Responses of python gastrointestinal regulatory peptides to feeding

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In the Burmese python (*Python molurus*), the rapid up-regulation of gastrointestinal (GI) function and morphology after feeding, and subsequent down-regulation on completing digestion, are expected to be mediated by GI hormones and neuropeptides. Hence, we examined postfeeding changes in plasma and tissue concentrations of 11 GI hormones and neuropeptides in the python. Circulating levels of cholecystokinin (CCK), glucose-dependent insulinotropic peptide (GIP), glucagon, and neurotensin increase by respective factors of 25-, 6-, 6-, and 3.3-fold within 24 h after feeding. In digesting pythons, the regulatory peptides neurotensin, somatostatin, motilin, and vasoactive intestinal peptide occur largely in the stomach, GIP and glucagon in the pancreas, and CCK and substance P in the small intestine. Tissue concentrations of CCK, GIP, and neurotensin decline with feeding. Tissue distributions and molecular forms (as determined by gel-permeation chromatography) of many python GI peptides are similar or identical to those of their mammalian counterparts. The postfeeding release of GI peptides from tissues, and their concurrent rise in plasma concentrations, suggests that they play a role in regulating python-digestive responses. These large postfeeding responses, and similarities of peptide structure with mammals, make pythons an attractive model for studying GI peptides.

hormones | neuropeptides | postprandial | *Python molurus*

The gastrointestinal (GI) tract synthesizes and releases peptides that regulate digestion. These peptides originate from endocrine cells and are released into circulation as hormones or intercellularly as paracrines, or originate from neurons and are released as neuropeptides. In recent decades, the tissue(s) of origin, tissue target(s), receptors, physiological roles, and synergistic relationships with other regulatory peptides have been discussed for each GI peptide, mostly on the basis of studies of humans and traditional laboratory mammalian models (mice, rats, rabbits, and dogs) (1–3).

But mammalian models suffer from a big potential drawback in studying digestive regulatory mechanisms; mammals are adapted to consuming small meals frequently and to digesting nearly constantly, hence their guts are rarely empty, and regulatory spans for digestive responses are modest (4). The resulting low signal-to-noise ratio of mammalian digestive responses makes it difficult to identify the underlying regulatory processes and may contribute to the controversies surrounding the roles of several GI peptides (2). Hence, we used the Burmese python (*Python molurus*) as an animal model for investigating digestive regulatory mechanisms (4–7). Because pythons are adapted to consuming large meals at infrequent intervals, their digestive regulatory responses exhibit much larger factorial changes and signal-to-noise ratios than do those of mammals. For example, pythons upon feeding experience up to a 2.2-fold increase in intestinal mucosal mass, 6-fold increase in intestinal microvillus length, and 40-fold increase in intestinal nutrient transport rates and metabolism. Corresponding factorial magnitudes of these same responses in mammals are typically less than two (4).

A role of GI peptides in mediating pythons' digestive responses is suggested by the following facts. (*i*) Python small intestine begins to respond (in function and morphology) to feeding within a few hours, before any of the ingested meal has reached the intestine (5). (*ii*) Python intestinal segments that have been surgically isolated from contact with intestinal nutrients but still retain their neurovascular supply continue to up-regulate nutrient transport after feeding (6). (*iii*) Eight python GI peptides have been identified and sequenced (8, 9). Hence, the python model offers an attractive alternative to mammals for resolving uncertainties about actions of GI hormones and neuropeptides.

In this article, we describe plasma concentrations of four peptides [cholecystokinin (CCK), glucose-dependent insulinotropic peptide (GIP), glucagon, and neurotensin] from fasted pythons and at 10 time points after feeding; tissue concentrations of 11 peptides [CCK, calcitonin gene-related peptide (CGRP), gastrin, GIP, glucagon, motilin, neurotensin, peptide YY (PYY), somatostatin, substance P, and vasoactive intestinal peptide (VIP)] from digesting pythons; postfeeding responses in tissue concentrations of three peptides (CCK, GIP, and neurotensin); and molecular characteristics of seven peptides in python tissues (CCK, GIP, glucagon, neurotensin, somatostatin, substance P, and VIP). We conclude by proposing four further studies on GI peptides in pythons.

