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Incretin release from gut is acutely enhanced by sugar but not by sweeteners in vivo

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Fujita Y, Wideman RD, Speck M, Asadi A, King DS, Webber TD, Haneda M, Kieffer TJ. Incretin release from gut is acutely enhanced by sugar but not by sweeteners in vivo. *Am J Physiol Endocrinol Metab* 296: E473–E479, 2009. First published December 23, 2008; doi:10.1152/ajpendo.90636.2008.—Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are released during meals from endocrine cells located in the gut mucosa and stimulate insulin secretion from pancreatic β-cells in a glucose-dependent manner. Although the gut epithelium senses luminal sugars, the mechanism of sugar sensing and its downstream events coupled to the release of the incretin hormones are not clearly elucidated. Recently, it was reported that sucralose, a sweetener that activates the sweet receptors of taste buds, triggers incretin release from a murine enteroendocrine cell line in vitro. We confirmed that immunoreactivity of α-gustducin, a key G-coupled protein involved in taste sensing, is sometimes colocalized with GIP in rat duodenum. We investigated whether secretion of incretins in response to carbohydrates is mediated via taste receptors by feeding rats the sweet-tasting compounds saccharin, acesulfame potassium, D-tryptophan, sucralose, or stevia. Oral gavage of these sweeteners did not reduce the blood glucose excursion to a subsequent intraperitoneal glucose tolerance test. Neither oral sucralose nor oral stevia reduced blood glucose levels in Zucker diabetic fatty rats. Finally, whereas oral glucose increased plasma GIP levels ~4-fold and GLP-1 levels ~2.5-fold postadministration, none of the sweeteners tested significantly increased levels of these incretins. Collectively, our findings do not support the concept that release of incretins from enteroendocrine cells is triggered by carbohydrates via a pathway identical to the sensation of "sweet taste" in the tongue.

sugar sensing; glucose-dependent insulinotropic polypeptide; glucagon-like peptide-1; K cell; L cell

GLUCOSE-DEPENDENT INSULINOTROPIC polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are gastrointestinal hormones with pleiotropic activities, many of which facilitate nutrient metabolism (3). Both hormones are known as "incretin" hormones, as they are released from the gut in response to dietary nutrients (1, 2, 6, 10, 14, 15, 23, 28, 29, 34, 45, 48, 49, 51) and stimulate synthesis and release of insulin from pancreatic β-cells, while also promoting β-cell growth and survival (4, 58). In view of these actions, there is significant interest in harnessing incretins to treat diabetes. However, the usefulness of the native peptides as therapeutic agents is limited by their short circulating half-life, which results from rapid cleavage by dipeptidyl peptidase-4 (DPP-4; Ref. 33). Approaches to increase the circulating bioactive levels of GIP and GLP-1 include the use of DPP-4-resistant analogs such as exendin-4 and NN2211 or the administration of DPP-4 inhibitors (3, 4, 58). Another strategy is to increase endogenous incretin release from enteroendocrine cells, perhaps through the development of oral drugs that activate nutrient-sensing pathways in incretin-producing cells. It has been demonstrated that fatty acids bind to G-protein-coupled receptors (GPRs) and induce incretin release in vitro and in vivo (8, 24, 30). In particular, GPR40 and GPR120 have been reported to be involved in "fat" sensing in enteroendocrine cells through increasing calcium influx. In contrast, the mechanism by which gut incretin-secreting cells sense dietary sugars remains relatively unknown.

