XENIN-25 POTENTIATES GIP ACTION VIA A NOVEL CHOLINERGIC RELAY MECHANISM
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The intestinal peptides GLP-1 and GIP potentiate glucose-mediated insulin release. Agents that increase GLP-1 action are effective therapies in type 2 diabetes mellitus (T2DM). However, GIP action is blunted in T2DM and GIP-based therapies have not been developed. Thus, it is important to increase our understanding of the mechanisms of GIP action. We developed mice lacking GIP-producing K cells. Like humans with T2DM, “GIP/DT” animals exhibited a normal insulin secretory response to exogenous GLP-1 but a blunted response to GIP. Pharmacologic doses of xenin-25, another peptide produced by K cells, restored the GIP-mediated insulin secretory response and reduced hyperglycemia in GIP/DT mice. Xenin-25 alone had no effect. Studies with islets, insulin-producing cell lines, and perfused pancreata indicated xenin-25 does not enhance GIP-mediated insulin release by acting directly on the β-cell. The in vivo effects of xenin-25 to potentiate insulin release were inhibited by atropine sulfate and atropine methyl bromide but not by hexamethonium. Consistent with this, carbachol potentiated GIP-mediated insulin release from in situ perfused pancreata of GIP/DT mice. In vivo, xenin-25 did not activate c-fos expression in the hindbrain or paraventricular nucleus of the hypothalamus indicating that CNS activation is not required. These data suggest that xenin-25 potentiates GIP-mediated insulin release by activating non-ganglionic cholinergic neurons that innervate the islets- presumably part of an enteric-neuronal-pancreatic pathway. Xenin-25, or molecules that increase acetylcholine receptor signaling in β-cells, may represent a novel approach to overcome GIP resistance and therefore treat humans with T2DM.

The entero-insulin axis is a physiological system comprised of peptides secreted from the gastrointestinal tract that play an important role in regulating insulin secretion from pancreatic islet β-cells (1,2). To date, attention has focused on two intestinal peptides, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). GIP is produced predominantly by intestinal K cells located in the proximal small intestine whereas GLP-1 is produced primarily by intestinal L cells present in the distal bowel (3-5). Release of GLP-1 and GIP is controlled by nutrient levels in the lumen of the gut rather than the bloodstream. Both hormones are released into the bloodstream immediately after ingestion of a meal and then potentiate glucose-stimulated insulin release. This increase in insulin secretion has been termed the incretin effect. Importantly, the incretin effect occurs only in the presence of elevated blood glucose and does not cause postprandial hypoglycemia. An increase in circulating GLP-1 activity has significant therapeutic benefit in patients with T2DM (1,2,6-10). Two major classes of drugs that accomplish this goal have recently been introduced into the market with substantial success. Long-acting analogs of GLP-1 potentiate glucose-stimulated insulin secretion leading to improved glucose control. Inhibitors of dipeptidyl peptidase IV (DPP IV), the enzyme responsible for GLP-1 breakdown in the circulation, raise circulating levels of endogenous GLP-1 and thus increase insulin secretion. Although DPP IV also cleaves and inactivates GIP, GIP or its analogs have not been used in the treatment of diabetes since the GIP-mediated incretin effect is severely blunted in persons with T2DM (11-13). Strategies to overcome this defect in the GIP response would have significant therapeutic potential in T2DM.
Thus, it is critical to understand the basis of GIP resistance more fully.

To study the physiologic mechanisms for GIP action in vivo, two complementary strategies have been used to abolish GIP signaling. One approach produced knockout mice that lack GIP receptors (14,15). These GIPR−/− mice do not respond to GIP or to any other peptide that binds to and signals via the GIP receptor. The approach adopted by our laboratory (16) generated transgenic mice that completely lack GIP-producing K cells by expressing an attenuated diphtheria toxin A chain using transcriptional regulatory elements from the GIP promoter and gene. The GIP/DT mice lack GIP and therefore GIP signaling, as well as signaling activated by other products of the K cell. Studies using both of these experimental approaches have provided strong evidence that GIP signaling promotes obesity and insulin resistance in mice fed a high fat diet (16,17). The insulin secretory response, measured 15 minutes after administration of oral glucose, is only mildly reduced in GIPR−/− mice (14,18) which could be due to compensatory action of GLP-1 (18). In contrast to single and double incretin receptor knockout mice that still exhibit a robust insulin secretory response when challenged with oral glucose (14), plasma insulin levels failed to increase in the GIP/DT mice 15 minutes after administration of oral glucose even though GLP-1 release and action were not perturbed (16). These observations suggest that 1) the GIP/DT mice could be used to identify compounds that can reconstitute the incretin response in vivo and 2) K cells may produce peptides in addition to GIP that are essential for amplifying the insulin secretory response observed 15 minutes after administration of oral glucose.

It has been reported that xenin-25, a 25 amino acid peptide, is produced by a subset of K cells (19). Xenin-25 has also been found in many other tissues but only after treatment with pepsin indicating that these additional tissues probably do not produce significant levels of mature xenin-25 (20). It was also reported that xenin-25 is secreted in response to meal ingestion and sham feeding in humans (21). The response to sham feeding suggests that xenin-25 could prime the β-cell in preparation for the impending increase in circulating GIP and glucose levels that will occur after meal ingestion. Infusion or administration of supraphysiologic doses of xenin-25 in animals decreased gastric acid release and increased jejunal motility, secretion from the exocrine pancreas and plasma levels of pancreatic polypeptide, vasoactive intestinal peptide (VIP), and glucagon (22). Plasma levels of gastrin, somatostatin, gastrin releasing polypeptide, substance P, neurokinin A, peptide tyrosine tyrosine, and chromogranin B remained unchanged. Plasma insulin levels increased only ~25% (22). Perfusion of the rat pancreas with xenin-8, an 8 amino acid C-terminal fragment of xenin-25, has been reported to increase both basal and glucose-stimulated insulin release (23). However, the physiologic properties of xenin-25 and xenin-8 are not identical (24) and the authors did not report whether xenin-25 increased insulin release. Xenin-25 also inhibits food intake in chicks, rats, and mice (25-27). In the single published human study, intravenous infusion of xenin-25 increased intestinal motility (28). This study did not report the effect of xenin-25 infusion on plasma insulin levels. Since both xenin-25 and GIP were previously reported to be produced by a subset of GIP-producing K cells (19), studies were conducted to test the hypothesis that xenin-25, either alone or in combination with other incretins, may enhance the insulin secretory response to glucose and overcome the block in GIP action seen in GIP/DT mice and in mice with diabetes.

**EXPERIMENTAL PROCEDURES**

*Animals*-Production, housing, and initial characterization of the GIP/DT mice have been described (16). GIP/DT mice were generated and maintained on a pure C57BL/6J background. Non-transgenic littermates were used as wild type controls. These mice were maintained on standard chow. NONcNZO10/LtJ mice (29,30) were purchased from The Jackson Laboratories (Bar Harbor, Maine). Unless indicated otherwise, all experiments were conducted on male mice. All animal protocols were approved by the Washington University Animal Studies Committee.