Materials and Methods

Animal Maintenance and Plasma and Tissue Sampling. Pythons were purchased as hatchlings, housed individually, and fed laboratory rodents once every 2 weeks. Eight pythons (5.8 ± 0.4 kg, 3.5 yr old, four males and four females) were used to measure postfeeding changes in plasma concentrations of CCK, GIP, glucagon, and neurotensin. We obtained 5-ml blood samples (by cardiac puncture) from snakes postabsorptively (fasted for 1 month) (5) and again at 10 time points (0.25, 0.5, 0.75, 1, 3, 6, 9, 14, 30, and 45 days) after their consumption of rabbit and/or rat meals equivalent to 25.5 ± 0.5% of the snake's body mass. Blood samples were placed into chilled tubes containing aprotinin (400 kallikrein inhibitor unit/ml) and EDTA (2 mg/ml) and centrifuged at 4,000 rpm for 10 min at 4°C. The plasma was snap-frozen in liquid N_2 and stored at −80°C. Before RIA, peptide fractions were extracted from plasma samples on Sep-Pak C-18 cartridges (Waters) and eluted with 60% acetonitrile and 0.1% trifluroacetic acid. Eluates were lyophilized in a vacuum centrifuge, and the resulting pellet was dissolved in 2 ml of 60 mM phosphate buffer (pH 7.4), 10 mM EDTA, 7 mM sodium azide, and 44 μM bovine

Abbreviations: CCK, cholecystokinin; CGRP, calcitonin gene-related peptide; GI, gastrointestinal; GIP, glucose-dependent insulinotropic peptide; VIP, vasoactive intestinal peptide; NPY, neuropeptide Y; PYY, peptide YY.

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Table 1. Characteristics of the antisera used in RIAs

Peptide	Antibody	Regional specificity	Titer	Sensitivity, fmol/tube	Immunoreactivity specificity	Ref.
CCK	CCK-2	C terminal tyr-SO_4	1:28,000	0.2	CCKs with sulfated tyrosine, no gastrins	10
CCK	O2	C terminal	1:250,000	0.1	All forms of CCK and gastrin	10
CGRP	D11	N terminal	1:80,000	0.6	No cross reaction with IAPP	11
Gastrin	Gas179	C terminal	1:200,000	0.2	All forms of gastrin	12
GIP	Spec 19	C terminal	1:24,000	1.2	Highly specific	12
Glucagon	GL77	Mid to N terminal	1:4,000	0.4	Cross reacts with glicentin	12
Glucagon	mono	Mid to N terminal	1:70,000	0.3	Cross reacts with glicentin	12
Glucagon	RCS5	C terminal	1:200,000	0.2	Pancreatic glucagon specific	12
Insulin	M8309	Unknown	1:1,000,000	0.4	No cross reaction with IGF-I or IGF-II	13
Motilin	M1	N terminal	1:200,000	1.0	Recognizes porcine and human motilin	12
Motilin	RAS7160	C terminal	1:150,000	0.5	Recognizes rat, dog, and porcine motilin	12
Neurotensin	NT58	C terminal	1:100,000	0.2	No cross reaction with NT1–8	14
NPY	YN7	N terminal	1:120,000	1.5	No cross reaction with PYY or PP	15
Peptide YY	Y21	N terminal	1:80,000	0.4	No cross reaction with NPY or PP	16
Peptide YY	Y24	C terminal	1:100,000	0.5	No cross reaction with NPY or PP	16
PP	HPP3	Unknown	1:250,000	0.2	No cross reaction with PYY or NPY	12
Secretin	Sc10	Mid to C terminal	1:200,000	0.2	Highly specific	10
Somatostatin	K9	Ring structure	1:40,000	0.3	Cross reacts with 14 and 28 AA forms	12
Substance P	SPG1	N terminal	1:80,000	0.5	Highly specific	12
VIP	V9	C terminal	1:320,000	0.3	Highly specific	12

We assayed python plasma and tissues for GI peptides by means of antibodies (column 2) to mammalian peptides (column 1).

albumin for subsequent RIA. Recovery of measured peptides was 82–96%.

We used nine pythons to measure tissue concentrations of GI peptides. From three fasted pythons (869 ± 228 g, 2–3 yr old, two males and one female) we measured CCK, GIP, and neurotensin in the proximal and distal regions of the stomach and small intestine, pancreas, and large intestine. From three pythons (802 ± 116 g, 1–2 yr old, all females), killed 1 d after consumption of rat meals equivalent to 65.7 ± 0.7% of the snake's body mass, we measured all 11 peptides in the proximal and distal stomach, pancreas, large intestine, and proximal, middle, and distal regions of the small intestine. We did not detect insulin, neuropeptide Y (NPY), pancreatic polypeptide, or secretin in any of these tissues. From three pythons (837 ± 131 g, 1–2 yr old, one male and two females) killed 3 d after consuming rat meals equivalent to 63.8 ± 0.4% of snake body mass, we measured CCK, GIP, and neurotensin in the distal stomach, pancreas, and proximal intestine.