In recent years, the mechanisms by which the tongue senses sugars have been identified. In gustatory cells, two type 1 taste GPRs (T1R2 and T1R3) heterodimerize to form the sweet receptor (42, 47). Sugars and sweeteners bind to the sweet taste receptor and thereby activate the G-protein gustducin to induce signal transduction (39, 43, 60). It has been suggested that there may be significant similarity between the structural and functional components mediating nutrient sensing in gustatory cells in tongue and gut endocrine cells (16). Indeed, Höfer et al. (25) reported that the α-subunit of gustducin (α-gustducin) is expressed throughout the surface epithelium of the gut. In addition, T1R2, T1R3, and α-gustducin are coexpressed in GLP-1-positive cells in rodent and human gut (27). GIP-expressing cells also coexpress α-gustducin (27). The artificial sweetener sucralose has been reported to induce release of GLP-1 and GIP from the murine endocrine cell line GLUTag in vitro (40). Moreover, α-gustducin-null mice display an abnormal GIP and GLP-1 secretory response to oral glucose administration (27). Taken together, these studies provide evidence suggesting that the mechanism for signal transduction of sweetness through T1Rs and the α-gustducin cascade utilized by the gustatory system might also be involved in sugar sensing in gut endocrine cells. We rationalized that if this were correct, sweetener compounds could be utilized as agents to induce incretin release, in the absence of sugars, to lower blood glucose levels. We used intraperitoneal glucose tolerance tests (IPGTTs) in combination with oral sweetener administration in rats to determine: *1)* whether glucose excursions were improved in response to oral sweeteners, and *2)* whether GIP and GLP-1 were released in response to oral sweeteners. Our results suggest that

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sweeteners do not acutely enhance the release of incretin hormones in vivo.

MATERIALS AND METHODS

Animals. Male Wistar rats (290–530 g) and C57/BL6 mice were purchased from the University of British Columbia (UBC) Animal Care Centre (Vancouver, BC, Canada) or Charles River (Montreal, PQ, Canada or Yokohama, Kanagawa, Japan). Male Zucker diabetic fatty (ZDF) rats (17 wk) were purchased from Charles River (Montreal, PQ, Canada). All studies were performed in accordance with UBC and Asahikawa Medical College guidelines for the use of animals. Animals had ad libitum access to water and a standard rodent chow diet.

Immunohistochemistry. Rat small intestine and mouse tongue were extracted while the animals were under inhalable anesthesia (1.5% isoflurane). Tissues were fixed in 4% paraformaldehyde in PBS overnight at 4°C and then embedded in paraffin blocks. Antigen retrieval was performed by washing in citrate buffer (pH 6 containing 0.05% Triton X) followed by microwave heating. Sections were treated with a protein-blocking reagent (Dako Cytomation, Mississauga, ON, Canada) for 30 min and incubated with mouse anti-GIP antibody (1:5,000; kindly provided by Dr. Alison Buchan, University of British Columbia) or rabbit anti-α-gustducin (1:200; sc-395; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After washes, sections were incubated with secondary antibodies (Alexofluor 488 or Alexofluor 594, 1:500; Molecular Probes, Eugene, OR) for 1 h at room temperature. Slides were mounted in aqueous media (Vector Laboratories, Burlingame, CA).

Oral sweetener challenge and IPGTT. IPGTTs were performed on conscious, overnight-fasted Wistar rats or fed ZDF rats. Glucose or sweeteners were administered with a syringe attached to a stainless steel gavage tube. Blood was drawn from tail vein and collected to a microcentrifuge tube and centrifuged before plasma samples were stored at −20°C or −80°C until assays. For tests combining oral sweetener delivery with IPGTT, sweeteners were given 15 min before intraperitoneal injection of glucose. Blood glucose levels were measured via a hand-held glucometer (One Touch Ultra; Life Scan, Burnaby, BC, Canada).

Chemicals and assays. D-tryptophan (T9753), acesulfame K (0454), and saccharin (109185) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Sucralose was kindly provided by McNeil Nutritionals (Fort Washington, PA). Stevia (R9003) and glucose (D14-500) were purchased from the JG Group (Burlington, ON, Canada) and Fisher Scientific (Pittsburgh, PA), respectively. Plasma total GIP levels were assayed by ELISA (EZRMGIP-55K; Millipore, Billerica, MA). Although samples from seven animals were collected for each group, overnight fasted levels sometimes fell below the assay's limit of detection (8.9 pg/ml); data are reported only for animals whose plasma GIP levels fell within the assay's readable range. Plasma total GLP-1 levels were assayed using a kit (K110FCC) from Meso Scale Discovery (Gaithersburg, MD) using a 50-μl sample volume, a modification suggested by the manufacturer to increase the sensitivity of the assay.

Statistical analysis. Results are expressed means ± SE. Statistical significance was assessed using commercial software (Prism; GraphPad, San Diego, CA). $P < 0.05$ was deemed statistically significant by ANOVA or student's *t*-test as appropriate.