*In vivo metabolic studies in mice*- Studies were conducted essentially as described (16). Briefly, glucose tolerance tests were performed on animals that were fasted for 16-h but given free access to water. Blood was sampled for
measurement of glucose before (0 min) and at the indicated time after administration of glucose by intragastric gavage (3 g per kg body weight) for oral glucose tolerance tests (OGTTs) or by intraperitoneal injection (1 g per kg body weight) for intraperitoneal glucose tolerance tests (IPGTTs). As indicated, GIP, GLP-1, xenin-25, and pituitary adenylate cyclase-activating polypeptide(6-38) [PACAP(6-38)] were administered in PBS containing 0.1% bovine serum albumin [(BSA) 1 nmole peptide per mouse in 100 μL] by intraperitoneal injection 20 seconds prior to administration of glucose. Control animals (i.e. no peptide) received 100 μL vehicle alone (“BSA”). In vivo insulin, GIP and xenin-25 release assays were conducted similar to the glucose tolerance tests except blood was collected at the indicated time for measurement of peptides. Atropine sulfate (500 μg/kg), atropine methyl bromide (AMB; 400 μg/kg), hexamethonium chloride [15 mg/kg (31)], or normal saline as a control were administered by intraperitoneal injection 15 minutes prior to the injection of glucose and peptides. Blood glucose was measured using MediSense Precision Xtra Blood Glucose Test Strips (Abbott Laboratories, Alameda, CA). Plasma was prepared from blood and assayed for insulin or GIP by ELISA (Millipore Research, Inc., Saint Charles, MO) and for xenin using an RIA (Phoenix Pharmaceuticals, Inc, (Burlingame, CA) or a custom prepared ELISA (see below). Fold-increases in plasma insulin were calculated by dividing the plasma insulin levels in a treated group by the fasting insulin level in the same group. The incremental glucose area under the curve (AUC) was first calculated for each mouse following subtraction of fasting glucose level and then expressed as the average ± SEM for each group.

Cell Culture- MIN6 cells (32) were cultured as previously described (33). Panc1 cells were cultured in Dulbecco’s modified MEM containing 10% fetal bovine serum. In vitro insulin release assays- Islets were isolated from wild type C57BL/6J or GIP/DT mice, cultured overnight in RPMI 1640 containing 10% fetal bovine serum and then subjected to insulin release assays (typically 5 islets per sample and 6 samples per group) as previously described (34). MIN6 cells were plated in 12-well tissue culture dishes and then assayed for insulin release as previously described (33) (4-wells per group). Briefly, following a 60-minute pre-incubation in assay buffer containing 2.5 mM glucose, samples were switched to buffer containing the indicated concentration of glucose plus peptide(s). Sixty minutes later, buffer was collected for insulin determination.

In situ pancreas perfusion- The pancreas was perfused in situ in a humidified, temperature-controlled chamber essentially as described except a fixed dose of 10 mM glucose was maintained throughout the perfusion (35,36). Briefly, the perfusate was introduced through the aorta and collected from the portal vein. Following equilibration in buffer containing 10 mM glucose, the indicated drug(s) was perfused for 15 minutes followed by a 15 minute drug washout period. This cycle was repeated 3 times with additional test drugs. The final perfusion in each experiment involved exposure to 30 mM potassium chloride (KCl) to document that the pancreas retained the ability to respond to secretagogues. The flow rate was maintained at 1 mL/min throughout, fractions were collected every 2 min, and insulin was measured in every other fraction.

Peptides/Drugs- Atropine sulfate, which crosses the blood-brain barrier to inhibit central and peripheral muscarinic receptors, was purchased from Baxter Healthcare Corporation (Deerfield, IL). Atropine methyl bromide (AMB), which does not cross the blood-brain barrier and inhibits only peripheral muscarinic receptors (37-40), was purchased from Phaltz and Bauer, Inc. (Waterbury, CT). Hexamethonium chloride was purchased from Sigma-Aldrich (St. Louis, MO). GLP-1 was purchased from Bachem (Torrance, CA). GIP, xenin-25, and PACAP(6-38) were purchased from American Peptide Company, Inc., (Sunnyvale, CA).

Western blots- MIN6 cells were cultured overnight in media containing 2.5 mM glucose and 0.1% BSA without serum and then treated for 10 minutes with 7.5 mM glucose and 100 nM peptide. Panc1 cells were similarly treated except they were maintained in 25 mM glucose and treated with the indicated concentration of xenin-25. Cells were harvested and subjected to Western blot analysis as previously described (41) using antibodies 9101S or 9107 (Cell Signaling Technology, Danvers, MA) which are specific for
Erk1 and Erk2 when phosphorylated at Thr202 and/or Tyr204 of Erk1 (Thr185 and/or Tyr187 of Erk2) or for total Erk1 and Erk2, respectively.

**Antibody production, characterization and assay development**- A synthetic peptide MLTKFETKSARVKGLSFHPKRPWILC, (Biomolecules Midwest, Inc., Waterloo, IL), corresponding to xenin-25 with an additional non-native C-terminal cysteine was conjugated to KLH-maleimide (Pierce Chemical Co., Rockford, IL) as described (42). This immunogen was injected into rabbits (Harlan Bioproducts for Science, Inc., Madison, WI) and Armenian hamsters (Washington University Hybridoma Core) to generate polyclonal and monoclonal antibodies, respectively. The rabbit antiserum was affinity purified over the antigen conjugated to a SulfoLink resin (43) to yield Rabbit antibody 9728 and supernatant from hamster fusion products was purified using Protein A/G (both from Pierce Chemical Co.) to yield several monoclonal antibodies. Epitope mapping was performed on spot-peptide array membranes (MIT Biopolymers Laboratory, Cambridge, MA) with spot 1 corresponding to residues 1-10, spot 2 residues 2-11, spot 3 residues 3-12, etc (43). Primary antibodies were used at 4 μg/ml for 4h and secondary antibodies goat anti-rabbit alkaline phosphatase (111-055-047) or Goat anti-Armenian Hamster alkaline phosphatase (127-055-160) were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA) and used at a dilution of 1/1,000 for 1h.

A sandwich ELISA was developed on a Meso Scale Discovery (MSD, Gaithersburg, MD) 2400 sector imager. The most sensitive format used the hamster monoclonal as capture antibody and biotinylated rabbit antibody 9728 as capping antibody on a 96-well standard MSD plate. Detection was with ruthenium-labeled streptavidin (MSD). Standard curves were generated using synthetic xenin-25. Ninety μl samples were diluted with an equal volume of a 60/40 by volume mixture of HBR (Scantibodies Laboratory, Inc., Santee, CA) and casein-tris-tween blocker. Fifty μl aliquots were assayed in triplicate and xenin-25 concentrations determined with MSD instrument software. Our operational limit of detection (LOD) is defined as 2x signal/noise and ranged from 4.5 to 30 pg/ml with a coefficient of variation typically 10% or less. In the later stages of this work, we were able to decrease the amount of sample by ~40% by translating the assay to MSD Hi-Bind plates. No loss of assay performance occurred. The ELISA does not detect neurotensin. To estimate the minimum xenin-25 N-terminal fragment length that could be detected by our sandwich immunoassay, 3 additional xenin peptides were prepared: xenin(16-25), xenin(11-25), and xenin(6-25).

