Expression of 5-HT$_3$ receptors by extrinsic duodenal afferents contribute to intestinal inhibition of gastric emptying

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Submitted 5 July 2001; accepted in final form 21 October 2002

Raybould, Helen E., Jorg Glatzle, Carla Robin, James H. Meyer, Thomas Phan, Helen Wong, and Catia Sternini. Expression of 5-HT$_3$ receptors by extrinsic duodenal afferents contribute to intestinal inhibition of gastric emptying. Am J Physiol Gastrointest Liver Physiol 284: G367–G372, 2003. First published October 30, 2002; 10.1152/ajpgi.00292.2001.—Intestinal perfusion with carbohydrates inhibits gastric emptying via vagal and spinal capsaicin-sensitive afferent pathways. The aim of the present study was to determine the role of 1) 5-hydroxytryptamine (5-HT$_3$) receptors (5-HT$_3$R) in mediating glucose-induced inhibition of gastric emptying and 2) 5-HT$_3$R expression in vagal and spinal afferents in innervating the duodenum. In awake rats fitted with gastric and duodenal cannulas, perfusion of the duodenum with glucose (50 and 100 mg) inhibited gastric emptying. Intestinal perfusion of mannitol inhibited gastric emptying only at the highest concentration (990 mosm/kgH$_2$O). Pretreatment with the 5-HT$_3$R antagonist tropisetron abolished both glucose- and mannitol-induced inhibition of gastric emptying. Retrograde labeling of visceral afferents by injection of dextran-conjugated Texas Red into the duodenal wall was used to identify extrinsic primary afferents. Immunoreactivity for 5-HT$_3$R, visualized with an antibody directed to the COOH terminus of the rat 5-HT$_3$R, was found in $>$80% of duodenal vagal and spinal afferents. These results show that duodenal extrinsic afferents express 5-HT$_3$R and that the receptor mediates specific glucose-induced inhibition of gastric emptying. These findings support the hypothesis that enterochromaffin cells in the intestinal mucosa release 5-HT in response to glucose, which activates 5-HT$_3$R on afferent nerve terminals to evoke reflex changes in gastric motility. The primary glucose sensors of the intestine may be mucosal enterochromaffin cells.

NUTRIENTS IN THE INTESTINAL lumen produce a number of different feedback effects on gastrointestinal function, including inhibition of gastric emptying and gastric acid secretion, stimulation of the pancreas and gallbladder, and alteration of intestinal motility. These feedback mechanisms have been shown to be dependent on the extrinsic afferent innervation of the intestine (28). There is evidence that each macronutrient group (lipid, carbohydrate, and protein) can activate specific afferent pathways. For instance, in the rat, intestinal lipid inhibits gastric emptying via a vagal afferent pathway (13), whereas the inhibitory effects of intestinal carbohydrate on gastric emptying depend on both vagal and spinal capsaicin-sensitive afferent pathways (30).

Electrophysiological experiments have demonstrated that vagal and spinal afferents respond to both mechanical and chemical stimuli (10). Glucose and osmotic stimuli are effective stimulants of vagal and spinal afferent fiber activity (22, 23, 25). However, the mechanism by which these luminal factors activate visceral afferents is unclear. The current hypothesis for which there is experimental support is that visceral afferents are not directly activated by luminal nutrients but are activated indirectly via the release of peptide hormones and neurocrine agents present in enteroendocrine cells in the intestinal mucosa (9). Nutrients in the intestinal lumen release a number of different hormones and neurocrine mediators from endocrine cells in the intestinal mucosa (3). Studies using specific and potent receptor antagonists or antibodies have provided evidence that these substances are functionally important in mediating intestinal feedback inhibition of gastrointestinal function. For example, there is good evidence for a role for CCK in mediating intestinal feedback inhibition of gastric emptying, gastric acid secretion, and pancreatic secretion (13, 18, 20). In addition, it has been shown that serotonin 5-hydroxytryptamine (5-HT) mediates increases in pancreatic secretion in response to in-
testinal stimuli, such as high osmolarity and disaccharides (17).