Tissue samples were snap-frozen in liquid N_2 immediately after removal from snakes and stored at −80°C until analysis. Frozen tissues were chopped and plunged into preheated polypropylene tubes containing water (10 ml/g of tissue wet mass) and left to sit for 15 min. An aliquot of the resulting neutral extract was acidified with glacial acetic acid. Neutral and acid extracts were centrifuged, and the supernatants were stored at −80°C for subsequent RIA. Gastrin and CCK were measured in both neutral and acid extracts, whereas all other peptides were measured only in acid extracts.

RIA. We measured peptides by established RIA methods that had been previously standardized and optimized. Table 1 presents for each peptide the antibodies used, regional specificity, titer, sensitivity, and immunoreactivity specificity. All labels were produced by conventional chloramine T oxidation methods, with the exception of CCK, NPY, and CGRP, which were labeled by the nonoxidative Bolton and Hunter method. Mono-iodinated peptides were purified by high-resolution reverse-phase HPLC. Specific activities were 68–80 Bq·fmol^{-1}. Antisera were added at a dilution binding about 50% of the 1–1.5 fmol of labeled peptide in the absence of nonlabeled peptide. Free and bound hormones were separated by forming the latter into a visible precipitate by adding polyethylene glycol and γ-globulin. After removal of the supernatant, samples were counted on a 10-well auto gamma counter (model 1277, Amersham Pharmacia).

Characterization of Molecular Forms by Gel-Permeation Chromatography. We separated selected tissue samples by gel-permeation chromatography to determine the degree of similarity between the molecular forms of python and mammalian peptides. Tissue extracts were prepared by Sep-Pak as described above, and 100-μl samples were subjected to HPLC. Gel-permeation chromatography was performed on a Superdex peptide column (Amersham Pharmacia) eluted with 30% acetonitrile/water and 0.1% trifluoroacetic acid at 0.5 ml·min^{-1}. Synthetic human and porcine peptides were chromatographed in identical fashion for comparison.

Results

Postfeeding Responses of Plasma Peptides. We found plasma levels of CCK, GIP, glucagon, and neurotensin to vary significantly (repeated measures ANOVA, F values = 16.1–47.2, P values < 0.0001) among the 11 prefeeding and postfeeding sampling times. Within 6 h after feeding (the first postfeeding sample), pythons experienced significant (*a priori* planned pairwise mean comparisons, $P < 0.002$) increases in plasma concentrations of CCK (assayed using CCK-2 antibody), GIP, and glucagon (RCS5 antibody) by factors of 5.8, 2.2, and 3.3, respectively (Fig. 1). Plasma levels of neurotensin were significantly ($P < 0.0001$) elevated by 117% within 12 h after feeding (Fig. 1). All four peptides peaked in concentration at 24 h postfeeding, at which time plasma CCK, GIP, glucagon, and neurotensin concentrations had increased by factors of 25, 6, 6, and 3.3 over fasting values, respectively. After the 24-h peak, plasma concentrations of each peptide had declined significantly by either 3 or 6 d and continued to decline thereafter, eventually returning to prefeeding values by either 14 or 30 d.

Tissue Concentrations. Python CCK was sequestered within the small intestine (31–107 pmol/g) and marginally present in other tissues (0.4–2.4 pmol/g) (Fig. 2). Glucagon was almost entirely

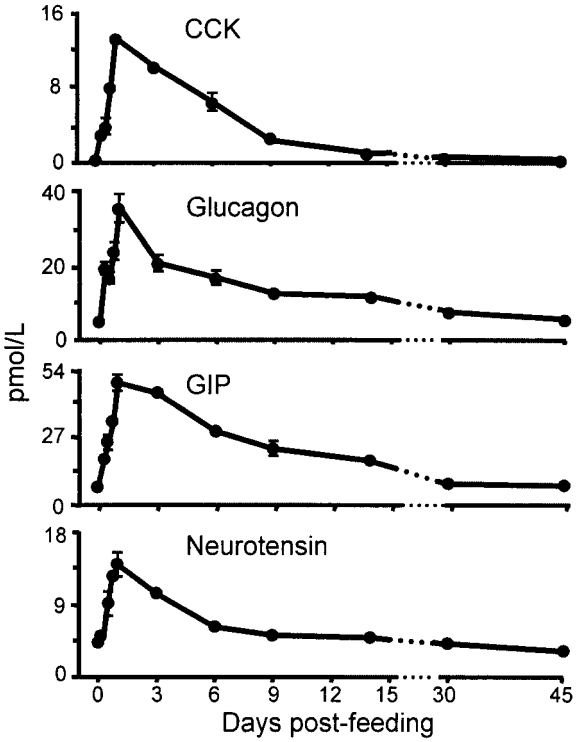

Fig. 1. Plasma concentrations (pmol/liter) of CCK, glucagon, GIP, and neurotensin during fasting (day 0) and at 10 times (0.25–45 d) after pythons ($n = 8$) had consumed meals equivalent to 25% of the snake's body mass. CCK, glucagon, GIP, and neurotensin concentrations peaked 1 d postfeeding at 25, 6, 6, and 3.3 times fasting levels, respectively. In this and subsequent figures, the vertical error bars represent ± 1 SEM.