**c-fos Immunohistochemistry.** Overnight fasted GIP/DT mice were injected with saline or glucose (1 g/kg) plus the indicated peptide(s) (1 nmole each peptide per mouse) as described in Figure 1. Insulin-induced (3.5 IU per mouse) hypoglycemic mice were used as a positive control for c-fos staining. All mice were anesthetized and then perfused transcardially with 0.1% heparin followed by 4% paraformaldehyde. Brains were fixed, cryoprotected with 25% sucrose, and embedded in OCT. Hypothalamic and hindbrain sections (20-30μm) were taken from 0.58 to 1.82mm and 6.24 to 8.00mm caudal to bregma, respectively. Free-floating sections were blocked with 10% normal goat serum in PBS containing 0.1% Triton-100 and then incubated with primary c-fos antibody in the same buffer (1:2000, Ab-5, Calbiochem, La Jolla, CA). Bound primary antibodies were detected using the Elite ABC kit (Vector Laboratories, Burlingame, CA). The sum number of c-fos positive cells counted on both sides of the brain on four to six anatomically matched sections was used for statistical analyses.

**Statistical analyses**- All values are expressed as the average ± SEM. Statistical significance was determined at the 5% level using the two-tailed Student’s t test.

**RESULTS**

**GIP-mediated reduction in hyperglycemia is blunted in GIP/DT mice.** The expected increase in plasma insulin levels 15 minutes after administration of oral glucose (incretin effect) is essentially absent in GIP/DT mice (16). IPGTTs were therefore performed to determine whether exogenously administered peptides could induce an incretin response in GIP/DT mice in vivo. The glucose was administered by intraperitoneal injection rather than intragastric gavage in order to bypass release of endogenous gut incretin hormones and to eliminate potential artifacts that
could arise from alterations in gastric emptying and/or gut motility. As shown in figures 1A and E, GLP-1 efficiently reduced hyperglycemia (as judged by glucose AUC 120 minutes after glucose administration) as expected in response to glucose in both wild type (2.5-fold; \( p < 0.001 \)) and GIP/DT mice (2.0-fold; \( p = 0.0002 \)). In contrast, GIP reduced hyperglycemia in the wild type animals (1.7-fold; \( p = 0.029 \)) but had little effect on blood glucose levels in the GIP/DT mice (Figs 1B and F).

Xenin-25 restores the GIP-mediated reduction in hyperglycemia in GIP/DT mice. Because a sub-set of K cells have been reported to produce xenin-25 as well as GIP (19), IPGTTs were performed to examine the effects of xenin-25 on the incretin response. As shown in figures 1C and G, xenin-25 alone had no effect on blood glucose levels relative to mice receiving vehicle alone. In contrast, co-administration of GIP plus xenin-25 reduced hyperglycemia 2.7-fold (\( p = 0.002 \)) in the GIP/DT mice (Figs 1D and H). This effect is comparable to or even greater than, that observed in wild type animals receiving either GIP alone or GIP plus xenin-25.

Xenin-25 potentiates GIP-mediated insulin release in vivo. Next, in vivo insulin release assays, with and without intraperitoneal injections of the indicated peptide(s), were performed on male GIP/DT mice (Fig 2A) to determine whether the effects of xenin-25 plus GIP on hyperglycemia were mediated by insulin. Injection of glucose alone or together with xenin-25 did not cause statistically significant changes in plasma insulin levels in either wild type or GIP/DT mice. Consistent with its known incretin action, GIP caused a 5-fold (\( p < 0.005 \)) increase in plasma insulin levels in wild type mice. When wild type mice were treated with GIP plus xenin-25, insulin levels did not increase significantly compared to animals receiving GIP alone (\( p = 0.45 \)). Compared to the 5-fold increase in wild type mice, GIP induced a blunted insulin secretory response in the GIP/DT mice (3-fold increase versus fasting mice, \( p < 0.005 \)). Xenin-25 plus GIP augmented insulin levels 5.5-fold in GIP/DT mice compared to animals receiving BSA alone (\( p < 0.005 \)). The difference in plasma insulin levels in the GIP/DT mice receiving GIP versus GIP plus xenin-25 was also statistically significant (\( p = 0.04 \)). The insulin response to GIP plus xenin-25 in GIP/DT mice (5.5-fold) was similar (\( p = 0.3 \)) to that in wild type mice receiving GIP alone (5-fold). Thus the GIP-mediated increase in insulin release can be enhanced 1.8-fold by co-administration of xenin-25. Xenin-25 also potentiated GIP-mediated insulin release in female GIP/DT mice (Fig 2B).

Xenin-25 does not potentiate the incretin action of GLP-1 in vivo. Xenin-25 did not potentiate the GLP-1-mediated reduction in hyperglycemia in response to glucose when a sub-maximal dose of GLP-1 (0.1 nmole) was administered to either wild type or GIP/DT mice (Figs 3A-C). Additionally, xenin-25 failed to potentiate insulin release in wild type or GIP/DT mice injected with glucose plus GLP-1 (Fig 3D). GIP/DT and wild type mice secrete similar amounts of GLP-1 fifteen minutes after administration of oral glucose whereas GIP/DT mouse produce and release almost no GIP (16). However, xenin-25 alone did not reduce hyperglycemia in GIP/DT mice given oral glucose (Figs 3E and F). Taken together, these observations indicate that xenin-25 does not potentiate the incretin response to GLP-1. Moreover, since xenin-25, but not GIP, increases gut motility (22,24,28,44-46) and xenin-25 had no effect on hyperglycemia in GIP/DT mice during an OGTT (Figs 3D, E), the absence of an insulin secretory response 15 minutes after administration of oral glucose to GIP/DT mice cannot be explained by reduced GIP and/or xenin-25 release leading to decreased gut motility and diminished GLP-1 release.

