−) and was prevented by pretreatment with a Y2 receptor-selective antagonist. In contrast, lack of CCK1Rs and inactivation of Y2 receptors did not affect the ability of NTS neurons to respond to PTC, a preferred ligand for the mT2R138. These findings suggest a selectivity of the neuronal response in the NTS to agonists acting at different T2Rs. The involvement of CCK1Rs and Y2 receptors in the activation of vagal afferents in response to DB implies that activation of DB-preferred T2R evokes CCK and PYY release. This is consistent with the observation that DB potently stimulates CCK release from an intestinal endocrine cell line of STC-1 cells (14) and is supported by the expression of Gogust, a G-protein subunit that mediates gustatory signals in the tongue, in enteroendocrine L cells that express PYY in the human colonic mucosa (47). Furthermore, our findings for selectivity of different bitter taste receptors with DB and PTC are consistent with receptor selectivity for bitter tastants in the human tongue. Indeed, in humans, detection of the bitter compounds, such as salicin, PTC, and DB, is thought to be mediated by three different receptors of the T2R family, hT2R16 (11), hT2R38 (27), and hT2R44 (40), respectively. The latter two receptors are the homologues to mouse T2R138 (PTC receptor) and mT2R108 (DB receptor), respectively (56).

It is interesting to note that either CCK1R or Y2 receptor blockade abolished the response to DB. These data are not readily explained in terms of our current understanding of the vagal afferent pathway. However, the majority of vagal afferent neurons expressing the Y2 receptor also express the CCK1R (G. J. Dockray, personal communication), so an interaction at the level of the vagal afferent pathway is possible. In addition, CCK regulates PYY release via CCK1Rs (16, 32), and this could account for the observation. Alternatively, it has been shown that CCK and PYY have direct effects on NTS neurons (6, 10). Thus it is possible that the action of CCK is at the level of the vagus nerve, and the action of the Y2 receptor is at the level of the NTS via the Y2 receptor.

Taken together, the present findings suggest an involvement of vagal afferents, including those expressing CCK1Rs and Y2 receptors, in mediating the activation of neurons in the NTS by T2R agonists. Activation of the vagal afferent pathway is important for the regulation of GI function when food is in the intestinal lumen. Perfusion of the intestine with nutrients activates neurons in the NTS, and this is mediated by CCK1Rs likely located on the peripheral terminals of vagal afferents that terminate close to the base of CCK-secreting enteroendocrine cells. CCK and PYY have been shown to exert a number of biological actions on GI functions, including effects on gastric motility (42), gastric emptying (41, 43, 52), gastric acid secretion (33, 52), pancreatic secretion (30), gallbladder contraction (31), and control of food intake (7, 13, 15). It is likely that activation of the vagal afferent pathway by bitter taste receptor ligands and subsequent release of regulatory peptides such as CCK and PYY will produce changes in GI function; however, this remains to be investigated. It should be noted that the present experiments do not provide definitive evidence that...
these ligands are acting at the T2R receptor; it is consistent with the known localization to CCK and PYY endocrine cells in the gut wall.

Perspectives and significance. This study demonstrates that bitter taste agonists acting at multiple T2Rs, as well as individual agonists like DB and PTC that preferentially bind mT2R108 and mT2R128, respectively, induce activation of neurons in the NTS, likely via release of either PYY or CCK from endocrine cells and subsequent activation of vagal afferent nerve terminals expressing CCK1Rs and Y2 receptors. Stimulation of different T2R receptor subtypes activate different neuronal pathways, as supported by the observation that DB-induced neuronal activation is mediated by CCK and PYY, whereas the neuronal pathway stimulated by PTC-sensitive T2R does not appear to involve CCK or PYY. These findings support a role of T2Rs expressed in the GI tract in regulating GI function through the activation of vagal afferent neurons involving the release of regulatory peptides that differ, depending upon the stimuli.

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REFERENCES