RESULTS

α-Gustducin is coexpressed with GIP in rat duodenum. Several preceding studies (25, 27, 40) reported expression of α-gustducin in the gut; more recently, the sweet receptor components and α-gustducin have been colocalized to GLP-1-expressing L cells and/or GIP-expressing K cells. To confirm previous observations, we used immunohistochemical methods to analyze α-gustducin expression in the gut and tongue. We observed cytoplasmic immunoreactivity of α-gustducin in the gustatory receptor cells of murine taste buds (Fig. 1, *A* and *B*). We also detected flask-shaped α-gustducin-immunoreactive cells scattered throughout the intestinal mucosa by immunostaining of rat duodenum (Fig. 1, *C* and *E*). Double immunofluorescent staining for α-gustducin and GIP revealed that approximately one-third of GIP-immunoreactive cells coexpressed α-gustducin (Fig. 1).

Oral administration of sweeteners does not reduce blood glucose levels. Since the sweetener sucralose has been reported to stimulate GLP-1 and GIP secretion from enteroendocrine cells in vitro (40), we next examined the possibility that oral sweeteners might improve glucose disposal in vivo through induction of incretin release from the gut. We rationalized that if sweetener ingestion promotes release of GIP and GLP-1, then it should reduce the glycemic excursion to an IPGTT in the same way that an oral glucose load accompanied by incretin release yields a smaller glucose excursion compared with the same glucose load delivered either via intraperitoneal or intravenous routes. We chose five sweeteners to administer to rats (D-tryptophan, acesulfame K, saccharin, sucralose, and stevia), excluding aspartame as it does not activate the rodent sweet receptor (61). Peak blood glucose levels were observed

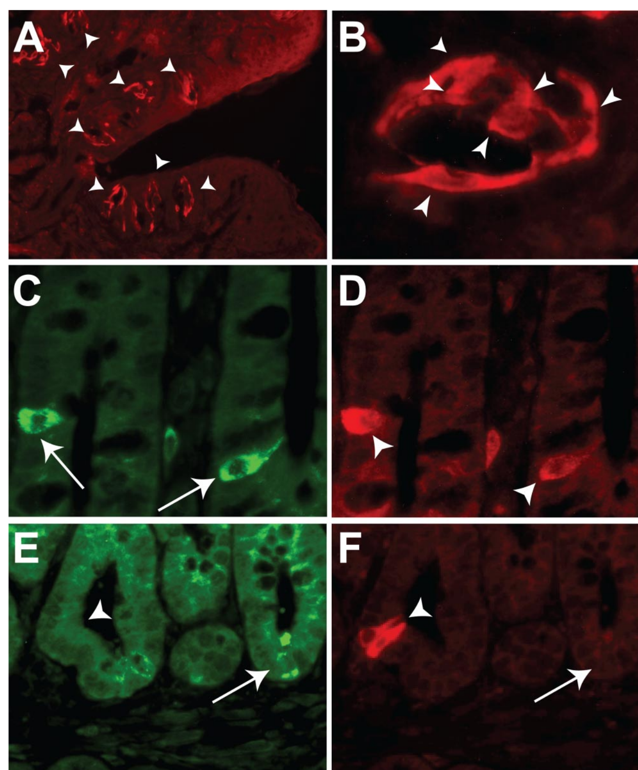

Fig. 1. Immunohistochemical analyses of α-gustducin expression in tongue and gut. α-gustducin-positive cells were identified in mouse taste buds (*A* and *B*). *A*: mouse circumvallate papillae in tongue (arrowheads indicate taste buds). *B*: mouse taste bud (arrowheads indicate α-gustducin positive cells). *C–F*: rat duodenum. Arrows indicate α-gustducin positive cells (*C* and *E*; in green) and arrowheads indicate glucose-dependent insulinotropic polypeptide (GIP)-positive cells (*D* and *F*; in red). Approximately one-third of GIP-positive cells coexpress α-gustducin (*C* and *D*), although α-gustducin-positive/GIP-negative cells are also found (*E* and *F*). *A* and *C–F* = ×200 magnification; *B* = ×400 magnification.