Xenin-25 increases the GIP-mediated insulin response in NONcNZO10/LtJ mice. The action of GIP, but not GLP-1, is blunted in humans with T2DM (11-13). We determined whether xenin-25 can potentiate GIP action in NONcNZO10/LtJ mice (29,30), a polygenic, spontaneous, progressive model of human T2DM. NONcNZO10/LtJ mice were first subjected to IPGTTs with and without the indicated peptide(s). In 5-week old mice (Figs 4A, C), GIP alone or xenin-25 alone failed to significantly reduce hyperglycemia in response to injection of glucose (\( p = 0.14 \) and \( p = 0.53 \), respectively). In contrast, GIP plus xenin-25 caused a 1.6-fold reduction in the area under the curve compared to mice treated with BSA (\( p < 0.05 \)). When the IPGTTs were repeated in 16- and 18-week old mice (Figs 4B,
C), fasting blood glucose levels had increased from 80 mg/dL to 120 mg/dL ($p = 2.5 \times 10^{-6}$ and $1.3 \times 10^{-7}$ for 16- and 18-week old mice, respectively) and the area under the curve in the absence of peptide increased more than 2-fold ($p < 10^{-3}$ and $< 10^{-2}$ for 16- and 18-week old mice, respectively versus 5-week old mice). In spite of this, GIP plus xenin-25 reduced hyperglycemia in response to the glucose injection 2.3- and 2.1-fold in 16-week ($p = 10^{-3}$) and 18-week ($p < 10^{-2}$) old mice, respectively, compared to BSA. GIP alone reduced hyperglycemia 1.5-fold ($p = 0.04$) in the 16-week old mice and 1.37-fold in the 18-week old animals ($p = 0.19$).

Next, in vivo insulin release assays were performed with the NONcNZO10/LtJ mice. As shown in figure 5, fasting plasma insulin levels increased 1.7-fold ($p < 10^{-5}$) in 18-week (0.65+/-.04 ng/mL; n=29) versus 8-week old (0.37+/-0.03 ng/mL; n=15) mice. Fifteen minutes after intraperitoneal injections of glucose alone, plasma insulin levels increased 1.4-fold in the younger animals ($p = 0.03$) but remained unchanged in the older mice. When GIP was administered alone with the glucose, plasma insulin levels increased 1.9-fold in the younger mice and 1.6-fold in the 18-week old animals. However, neither increase reached statistical significance. In contrast, administration of GIP plus xenin-25 increased insulin levels 2.8-fold in the 8-week old mice ($p < 0.001$). This increase was statistically significant compared to the increases measured in mice injected with glucose plus BSA ($p < 0.005$) or glucose plus GIP ($p = 0.04$) alone. In the older mice, GIP plus xenin-25 still elicited a 2.5-fold ($p = 0.03$) increase in insulin release compared to mice receiving BSA alone. Thus, following the intraperitoneal administration of glucose, xenin-25 potentiated the GIP-mediated increase in plasma insulin levels resulting in a concomitant reduction in the level of hyperglycemia.

**Intraperitoneal injection of xenin-25 results in pharmacologic levels of circulating peptide.** To determine if the effects of exogenously administered xenin-25 represent physiologic or pharmacologic responses, plasma immunoreactive xenin levels were determined in wild type and GIP/DT mice under a variety of different nutritional conditions. Since commercially available assays were not available when these studies were initiated, an ELISA specific for xenin was developed (See Materials and Methods). The C-terminal 6 amino acids of xenin-25 are highly homologous to those of neurotensin and other neurotensin-related peptides (47). Moreover, antibodies to xenopsin, a highly related amphibian octapeptide, cross-react with antral G-cells and may recognize the N-terminus of human gastrin-17 (48). Thus, a sandwich ELISA was constructed that required multiple regions of xenin-25 for immunoreactivity. This ELISA detects full length xenin-25. N-terminally truncated xenin(6-25), xenin(11-25) and xenin(16-25) react with 100%, 30%, and <10% efficiency respectively. Thus, between 16 and 20 C-terminal amino acids of xenin are required for full immunoreactivity. After our ELISA was developed, a commercial RIA for xenin also became available.

In initial studies, wild type mice were injected with 1 nmole of xenin-25, human gastrin-17, or neurotensin. Blood was collected and plasma prepared 5 minutes later. Note that this is the same dose of xenin-25 that increases GIP action (e.g. figs 1, 2, 4 and 5). As shown in figure 6, immunoreactive xenin levels were $>2435$ pg/mL and 2160+/- 715 pg/mL when assayed using the RIA and ELISA, respectively. Following injection of neurotensin or gastrin, the RIA detected 203+/-97 and <59.1 pg/mL of immunoreactive xenin equivalents, respectively. Neither neurotensin nor human gastrin-17 circulating in the blood was detected by our ELISA (lower limit of detection was 29.3 pg of xenin-25 equivalents per mL).

Feurle et al previously reported that plasma immunoreactive xenin levels in humans are ~20 pM (60 pg/mL) under fasting conditions and increase to ~120 pM (360 pg/mL) with ingestion of nutrients or sham feeding (21). Since xenin-25 was reported to be expressed by K cells (19), we next compared the circulating levels of GIP versus xenin-25 in plasma from wild type versus GIP/DT mice. As shown in Figure 7A, GIP levels are extremely low in wild type mice following an overnight fast (68+/-12 pg/mL) and increase profoundly following oral administration of glucose (1036+/-89 pg/mL). As expected, GIP levels are extremely low in GIP/DT mice that were fasted (13+/-4 pg/mL) or administered oral glucose (35+/-14 pg/mL). Using the RIA (Fig 7B), immunoreactive xenin levels were below the
limit of detection (40 pg/mL) in both wild type and GIP/DT mice even following administration of oral glucose. A similar experiment was conducted using wild type mice except blood was collected from the portal vein and assayed for immunoreactive xenin using the custom prepared ELISA. As shown in Figure 7C, plasma immunoreactive xenin was still undetectable (lower limit of detection = 4.5 pg/mL) following administration of oral glucose. Thus, the amount of xenin-25 that potentiates GIP action in GIP/DT mice represents a pharmacologic dose.

_Xenin-25 does not act directly on the islet β-cell._ Experiments were conducted to determine how xenin-25 stimulates insulin secretion from β-cells. First, insulin release assays were conducted with isolated islets. GIP, GLP-1, and xenin-25 had no effect on insulin release in islets incubated in non-stimulating (2.5 mM) concentrations of glucose. In the presence of sub-maximal but stimulating (7.5 mM) concentrations of glucose, insulin release was potentiated by both GLP-1 and GIP (Fig 8A). In contrast, xenin-25 failed to potentiate insulin release from islets either alone (Fig 8A and B) or in combination with GIP (Fig 8B). MIN6 cells are glucose-responsive, insulin-producing cells (32). As with the primary mouse islets, glucose-stimulated insulin release was amplified by GLP-1 or GIP, but not by xenin-8 (Not Shown) or xenin-25 (Fig 8C). Xenin-25 also failed to potentiate GIP-mediated insulin release (Fig 8D).