Electrophysiological studies have shown that 5-HT directly stimulates the discharge of vagal afferents, an effect that is not secondary to changes in smooth muscle tone (11, 12). These terminals are in the intestinal mucosa, because the response to 5-HT is abolished by intraluminal lidocaine and they are unresponsive to changes in tension in the smooth muscle. This activation of vagal afferents by 5-HT is abolished by antagonists of the 5-HT$_3$ receptor (5-HT$_3$R) (11). Recently, a study (34), recording from vagal afferent cell bodies, demonstrated that carbohydrates and osmotic stimuli elicit a marked vagal afferent activity, which was abolished by the administration of a 5-HT$_3$R antagonist, tropisetron. These results suggest that vagal afferent fiber responses to luminal osmotic stimuli and to the products of carbohydrate digestion are dependent on the release of 5-HT from enterochromaffin (EC) cells and the consequent activation of 5-HT$_3$Rs on vagal afferent terminals to stimulate nerve fiber impulses.

The majority of 5-HT in the body is contained in EC cells of the intestinal mucosa. This pool of 5-HT has been shown to be released by various neurotransmitters, such as muscarinic and β-receptor agonists (26). 5-HT can also be released by physiological stimuli, such as an increase in intraluminal pressure (5), mechanical stimulation of the mucosa (14), and hyperosmotic solutions (21). In addition, there is some evidence that glucose can release 5-HT (4). 5-HT released from EC cells is involved in activation of enteric neurons in response to mechanical stimulation of the mucosa (8, 16, 24).

In the present study, we tested the hypothesis that 5-HT acting at 5-HT$_3$Rs on both vagal and spinal afferents mediates inhibition of gastric motility induced by duodenal perfusion with glucose. In addition, to demonstrate that vagal and spinal afferents innervating the intestine express the 5-HT$_3$R, we used immunocytochemistry with an antibody raised to the 5-HT$_3$R together with retrograde labeling of afferent neuronal cell bodies. These studies provide evidence that EC cells in the intestinal mucosa may act as sensors, detecting the presence of glucose in the intestinal lumen, and releasing 5-HT, which stimulates the terminals of extrinsic afferents that express 5-HT$_3$Rs resulting in reflex alteration of gastric motor function.

**MATERIALS AND METHODS**

**Animals**

Experiments were performed by using male Sprague-Dawley rats (Harlan Sprague Dawley, San Diego, CA) of initial weight of 240–280 g maintained on regular laboratory chow. Rats were fasted overnight but allowed water ad libitum before all surgical and experimental procedures. The institutional guidelines for the care and use of laboratory animals were followed throughout the study.

**Materials**

Glucose and mannitol were obtained from Sigma (St. Louis, MO) and dissolved in distilled water. The 5-HT$_3$R antagonist tropisetron was obtained from Research Biochemicals (Boston, MA) and dissolved in 0.9% saline. The 5-HT$_3$R antagonist ondansetron (GR38032F) and the 5-HT$_1$R antagonist GR113808A (8) were gifts from Glaxo Research Group (Greenford) and dissolved in 0.9% saline. Dextran-conjugated Texas Red (10,000 mol wt; Molecular Probes) was dissolved as a 5% solution in 0.1 M phosphate buffered saline.

**Surgical Procedures**

**Implantation of gastrointestinal cannulas.** This procedure has been described in detail elsewhere (13). Briefly, rats (n = 8) were anesthetized with pentobarbital sodium (50 mg/kg ip; Nembutal, Abbott Laboratories, North Chicago, IL). A small stainless steel Thomas cannula was placed into the forestomach, exteriorized through the abdominal wall, and capped. The duodenal cannula (Intramedic PE -90; Clay Adams, Parsippany, NJ) was inserted into the duodenum 1–2 cm distal to the pylorus. Rats were allowed to recover for 2 wk before being used in experiments and were used for a period of ≤3 mo after surgery.

**Retrograde labeling of visceral afferents.** Rats (n = 5) were anesthetized with pentobarbital sodium (50 mg/kg ip), and the duodenum was exposed by using a midline abdominal incision. Dextran-conjugated Texas Red was injected into the wall of the duodenum by using a 26-gauge needle attached to a 10-μl Hamilton glass syringe (29). Injections (6–8 of 1–2 μl) were made into the wall of the duodenum. The tip of the needle was advanced by ~5 mm under the serosa, and the needle was left in-place for 2 min after injection to help avoid leakage of dye from the injection site. The visceras were rinsed thoroughly with 0.9% saline before being returned to the peritoneal cavity. The abdomen wall was sutured, and tissue was harvested after 14–21 days. Subdiaphragmatic vagotomy was performed immediately before injection in two additional rats.