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10 min after glucose delivery, and, as anticipated, were significantly lower in rats administered glucose orally compared with those given the same glucose load by intraperitoneal injection (Fig. 2; 6.5 ± 0.4 mM for oral gavage compared with 11.2 ± 0.6 mM for intraperitoneal injection 10 min after glucose administration). However, the peak glucose levels were not significantly different in rats given an IPGTT alone compared with rats given IPGTT in combination with any of the oral sweeteners tested (Fig. 2).

To determine if the dose of sweeteners administered in our initial studies was inappropriate to improve the glucose excursion, we conducted additional experiments with a range of doses of orally administered stevia (5 mg/kg to 1 g/kg). As shown in Fig. 3, absolute blood glucose levels were not lower than intraperitoneal glucose alone at any time point; that is, none of doses of oral stevia tested significantly decreased blood glucose levels.

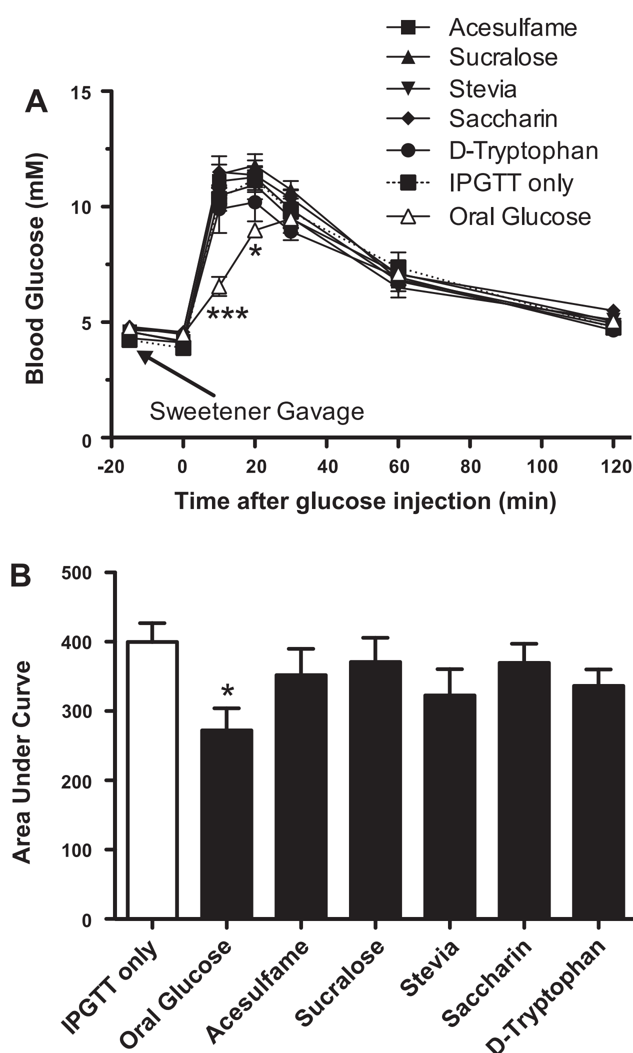

Fig. 2. Oral administration of sweeteners does not improve glucose excursion in normal rats during intraperitoneal glucose tolerance tests (IPGTT). Normal rats were given sweeteners at 50 mg/kg (D-tryptophan) or 1 g/kg (all others) or water (control IPGTT group) by oral gavage 15 min before glucose injection (1 g/kg) or received oral glucose only (1 g/kg) at time 0 min. *A*: blood glucose was measured via tail vein at −15, 0, 10, 20, 30, 60, and 120 min; △, glucose excursion of rats receiving oral glucose only. *B*: area under curve, calculated using blood glucose at *time 0* as baseline. $*P < 0.05$, $***P < 0.001$ vs. IPGTT using Student's *t*-test; $n = 5–7$ per group.