In situ pancreas perfusions were next conducted in GIP/DT mice. Preliminary studies determined that perfusion with 0.3 nM GIP resulted in a sub-maximal GIP-mediated insulin secretory response in the presence of 10 mM glucose. These doses of GIP and glucose were therefore used in subsequent perfusions to ensure that any further amplification of GIP-mediated insulin release would be detectable. Perfusion with GIP alone caused an increase in insulin release which returned to baseline levels when the peptide was removed (Fig 9A). The increase in insulin secretion was similar with GIP plus xenin-25 and GIP alone (Figs 9A and B). Thus, xenin-25 did not potentiate GIP-mediated insulin release. Xenin-25 alone had no effect on insulin release (Figs 9A and B). The large insulin secretory response to KCl at the end of the perfusion confirmed that islet function was retained throughout the procedure.

GIP, GLP-1, VIP, and PACAP (1-27 or 1-38) each can activate Erk1/2 signaling and potentiate glucose-stimulated insulin release in β-cells (49-53). Therefore, MIN6 cells were treated with the indicated peptide and the degree of Erk1/2 phosphorylation was determined by Western blots. GLP-1 and GIP, but not xenin-25, caused profound increases in Erk1/2 phosphorylation but had no effect on total Erk1/2 levels (Fig 10A). In contrast, xenin-25, which can bind and activate neurotensin receptors (54), did increase phosphorylation of Erk1/2 in Panc1 cells, a human exocrine pancreatic adenocarcinoma cell line known to be activated by neurotensin (Fig 10B). In the Panc1 cells, near maximal stimulation was observed with 1 nM xenin-25. Xenin-25 had no effect on total Erk1/2 expression. Thus, MIN6 cells, islets, and the perfused pancreas were treated with doses of xenin-25 that are sufficient to activate neurotensin/xenin signaling in Panc1 cells. These _in vitro_ experiments suggest that xenin-25 does not increase insulin release by directly stimulating the islet β-cell.

_Cholinergic neurons relay the xenin-25 signal to β-cells in vivo._ Activation of parasympathetic neurons that innervate the pancreas can increase glucose-mediated insulin release _in vivo_ (55-57). Thus, _in vivo_ insulin release assays were conducted to determine if the stimulating effect of xenin-25 on GIP-mediated insulin secretion was relayed by cholinergic neurotransmitters. Glucose in combination with GIP plus xenin-25 was administered to all mice. Some animals also received atropine sulfate or saline 15 minutes before the glucose. Atropine sulfate, a competitive antagonist of muscarinic acetylcholine receptors, produced a 1.8-fold (p = 0.04) decrease in plasma insulin levels in GIP/DT mice (Fig 11A). The magnitude of this reduction is similar to the 1.8-fold increase in plasma insulin levels in GIP/DT mice receiving GIP plus xenin-25 versus GIP alone (Fig 2). Atropine sulfate had no statistically significant effect on insulin release from wild type mice injected with GIP plus xenin-25 (Fig 11A). Likewise, xenin-25 had little effect on plasma insulin levels in wild type mice that received GIP plus xenin-25 versus GIP alone (Fig 2). Atropine sulfate had no effect on plasma insulin levels in either wild type or GIP/DT mice.
when GIP was administered without xenin-25 (Fig. 11B). Thus, atropine sulfate inhibits the contribution of xenin-25 to the combined effects of GIP plus xenin-25 in increasing plasma insulin. In contrast to atropine sulfate, atropine methyl bromide (AMB) does not cross the blood brain barrier and thus, only inhibits muscarinic receptors outside the central nervous system. AMB also inhibited insulin release 1.8-fold in the GIP/DT mice receiving glucose, GIP, and xenin-25 (Fig 11C). Thus, the muscarinic receptors that mediate the effects of xenin-25 are not located within the central nervous system.

Parasympathetic neurons do not mediate the effect of xenin-25 on insulin release. Subsets of parasympathetic neurons that innervate the islets also release VIP and PACAP (55-57). N-terminally truncated forms of PACAP [PACAP(6-38) or PACAP(6-27)] are competitive inhibitors of both VIP receptors (VPAC1 and VPAC2) as well as the PACAP-specific receptor Pac1 (58,59). Unlike atropine, PACAP(6-38) did not inhibit xenin-25 potentiation of the GIP-mediated increase in plasma insulin levels (Fig 11D). Signaling via parasympathetic neurons involves activation of post-ganglionic neurons, a pathway that would be blocked by hexamethonium, a nicotinic cholinergic antagonist. As shown in figure 11E, hexamethonium did not inhibit xenin-25 potentiation of GIP-mediated insulin release in wild type or GIP/DT mice.

If the xenin-25 effects on insulin release are mediated by a neuronal pathway involving the central nervous system (CNS), increased afferent and efferent signaling should be detected. Gut hormone-mediated afferent signaling to the CNS is routinely determined by noting increased c-fos expression in the nucleus of the solitary tract [NTS; (60,61)]. With regard to efferent signaling, cells within the hypothalamic paraventricular nucleus (PVN) provide a major descending pathway to the dorsal motor vagal nucleus (DMV) which is the site of origin for parasympathetic innervation of the endocrine pancreas (62,63). Activation of this efferent pathway can be detected by increased c-fos expression in the PVN and DMV (63). To determine the extent to which CNS activation is required for the effects of GIP plus xenin-25 on insulin release, GIP/DT mice were fasted overnight and then injected with glucose plus GIP or glucose plus GIP plus xenin-25. Negative control animals were injected with saline alone. Mice were sacrificed 60 minutes later and brains processed for c-fos immunohistochemistry (see Material and Methods). To serve as a positive control, a fourth group of mice was injected with insulin to induce hypoglycemia which, as expected, increased the number of c-fos positive cells in the NTS, PVN, and DVM (Fig 12). The numbers of c-fos positive cells in the NTS, PVN, and DVM were not increased in GIP/DT mice injected with GIP plus xenin-25 compared to the saline or GIP-treated mice. GIP plus xenin-25 did however, increase the number of c-fos positive cells in the arcuate nucleus (ARC) of GIP/DT mice.

Carbachol potentiates GIP-mediated insulin release in the perfused pancreas. The preceding results strongly suggest that activation of non-parasympathetic, cholinergic neurons that innervate the islets can potentiate GIP-mediated insulin release in GIP/DT mice. To determine if muscarinic receptors present on the β-cell can potentiate GIP-mediated insulin release, pancreas perfusion experiments were repeated using carbachol instead of xenin-25. Preliminary experiments established that 40 nM carbachol elicited a sub-maximal insulin secretory response in the presence of 10 mM glucose. As shown in figures 13A and B, perfusion with a low dose of either GIP alone or carbachol alone caused only a small increase in insulin release. In contrast, when the same low concentrations of both GIP and carbachol were co-perfused, there was a synergistic increase in insulin release. Thus, carbachol, but not xenin-25, can potentiate GIP-mediated insulin release in vitro in GIP/DT mice.