**Measurement of Gastric Emptying**

In the period during which rats were recovering from surgery, they were accustomed to light restraint in Bollman cages. The duodenal cannula were flushed daily with 0.9% saline and plugged with petroleum jelly. On experimental days, fasted rats were placed in Bollman cages, the gastric cannulas were opened, and any residual gastric contents were flushed out with warm 0.9% saline. The stomach was then allowed to drain freely for 30–45 min. Three milliliters of 0.9% saline containing the nonabsorbable marker phenol red (60 mg/l) was instilled into the stomach, and the cannula was closed. After 5 min, the contents of the stomach were collected, and the volume was measured and centrifuged. The concentration of phenol red was determined in the instilled and recovered fluid and the rate of gastric emptying was calculated by using the method of Hunt and Spurrell, which calculates the volume emptied from the stomach including any gastric secretions (30).

**Experimental Protocols**

Gastric emptying was measured in two consecutive control periods with no perfusion of the duodenum and with 10 min allowed between procedures. Perfusion of the duodenum with glucose or mannitol (330, 660, or 990 mM) at a rate of 0.056 ml/min (total amounts 25, 50, and 100 mg) was started and continued for 10 min. Gastric emptying was measured in the final 5 min of the perfusion period. On some days, rats were pretreated with tropisetron (0.1 mg/kg ip) 15 min before the
gastric emptying studies. Only one perfusion test was performed per day, and animals were used no more than every third day. Rats were randomized for treatment. Not all rats received each treatment; the full protocol could not be completed because one rat, which had a leaking gastric fistula, was euthanized.

Measurement of Gastric Motility

Gastric motility was measured in urethane-anaesthetized rats as previously described (27). Intraluminal pressure was measured manometrically in the body of the stomach. Signals were collected and stored onto a personal computer for later analysis. Baseline intraluminal pressure was standardized between rats at <5 cmH₂O. A segment of duodenum was isolated by inserting a second catheter into the proximal duodenum 0.5 cm distal to the pylorus and a third catheter, 5 cm distal to the pylorus. The proximal duodenal cannula was used to perfuse glucose, and the more distal cannula was allowed to drain freely. Glucose (990 mM) was perfused for 10 min at the rate of 0.05 ml/min (total amount of glucose 100 mg). Glucose was perfused into the duodenum before and again 10 min after administration of either ondansetron (100 µg/kg iv) or GR113808 (1 mg/kg iv). At least 30 min were allowed between consecutive duodenal perfusions with glu-

Effect of 5-HT3R Antagonist on Gastric Emptying

In untreated or vehicle-treated rats, 75 ± 2% of the volume of liquid emptied in 5 min. Duodenal perfusion with glucose produced a load-dependent inhibition of gastric emptying (Fig. 1). Perfusion of the intestine with 50 and 100 mg glucose inhibited gastric emptying by 33 and 38%, respectively (n = 8, P < 0.05), and there was no significant difference in the inhibition produced by 50 and 100 mg (not significant). Gastric emptying during saline infusion of the intestine was unaltered by pretreatment with tropisetron. However, inhibition of gastric emptying in response to glucose was completely abolished by pretreatment with tropisetron (n = 8, P < 0.01); gastric emptying during perfusion of glucose in the presence of tropisetron was not significantly different from that obtained during perfusion of the intestine with saline.

To determine the specificity of the glucose response, gastric emptying was measured during duodenal perfusion of mannitol in the same osmotic concentrations as glucose. Perfusion of the intestine with mannitol (330 or 660 mosmol/kgH₂O) had no significant effect on the rate of gastric emptying (n = 8; P value not significant). However, perfusion of the intestine with 990 mM mannitol significantly inhibited gastric emptying by 35% (n = 8, P < 0.05). Inhibition of gastric emptying induced by mannitol was reversed by the administration of tropisetron (Fig. 2).