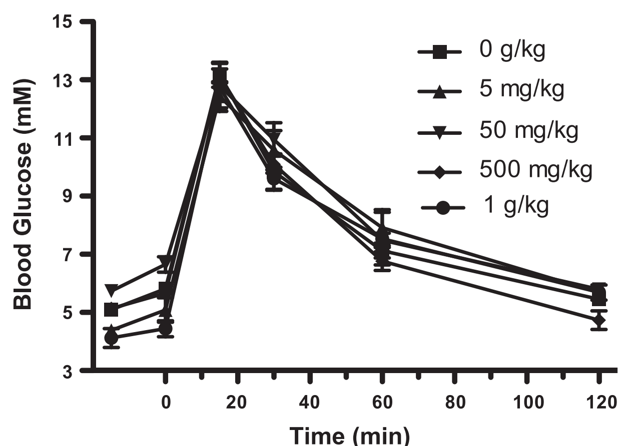

Fig. 3. Oral administration of stevia does not improve glucose excursion in normal rats during intraperitoneal glucose challenge. Normal rats were given stevia at the indicated dose 15 min before glucose injection (1 g/kg) at *time 0*. Blood glucose was monitored via tail vein; $n = 5–7$ except for 500 mg/kg dose, where $n = 3$.

Although we did not observe any effect of sweetener gavage on blood glucose levels in Wistar rats, we rationalized that any effect of possible incretin release on blood glucose levels might be limited by the glucose-dependent nature of the insulinotropic action of the incretins or be masked by counterregulatory mechanisms maintaining fasted blood glucose levels in normal rats. We therefore utilized ZDF rats, a model of type 2 diabetes, to examine the effect of a sweetener on blood glucose levels in hyperglycemic animals. Sucralose or stevia was administered orally to nonfasted ZDF rats without additional glucose load. Blood glucose levels were ~20–22 mM before sweetener administration and did not change significantly during the 120 min after sweetener delivery (Fig. 4).

GIP and GLP-1 release is stimulated by glucose but not sweeteners in vivo. Since we failed to observe any effect of oral sweetener delivery on glucose handling in vivo, we sought to directly test whether oral sweeteners induce incretin release. Plasma GIP levels increased robustly in normal rats administered an oral glucose load (2 g/kg), rising within 5 min of glucose delivery and peaking at ~4-fold basal levels at 30 min (Fig. 5). However, compared with basal levels, no increases in plasma GIP were observed 30 min after oral administration of any of the sweeteners tested (Fig. 6*A*), and none of the oral sweeteners tested induced any changes in blood glucose levels (data not shown). Similarly, while plasma GLP-1 levels increased ~2.5-fold 10 min after glucose gavage, there was no increase in plasma GLP-1 concentration in response to gavage of any of the sweeteners tested (Fig. 6*B*).

DISCUSSION

GIP and GLP-1 are released from the gut in response to nutrient intake and in turn stimulate insulin secretion from pancreatic β-cells (1, 2, 6, 10, 14, 15, 23, 28, 29, 31, 32, 34, 45, 48, 49, 51). Increasing the release of these incretin hormones by oral agents may be an attractive approach to improve glucose homeostasis in subjects with diabetes mellitus. Such efforts will benefit from an increased understanding of the

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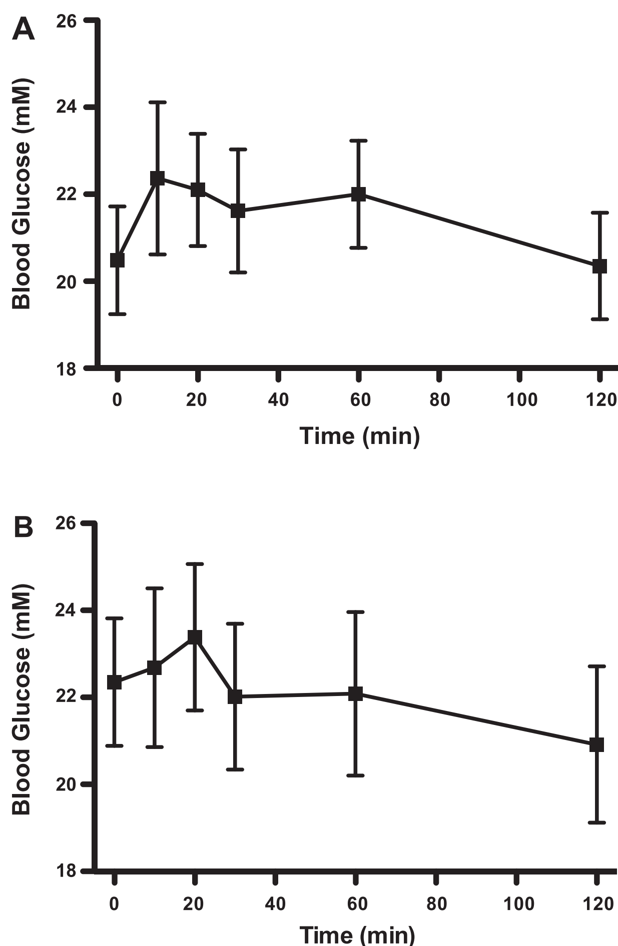