DISCUSSION

These studies have demonstrated for the first time that xenin-25, a peptide reportedly produced by intestinal K cells, potentiates the insulin secretory response to GIP in vivo. This effect was observed in two mouse models in which the response to GIP is blunted. The first model is a transgenic mouse in which intestinal K cells have been eliminated by diphtheria toxin whose expression is limited to GIP-producing cells. The second is the NONcNZO10/LtJ mouse- a polygenic model of type 2 diabetes.
GIP/DT mice, xenin-25 plus GIP significantly lowered the 2 hour glucose area under the curve and increased the 5 minute insulin response compared to mice receiving GIP alone or xenin-25 alone (Figs 1 and 2). These results are consistent with data showing that the insulin response during the first 10-minutes following meal ingestion is a very important determinant of the subsequent level of glycemia (64).

Using a commercial RIA and a custom prepared ELISA, we have been unable to detect physiologic levels of immunoreactive xenin in mouse plasma under conditions that profoundly increased GIP release. In contrast, exogenously delivered xenin-25 was readily detectable in mouse plasma. These observations indicate that the effects of xenin-25 on GIP-mediated insulin release in our mouse experiments occur at a dose that is pharmacologic. However, it should also be noted that xenin-25 probably amplifies GIP action by stimulating enteric neurons that directly connect the intestine and pancreas (see below). Since enteric nerve terminals come in close contact with enteroendocrine cells in the intestinal epithelium, it is possible that these terminals could be exposed to high concentrations of endogenously released xenin-25 in the interstitial fluid even though we cannot detect circulating xenin-25 in the plasma. Moreover, even though we cannot detect endogenously released xenin-25 in mice, our results raise the possibility that pharmacologic doses of xenin-25 could be used to increase GIP-mediated insulin release and reduce hyperglycemia in humans with T2DM.

The observation that GIP action is blunted in the 2 mouse models studied is particularly interesting in light of the fact that insulin responses to GIP are blunted in humans with T2DM. The underlying cause for this abnormality is not known although the response to GIP can be enhanced by improving glucose control (65). In vitro experiments showed that xenin-25 does not directly stimulate or potentiate insulin secretion from mouse islets, insulin-producing cell lines, or the in situ perfused pancreas. In vivo studies demonstrated that atropine had no effect on GIP-mediated insulin release but completely blocked the ability of xenin-25 to potentiate the GIP-mediated increase in plasma insulin levels in response to an intraperitoneal injection of glucose. Thus, muscarinic receptors are involved in relaying the xenin-25 signal to the β-cell.

Pancreatic islet β-cells are known to express muscarinic acetylcholine receptors on their cell surface (66). Studies using genetically modified mice and/or islets from such animals indicate that M3 muscarinic receptors mediate acetylcholine potentiation of glucose-stimulated insulin release (67-72). Acetylcholine is not systemically active since it is rapidly hydrolyzed by acetylcholinesterase and plasma butyrylcholinesterase (73). Since atropine methyl bromide, which does not cross the blood brain barrier, also prevented xenin-25 potentiation of GIP-mediated insulin release, acetylcholine is most likely acting directly on muscarinic receptors that reside on the β-cell. Therefore, acetylcholine locally released from cholinergic neurons that innervate the islets probably potentiates GIP-mediated insulin release in vivo. This is further supported by the observation that carbachol, but not xenin-25 itself, potentiates GIP-mediated insulin release in the perfused pancreas of GIP/DT mice (Fig 13). These findings are intriguing since the cephalic insulin response is known to be partially regulated by a cholinergic mechanism (64). Although unlikely, we cannot exclude the possibility that the acetylcholine acts on another cell population which in turn, increases insulin release.

These in vivo results also suggest that the effect of xenin-25 on GIP-mediated insulin is part of an enteric-neuronal-pancreatic pathway since 1) mice lacking intestinal K cells exhibit an impaired insulin secretory response to GIP, 2) this defect can be overcome by administration of GIP plus xenin-25, and 3) the effects of xenin-25 can be completely prevented by atropine sulfate and atropine methyl bromide. To further explore other aspects of this enteric-neuronal-pancreatic pathway, PACAP(6-38), which inhibits VIP and PACAP receptors, and hexamethonium, a post-ganglionic blocker, were administered but failed to inhibit the xenin-25 potentiation of GIP-mediated insulin release. These results suggest that the effects of xenin-25 are independent of VIP/PACAP neurotransmitters and not mediated by post-ganglionic neurons of the parasympathetic nervous system. Consistent with these findings, xenin-25 failed to increase the number of c-fos positive cells in the hindbrain (NTS and DMV)
and hypothalamus (PVN) indicating that central nervous system activation is also not required for xenin-25 to enhance GIP-mediated insulin release.

Xenin-25 has been shown to inhibit food intake (25-27,74) and this effect is associated with increased c-fos expression in the hypothalamus (26). However, in this previous report, the dose of xenin-25 was 400-500 times higher than that used to potentiate GIP-mediated insulin release in our experiments. Consistent with this other report, the low dose of xenin-25 that was administered along with GIP did increase c-fos expression in the arcuate nucleus (Fig 13) which indicates activation of hypothalamic regions important for regulating feeding behavior (26). However, amplification of insulin release by the relatively low dose of xenin-25 did not require activation of key nuclei within the central nervous system (i.e. NTS, PVN, and DMV) that are functionally linked to parasympathetic/cholinergic mediated insulin release. In another study using doses of xenin-25 slightly lower than those used in our experiments (46), xenin-25 was shown to stimulate gall bladder contractions and this response was inhibited by atropine, but not vagotomy. Taken together, these studies indicate that low doses of xenin-25 exert metabolic effects independent of central nervous system activation.

Kirchgessner and Gershon (75-77) have described an extensive network of enteric neurons that directly connects the pancreas and the stomach/duodenum. These interneurons function independently from the central nervous system and are capable of modifying pancreatic endocrine function. Our in vivo results are consistent with the possibility that a subset of non-ganglionated enteric neurons regulates the effects of xenin on GIP-mediated insulin release since 1) inhibition of muscarinic acetylcholine receptors with atropine or atropine methyl bromide inhibited the effects of xenin-25 on insulin release, 2) ganglionic blockade with hexamethonium did not prevent the effects of xenin-25 on insulin release in vivo, 3) denervation of the pancreas for in situ perfusion experiments abrogated the effects of xenin-25, and 4) the dose of xenin that stimulates insulin release in vivo did so independent of PVN, NTS, and DMV activation. Intriguingly, Feurle et al reported that atropine, but not hexamethonium, inhibits contraction of guinea pig jejunum in vitro suggesting the same neuronal sub-type regulates jejunal motility and xenin-25 mediated insulin release in GIP/DT mice (24). Studies are underway to identify the particular enteric neurons that mediate the effects of xenin on insulin release.