Data for gastric emptying are expressed as the percentage of the liquid emptied after 5 min as means ± SE. The signal from the intraluminal pressure trace was digitized and stored on a personal computer for later analysis by using Axotape software (Axon Instruments). The decrease in intragastric pressure was taken as the mean nadir of the trace over 2 min of nutrient perfusion compared with baseline (27). Values were compared by using two-way ANOVA followed by Tukey’s test and considered significantly different if P < 0.05.

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Effect of 5-HT₃R and 5-HT₄R Antagonist on Glucose-Induced Inhibition of Gastric Motility

Intraduodenal infusion of glucose decreased tonic intragastric pressure by 0.8 ± 0.1 cmH₂O as previously described (27). Administration of the specific 5HT₃R antagonist ondansetron (100 μg/kg iv) inhibited the response to glucose. In the presence of the antagonist, glucose decreased intragastric pressure by 0.1 ± 0.1 cmH₂O (P < 0.01 vehicle vs. ondansetron; n = 7). In contrast, the administration of the specific 5-HT₄R antagonist GR11808 (1 mg/kg iv) had no effect on inhibition of gastric motility induced by duodenal perfusion with glucose (0.7 ± 0.1 vs. 0.7 ± 0.1 cmH₂O, vehicle vs. GR11808; n = 5, P value not significant).

5-HT₃R Immunoreactivity in Vagal and Dorsal Root Ganglion Neurons

Immunoreactivity for the 5-HT₃R was found in neurons throughout the nodose and dorsal root ganglia (Fig. 3). Confocal microscopy revealed that the immunoreactivity was located predominately at the cell surface. The specificity of the immunoreactivity was demonstrated by the lack of staining after preadsorption of the antibody with an excess of the synthetic peptide (Fig. 3).

Retrograde labeling of nodose and dorsal root ganglia neurons was observed after injection of the retrograde tracer into the duodenum (Fig. 4). In the nodose,
labeled neurons were predominately found in the caudal part of the nodose ganglion. In the dorsal root ganglion, a retrograde label was observed in T7-L4 levels after injections into the duodenum. The specificity of the retrograde labeling was demonstrated by the absence of a label in the sacral or high thoracic dorsal root ganglia and by the absence of a label detected in the nodose ganglion after subdiaphragmatic vagotomy. Retrogradely labeled nodose and DRG neurons were immunopositive for the 5-HT3R, 83 and 86%, respectively.

DISCUSSION

The present study demonstrates that inhibition of gastric emptying induced by glucose in the intestine is mediated by 5-HT3Rs. Glucose-induced inhibition of proximal gastric motility, part of the gastroduodenal motility pattern predictive of decreased gastric emptying, was also inhibited by ondansetron, a 5-HT3R antagonist, but not by a 5-HT3R antagonist. In addition, we show for the first time that vagal and spinal afferents innervating the duodenum, that have previously been shown to mediate intestinal feedback inhibition of gastric emptying in response to the digestive products of dietary carbohydrate, express 5-HT3Rs (30). Taken together, these results suggest that glucose inhibits gastric emptying by release of 5-HT from EC cells and activation of 5-HT3Rs on the peripheral terminals of vagal and spinal afferents in the duodenum.