Fig. 4. Oral sucralose or stevia challenge does not change blood glucose levels in Zucker diabetic fatty rats. Random fed Zucker diabetic fatty rats (n = 6) were given oral sucralose (*A*; 1 g/kg) or stevia (*B*; 1 g/kg) at *time 0*. Blood glucose was measured via tail vein for 120 min after gavage.

mechanisms by which enteroendocrine cells detect and respond to various stimuli. In this regard, it has been suggested that incretin-producing cells possess the machinery necessary to respond to sweeteners (27, 40). GLP-1 release from the NCI-H716 model of human L cells was promoted by sugars and by the sweetener sucralose (27). Sucralose was also reported to induce GIP and GLP-1 release from murine gut endocrine GLUTag cells (40). Jang et al. (27) reported that α-gustducin null mice manifested deficiencies in secretion of GLP-1 and GIP and that the regulation of plasma insulin and also showed higher glucose excursion by oral glucose tolerance test. Based on these observations, we sought to determine whether artificial sweeteners could induce incretin release from the gut and thereby improve glucose homeostasis in normal and diabetic rats in vivo.

Collectively, our findings do not support the notion that sweeteners acutely induce the release of meaningful quantities of incretin hormones in rodents. Our results are in agreement with a clinical study (20) that reported no effect of acute oral stevioside on release of GIP and GLP-1 in subjects with type 2 diabetes. However, it remains possible that chronic sweetener administration may be capable of improving glucose homeostasis. Dietary sugar and artificial sweeteners increased sodium-dependent glucose-cotransporter (SGLT)1 mRNA and protein expression and improved glucose absorptive capacity in wild-type mice but not in sweet receptor T1R3 or α-gustducin null mice (40). Moreover, as SGLT1 has been implicated in the release of both GIP and GLP-1 (40), sweeteners might indirectly contribute to GIP and GLP-1 release by modulating expression of SGLT1. Indeed, a 4-wk treatment with the nondigestible sugar oligofructose showed an antidiabetic effect through the enhanced secretion of GLP-1 in high-fat-fed diabetic mice (5). These observations indicate that long-term treatment with sweeteners might be necessary to improve glucose homeostasis through enhanced incretin release, rather than the acute treatments used in our current study. It also remains possible that we did not test optimal doses for acute sweetener effects on incretin secretion and glucose handling, although we observed no effect of stevia on glycemic excursion over a wide range of doses. Moreover, the "sweetness" of the sweeteners we tested is up to several hundredfold higher than equal doses of glucose (12, 37, 47, 59, 62).

We observed some colocalization of immunoreactive α-gustducin and GIP, as has been reported previously (27, 40), but we noted that the majority of GIP-expressing K cells do not coexpress α-gustducin. It has been reported that α-gustducin is expressed in ∼15% of GLP-1-expressing cells (55). These observations are consistent with our previous findings (18) that indicate that there may not be one prototypical K or L cell. Moreover, the limited localization of α-gustducin in incretin-expressing cells suggests that this signaling pathway might not play a major role in regulating the release of either GIP or GLP-1 in response to "tasting" nutrients.