Silvestre et al (23) previously reported that xenin-8 increased both basal and glucose-stimulated insulin release in the perfused rat pancreas. In contrast, intravenous infusion of xenin-25 in dogs resulted in only a 25% increase in plasma insulin levels and had no effect on blood glucose levels (22). These later results are consistent with our data demonstrating that xenin-25 alone does not increase insulin release or reduce hyperglycemia in mice and does not appreciably increase insulin release from MIN6 cells, isolated islets, and the perfused mouse pancreas. Since xenin-25 activated Erk1/2 signaling in an exocrine pancreas cell line, but not MIN6 insulin-producing cells, the xenin-25 used in these studies is capable of binding and activating appropriate receptors. Therefore, xenin-25 appears to indirectly stimulate insulin secretion from pancreatic islet β-cells but directly acts on an exocrine pancreas cell line. This is consistent with data indicating that atropine does not inhibit xenin-25 stimulation of secretion from the exocrine pancreas (78). Thus, the discrepancy between our results and those of Silvestre et al could be due to the fact that we used intact xenin-25 whereas this other group infused an 8 amino acid, C-terminal fragment of xenin. Physiological studies have demonstrated that full length and truncated forms of xenin exhibit unique properties. For example, a greater than 10-fold higher concentration of xenin-8 versus xenin-25 is required for stimulating contraction of the jejunum. Alternatively, species-specific differences could account for the ability of rat, but not mouse or canine β-cells to respond to xenin-25 alone.

Like the GIP/DT mice, humans with T2DM are resistant to the action of exogenously administered GIP but retain the ability to respond to GLP-1. Since GIP production and release are normal in persons with T2DM, our observations raise the possibility that xenin-25 could overcome the blunted response to GIP observed in T2DM. Intriguingly, diabetes is a major cause of autonomic neuropathy (79,80) which in turn, could also contribute to the blunted GIP action observed in persons with T2DM. Since xenin-25 appears to
potentiate GIP action by indirectly activating M3 muscarinic receptors on the β-cell, drugs that directly activate muscarinic receptor signaling in β-cells could potentially restore GIP action in persons with diabetes and autonomic neuropathy. Interestingly, anti-psychotic medications associated with development of T2DM exhibit secondary affinity/antagonism to muscarinic M3 receptors (81). Our results by defining a novel effect of xenin-25 to potentiate insulin secretion in response to GIP have raised the importance of defining the pattern of xenin-25 release in healthy humans and in T2DM and the role of muscarinic receptors in the regulation of normal insulin secretion and its alterations in T2DM.

FOOTNOTES

BMW and KSP designed, analyzed and interpreted experiments and wrote the manuscript. DLC and LHD developed the ELISA for immunoreactive xenin. KAD and SJF conducted c-fos immunohistochemistry. The other authors conducted experimental manipulations. Portions of this research were supported by funds from a Career Development Award from the American Diabetes Association (BMW); a pilot and feasibility grant from the Washington University Clinical Nutrition Research Center (P30 DK056341) (BMW); the Washington University Digestive Diseases Research Core Center Morphology and Transgenic Mouse Cores (5P30 DK052574); the Washington University Diabetes Research and Training Center Radioimmunoassay, Transgenic and Mouse Phenotyping Cores (Grant number P60 DK020579); the Washington University Clinical and Translational Science Award (UL1 RR024992); NIH grant number DK31842 (KSP) and the Blum Kovler Foundation. Washington University is pursuing a patent related to the use of xenin-25 to treat T2DM. In the future, this could lead to personal financial benefit to BMW, KSP, and the University.

The abbreviations used are: AMB, atropine methyl bromide; ARC, arcuate nucleus; BSA, bovine serum albumin; DMV, dorsal motor vagal nucleus; DPP IV, dipeptidyl peptidase IV; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; IPGTT, intraperitoneal glucose tolerance test; KCl, potassium chloride; LOD, Limit of Detection; NTS, nucleus of the solitary tract; OGTT, oral glucose tolerance test; PACAP, pituitary adenylate cyclase-activating polypeptide; PVN, paraventricular nucleus; T2DM, type 2 diabetes mellitus; VIP, vasoactive intestinal peptide.
FIGURE LEGENDS

Figure 1. Xenin-25 potentiates the GIP-mediated reduction in hyperglycemia. Wild type (WT) or GIP/DT (DT) mice were fasted for 16-hours and then given intraperitoneal injections of glucose plus GLP-1 (Panel A), GIP alone (Panel B), xenin-25 alone (Xen; Panel C), or GIP plus xenin-25 (GIP+Xen; Panel D). Blood glucose levels were measured before and at the indicated times after glucose injection. Incremental areas under the curve (Panels E-H) were calculated from data presented in Panels A-D, respectively. All \( p \) values compare mice of the same genotype receiving peptide versus BSA without peptide. * and ** represent \( p \) values < 0.03 and \( \leq 0.002 \), respectively.

Figure 2. Xenin-25 potentiates GIP-mediated insulin release in vivo. Male (Panel A) and female (Panel B) mice were treated with BSA, GIP alone (GIP), xenin-25 alone (Xen) or GIP plus xenin-25 (G+X) as described in figure 1. Mice receiving saline plus BSA served as fasting controls. Plasma insulin was measured 5 minutes after the intraperitoneal injection of glucose or saline. \( p \) values compare mice with the same genotype receiving glucose plus the indicated peptide(s) versus animals receiving neither glucose nor peptides. * and ** represent \( p \) values < 0.05 and 0.005, respectively. The bars indicate \( p \) values for mice of the same genotype treated with GIP alone versus GIP plus xenin-25. NS indicates a non significant \( p \) value.

Figure 3. Xenin-25 does not amplify GLP-1 action in mice. Panels A and B: The reduction in hyperglycemia was measured in wild type (WT) or GIP/DT (DT) mice as described in figure 1 except animals received vehicle alone (BSA) or a sub-maximal dose of GLP-1 (0.1 nmole per mouse) with or without 1 nmole of xenin-25 as indicated. Incremental areas under the curve for panels A and B are shown in panel C. Panel D. Plasma insulin levels were measured in mice as described in figure 2 except animals were injected with either GLP-1 alone or GLP-1 plus xenin-25. Panel E. Wild type (WT) or GIP/DT (DT) mice were treated as described in figure 1 except glucose (3g/kg) was administered at time zero by intragastric gavage rather than intraperitoneal injection. The incremental glucose areas under the curve are shown in Panel F.

Figure 4. Xenin-25 potentiates the GIP-mediated reduction in hyperglycemia in NONcNZO10/LtJ mice. Animals of the indicated age were treated as described in figure 1. Areas under the curve for panels A and B, as well as for 18-week old mice, are shown in panel C. * and ** represent \( p \) values < 0.05 and < 0.005, respectively versus mice receiving BSA alone.

Figure 5. Xenin-25 potentiates GIP-mediated insulin release in NONcNZO10/LtJ mice. Mice of the indicated age were fasted overnight and plasma insulin was measured before (0) and 15 minutes after intraperitoneal injections of glucose plus the indicated peptide(s). Control mice were injected with glucose plus vehicle alone (BSA). * and # represent \( p \) values <0.05 and <0.03, respectively versus mice receiving BSA alone.