Extrinsic primary afferent neurons with mucosal terminal fields respond to both mechanical and chemical stimuli applied to the mucosa. Whether mucosal afferents respond specifically to the digestive products of dietary carbohydrate has been questioned. A recent study (34) recording from nodose neurons in the rat showed a clear response to glucose, a response unlikely to be due to an osmotic effect. It should be noted that some of the vagal afferents responding to glucose also respond to hyperosmotic solutions either of sodium chloride or the disaccharide maltose. However, a population of afferents was identified that responded only to glucose supporting the existence of specific glucose-sensitive extrinsic afferents. Because of the location of the terminals of vagal afferents in the lamina propria, it has been suggested that these afferents do not respond directly to events in the lumen but that the stimulus acts at a postabsorptive site (2). Afferents may respond directly to postabsorbed nutrients, such as monosaccharides. However, an alternate hypothesis is that responses are indirect, mediated via release of a neuroactive substance from endocrine or EC cells, which stimulates afferent nerve fiber terminals located in the lamina propria. Vagal afferents express a number of different subtypes of receptors for ligands that are contained in endocrine and EC cells, for example, CCK and 5-HT, lending support to this hypothesis; however, until recently there was no direct support for this hypothesis. The recent study by Zhu et al. (34) demonstrates that vagal afferents respond specifically to glucose and that this response is mediated by 5-HT3Rs. The present study provides strong functional evidence that this pathway mediates glucose-induced inhibition of gastric emptying. However, glucose-sensitive neurons have been described in the enteric nervous system, and these neurons might also play a role in intestinal feedback inhibition of gastric function by sensing glucose followed by release of 5-HT and activation of 5-HT3Rs on extrinsic nerve terminals in the myenteric plexus. These glucose-responsive enteric neurons express ATP-sensitive K+ channels and were hyperpolarized by the removal of extracellular glucose (19). In separate studies (31), we have shown that only analogs of glucose that are substrates of the sodium-glucose cotransporter SGLT-1 elicit inhibition of gastric emptying. In addition, analogs of glucose that are substrates of SGLT but are not metabolized, such as 3-O-methyl-glucose and α-methyl glucose, inhibit gastric motor function. This suggests that metabolism is not required to initiate activation of vagal afferents, and thus it is unlikely that these glucose-sensitive enteric neurons are playing a role in the reflex response being studied here.

The present studies support the hypothesis that 5-HT is released by EC cells and activates vagal and spinal afferents. However, we do not directly show that glucose releases 5-HT from EC cells. By using a cell line that expresses 5-HT, we have shown that substrates of SGLT can release 5-HT from these cells in culture (15), further supporting the hypothesis that 5-HT in EC cells represents an important pool in mediating intestinal feedback inhibition of gastric function. Whether this release occurs in native EC cells remains to be determined.

In the present study, using combined immunocytochemistry and retrograde tracing, we have shown that vagal and spinal afferents innervating both the duodenum express 5-HT3Rs on their cell bodies. Previous studies have shown that receptor proteins are synthesized in the cell bodies of these afferent neurons and are transported toward the central and peripheral terminals. We hypothesize that the 5-HT3Rs are transported from the cell bodies to the peripheral terminals. At this time, we cannot conclude that these afferents terminate in the duodenal mucosa. We have evidence that numerous myenteric neurons express this receptor and immunopositive fibers are distributed to the intestinal mucosa (7). These fibers are likely to be both of extrinsic and intrinsic origin. In the present study, we demonstrate that vagal and spinal afferents innervating the duodenum do express 5-HT3Rs, although we did not identify the exact site of termination.

The present study demonstrates that glucose can elicit specific effects on gastric emptying, independent of its osmolarity. However, hyperosmotic solutions can also inhibit gastric emptying, and this response is mediated by 5-HT3Rs. This confirms the functional importance of the vagal afferents recorded in electrophysiological studies in which afferent response to hyperosmotic sodium chloride or the disaccharide maltose were also inhibited by 5-HT3R antagonists (34). This is in-line with our findings that many neurons in the nodose and dorsal root ganglia express 5-HT3Rs. In other systems, responses to 5-HT3R desensitize rapidly, and it is not clear if this is occurring in the present system.
(6). If so, it would be hard to visualize how continued negative feedback via release of 5-HT and activation of the 5-HT2Rs on nerve terminals would result in a sustained delay of gastric emptying. However, gastric emptying in the postprandial period has been shown to occur in a pulsatile manner (1). Thus it is possible that the duodenum is not continuously exposed to nutrients and therefore desensitization would not occur.

In conclusion, we show that glucose evoked a nutrient-specific effect to inhibit gastric emptying mediated by 5-HT3Rs. We have previously shown that this response to glucose is mediated by both vagal and spinal afferents, and here we provide morphological evidence that both vagal and spinal afferents innervating the duodenum express 5-HT3Rs. The mechanism by which glucose releases 5-HT, in response to either glucose or hypertonic solutions is unclear, but the response to glucose may involve specific transporters expressed by the intestinal epithelium.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-41004 (to H. E. Raybould), DK-41301, DK-35740, and DK-57037 (to C. Stermini) and a Deutsche Forschungsgemeinschaft (Bonn, Germany) Grant GL 311/1-1 (to J. Glatzle).

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