Incretin levels increase severalfold after the ingestion of a mixed meal or glucose (1, 6, 14, 15, 28, 29, 34, 45). Unlike the sweeteners, oral glucose clearly elicited the well-documented release of GIP and GLP-1 in our studies. The sensing mechanism for this involves luminal contact with glucose, since GIP and GLP-1 release does not increase when glucose is admin-

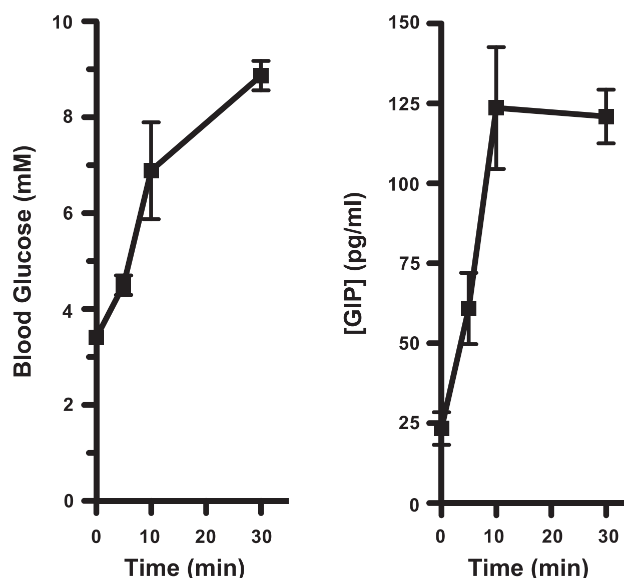

Fig. 5. Total GIP levels in response to oral glucose load in normal Wistar rats. *Left*: blood glucose levels (mM). *Right*: total plasma GIP levels (pg/ml) just before (*time 0*) and after oral gavage of glucose (2 g/kg) in normal rats (n = 3).

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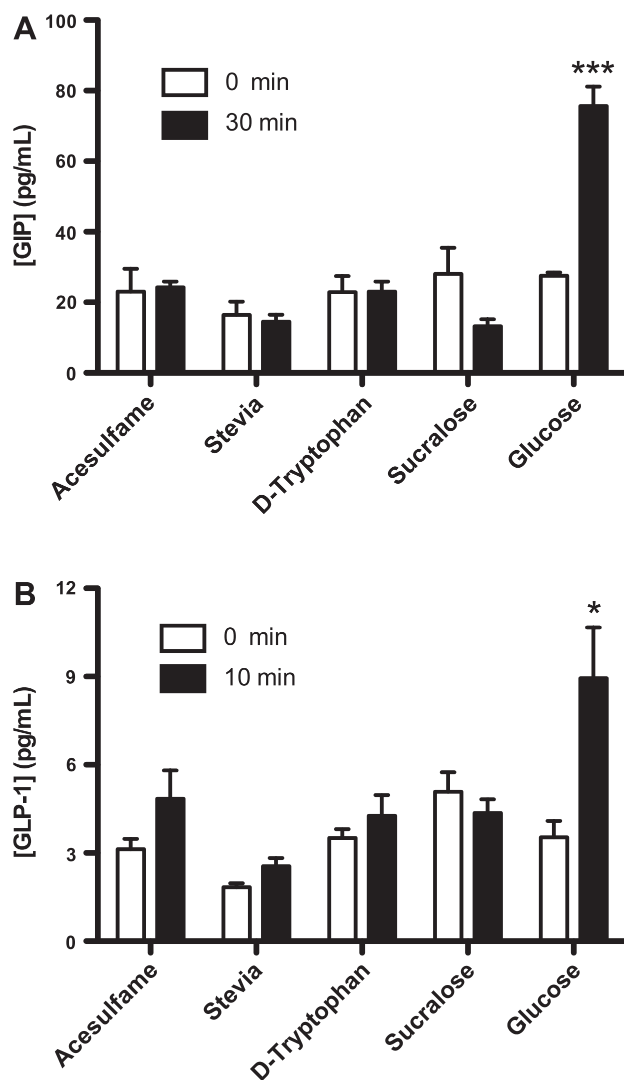

Fig. 6. Oral administration of sweeteners does not enhance GIP or GLP-1 release in rats. Sweeteners (50 mg/kg for D-tryptophan; 1 g/kg for all others) were given orally to normal fasted rats after an overnight fast. Blood samples were drawn before and 10 or 30 min after sweetener delivery. Glucose, but none of sweeteners, enhanced GIP release (*A*) and GLP-1 release (*B*). $*P < 0.05$, $***P < 0.001$ compared with basal using student's *t*-test; $n = 3–7$ for GIP (*A*); $n = 5–6$ for GLP-1 (*B*).