Figure 6. Immunoreactive (IR) xenin is detectable in mouse plasma following intraperitoneal injection of xenin-25. Wild type mice were injected with 1 nmole of human gastrin-17 (Gastrin), neurotensin (NT), or xenin-25. Five minutes later, blood was collected and plasma prepared. IR-xenin was assayed using a commercially available RIA (panel A) or a custom synthesized ELISA (panel B). The dotted lines represent the lower limit of detection for both assays and the maximal level detectable by the RIA.

Figure 7. IR-xenin-25 is not detectable following administration of oral glucose to wild type (WT) or GIP (DT) mice. Mice were treated as described in figures 1 and 2. Blood was collected following a 16-hour fast (Fasting) or 15 minutes after glucose administration (3 g/kg) by intragastric gavage (Glucose). Plasma was prepared and assayed for immunoreactive GIP or xenin using a commercially available ELISA or RIA, respectively. In panels A and B, blood was collected from the retroorbital sinus of female mice. In panel C, blood was collected directly from the portal vein of WT mice (4 male and 7 female mice).
fasting mice and 6 male and 2 female mice glucose-treated mice) and assayed for IR-xenin using the

custom prepared ELISA.

**Figure 8. Xenin-25 does not directly stimulate insulin release from the beta cell.** Insulin release from

islets isolated from wild type C57BL/6J mice (Panel A; n=6), GIP/DT mice (Panel B; n=6) or MIN6 cells

(Panel C and D; n=4) was determined in static incubations. 100 nM GIP, GLP-1, or xenin-25 (Xen) was

added to the indicated samples in Panels A and C. In panels B and D, islets (10 mM glucose) or MIN6

cells (7.5 mM glucose) were incubated with the indicated concentration of GIP in the presence or absence

of 10 nM xenin-25. *Represents p values <0.02 for groups receiving the indicated peptide versus no

peptides (None) at the same concentration of glucose (panels A and C only).

**Figure 9. Xenin-25 does not potentiate GIP-mediated insulin release from the in situ perfused pancreas.** The effects of xenin-25 (Xen or X) on GIP (GIP or G) mediated insulin release were studied using in situ pancreas perfusions in GIP/DT mice (n=4 perfusions). Glucose was maintained at 10 mM for the duration of the perfusion. Peptides were perfused for 15 minutes followed by a 15 minute washout period. Preliminary perfusions established that 0.3 nM GIP elicited sub-maximal insulin secretory responses in the presence of 10 mM glucose which ensured that a xenin-25 mediated increase in insulin release could be detected. The dose of xenin-25 used for the perfusion (3 nM) caused profound activation of xenin-25 signaling in Panc1 cells (see Fig 10). The amount of insulin release, normalized to that measured in the presence of GIP alone, is shown for each condition in Panel B. *Represents a p value <0.03 compared to perfusion with GIP alone.

**Figure 10. Incretins, but not xenin-25, increase MAPK signaling in MIN6 Cells.** Panel A, MIN6 cells were treated with the indicated peptide and subjected to Western blot analysis using antibodies specific for activated (P-Erk) or Total Erk1/2 (Total Erk). Panel B. Panc-1 exocrine pancreatic cells, which are known to express neurtrotensin receptors, were treated with the indicated concentration of xenin-25 as described in Panel A. Western blots were probed with antibodies for P-Erk or total Erk.

**Figure 11. Xenin-25 potentiation of GIP action is relayed by non-ganglionic, cholinergic neurons.** Wild type (WT) or GIP/DT (DT) mice were treated as described in figure 2. As indicated, atropine sulfate (Panels A and B; Atrop), AMB (Panel C), PACAP(6-38) [Panel D; P(6-38)], or hexamethonium chloride (panel E) was administered to some animals along with the indicated peptide(s). Control mice received vehicle instead of drug. Blood was collected for insulin assays 5 minutes after injection of glucose. * and ** represent p values < 0.05 and < 0.005, respectively. Neither atropine compound exerted a statistically significant effect on WT mice.

**Figure 12. Afferent/Efferent signaling does not regulate the effects of xenin-25 on GIP action.** Following an overnight fast, mice (n=4 per treatment group) were given an intraperitoneal injection of saline alone (“Saline”), glucose plus GIP (“GIP”), or glucose plus GIP plus xenin-25 (GIP+Xen or G+X). One hour later, brains were harvested for c-fos immunohistochemistry. Representative images of c-fos staining in the DMV and NTS, PVN or ARC are shown. Brains from insulin injected hypoglycemic mice (“Insulin” or Ins) served as a positive control for induction of c-fos levels in the NTS, PVN and ARC. Panels A-D. The number of c-fos positive cells was quantitated by morphometry. *One-way analysis of variance indicates that variation among column means is significant (p < 0.01) and the Tukey-Kramer Multiple Comparisons Test indicates that the differences in c-fos measured in the presence of insulin compared to those obtained with saline, GIP plus glucose, or GIP plus glucose plus xenin in the PVN or insulin and GIP plus glucose plus xenin compared to those obtained with saline or GIP plus glucose in the ARC are statistically significant (p < 0.05).

**Figure 13. Carbachol potentiates GIP-mediated insulin release from the in situ perfused pancreas.** The effects of carbachol (Carb or C) on GIP (GIP or G) mediated insulin release were studied using in situ
pancreas perfusions in GIP/DT mice as described in figure 9 (n=4 perfusions). Preliminary perfusions established that 40 nM carbachol elicited sub-maximal insulin secretory responses in the presence of 10 mM glucose. The amount of insulin release, normalized to that measured in the presence of GIP alone, is shown for each condition in panel B. *One-way analysis of variance indicates that variation among column means is significant ($p = 0.001$) and the Tukey-Kramer Multiple Comparisons Test indicates that the differences in insulin release measured in the presence of GIP plus carbachol compared to those obtained with GIP alone, carbachol alone, or the sum of the values obtained with GIP alone and carbachol alone are statistically significant ($p < 0.05$).

REFERENCES

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Figure 1
Figure 2

A. Males

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B. Females

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**P = 0.04**

**P = 0.03**
Figure 3
Figure 4
Figure 5

A. 8-Week Old Mice (n=5)

B. 18-Week Old Mice (n=9)

Plasma Insulin (ng/mL) vs. Minutes After Intraperitoneal Glucose
Figure 6

Plasma Immunoreactive Xenin (pg/mL) by peptide injected:
- Gastrin (n=6)
- NT (n=8)
- Xenin-25 (n=7)

A. RIA
B. ELISA
Figure 7
Figure 8
Figure 9
A. MIN6 cells

100 nM Peptide

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B. Panc1 cells

nM Xenin    0  0.1  1.0  10  100

Figure 10
Figure 11
Figure 12
Figure 13

A. Insulin Released (ng/mL Perfusate)

B. Insulin Released (Fold-increase vs GIP)

18 mM Glucose
Xenin-25 potentiates GIP action via a novel cholinergic relay mechanism

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