istered intravenously (2, 6, 10, 49, 50). Experiments with both cell lines and isolated primary cells suggest that K cells may be capable of directly sensing glucose (31, 32), although it remains possible that messages are transmitted by adjacent glucose-transporting mucosal cells (56). Studies in rodents, dogs, and humans suggest that GIP secretion is dependent on active transport of monosaccharides (19, 52, 53, 56). Thus treatment with compounds such as phloridzin, guar, and gymnemic acid can suppress the release of GIP (19, 44, 56). Blocking glucose transport with phloridzin also impairs the release of GLP-1 from the GLUTag model of L cells (21). Thus there is abundant evidence for nontaste receptor-mediated induction of incretin release from enteroendocrine cells.

There may be some similarities between the glucose-sensing pathways of incretin-producing enteroendocrine cells and pancreatic β-cells. This is not particularly surprising given the evolutionary relationship between the cells and the large number of gene-regulating transcription factors that the cells share in common (17). The glucose-phosphorylating enzyme glucokinase plays a key role in the metabolism of glucose by pancreatic β-cells and is often referred to as the "glucose sensor" (41). Glucokinase is also abundant in hepatocytes where it functions in the clearance of glucose. Activators of glucokinase enhance glucose-stimulated insulin release from pancreatic islets and glucose disposal by the liver and thus are being actively explored as novel treatments for diabetes (22, 41). Interestingly, both K cells and L cells also express glucokinase (7, 57). However, it has recently been reported that patients carrying a heterozygous glucokinase gene mutation had normal GIP and GLP-1 secretion in response to an oral glucose load (46). Carriers of homozygous glucokinase gene mutations were not studied, and it remains possible that there may be several redundant mechanisms controlling glucose sensing in the gut. It will thus be of interest to determine whether activators of glucokinase can be utilized to increase the release of incretin hormones.

In addition to glucose, fat is a potent GIP and GLP-1 secretagogue. Like that for sugars, the gustatory sensing of fat may be different than that of enteroendocrine cells. Lingual sensing of fat is believed to be mediated by CD36 (36), although the mechanisms by which CD36 promotes fatty acid uptake and signal transduction are currently not fully understood. In contrast, enteroendocrine cells express several recently deorphanized GPRs that are activated by fatty acids. GPR40, originally identified as a mediator of fatty acid induced insulin secretion from pancreatic β-cells (26), was subsequently identified on intestinal incretin-producing cells (13). Moreover, mice lacking GPR40 have reduced GIP and GLP-1 responses to an acute high-fat diet challenge (13). Similarly, GPR120 is expressed in GLP-1-secreting L cells, where it is reported to function as a receptor for dietary unsaturated long-chain free fatty acids (24, 30). GPR119 was identified as an additional long-chain fatty acid receptor expressed in β-cells (9) that, in contrast to the $G_{\alpha}q$ coupling of GPR40 and GPR120 (Ca^{2+} messenger), couples through $G_{\alpha}s$ (cAMP messenger). Interestingly, agonists of GPR119 were shown to enhance glucose-dependent insulin secretion, preferentially when glucose was administered orally with the agonist vs. via intraperitoneal injection, suggesting that the agonist might modulate incretin signaling (9). However, in a followup study (8), GPR119 was localized to L cells and oral treatment with a GPR119 agonist increased release of GLP-1 as well as GIP in normal but not GPR119 knockout mice. Therefore, agonists of fatty acid receptors may beneficial in treating type 2 diabetes, both by directly stimulating glucose-dependent insulin secretion from pancreatic β-cells and by boosting secretion of incretin hormones from the intestine (35, 38).

In summary, we were unable to find any evidence that artificial sweeteners acutely induce incretin release in vivo. Therefore, while parallels have been drawn between nutrient-sensing pathways in the oral cavity and gut (11, 54), our findings do not support the concept that gut incretin-secreting cells sense luminal carbohydrates via a pathway analogous to that by which the tongue senses sweetness. The physiological role of the taste receptor machinery in gut endocrine cells therefore remains to be clarified.

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