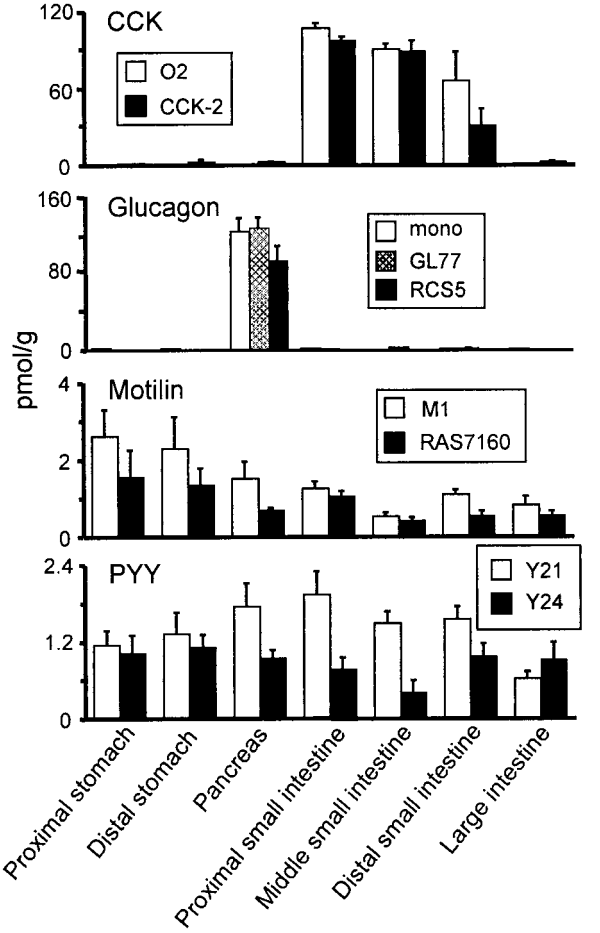

Fig. 2. Concentrations (pmol/g wet mass) of CCK, glucagon, motilin, and PYY measured by two or three different antisera (02, CCK2, mono, etc.) in the proximal and distal halves of the stomach, pancreas, proximal and middle and distal thirds of the small intestine, and large intestine of three pythons 1 d after they had consumed meals equivalent to 65.7% of their body mass. Note that CCK and glucagon are at high levels in the stomach and small intestine, respectively, but that motilin and PYY levels are apparently low throughout the gut (probably because of poor binding of our mammalian antibodies to these two python peptides).

confined to the pancreas and lacking from stomach and intestine (Fig. 2). Digesting pythons had only low motilin-like immunoreactivity (0.5–2.5 pmol/g, highest in the stomach) and PYY-like immunoreactivity (0.4–1.9 pmol/g) throughout the gut, suggesting poor binding between the antibodies used and these python peptides (Fig. 2).

At 1 d after feeding, python gastrin-like immunoreactivity was present at low levels (0.9–2.5 pmol/g) in the small intestine and significantly nonexistent in other tissues (Fig. 3). In contrast, GIP was highly concentrated in the pancreas (90 ± 13 pmol/g), with significantly ($P < 0.001$) smaller amounts (4–7 pmol/g) in the small and large intestines (Fig. 3). Neurotensin concentrations in the stomach were 3–10 times greater ($P < 0.04$) than in the pancreas and small and large intestines (Fig. 3). Somatostatin concentrations in the stomach and pancreas were 10-fold greater than in either the small or large intestine (Fig. 3).

Among neuropeptides assayed, only CGRP exhibited no significant variation in concentration among or between tissues (Fig. 3). Substance P concentrations in the small and large intestines were several times greater ($P < 0.003$) than in the stomach or pancreas (Fig. 3). Levels of VIP were significantly higher ($P < 0.017$) in the stomach than in the small intestine (Fig. 3).

Postfeeding Response in Tissue Concentrations. Feeding induced significant decreases (by 92%) in gastric and pancreatic CCK concentrations but had no significant effect on intestinal levels (Fig. 4). The pancreas was the only tissue to experience a significant ($P < 0.01$) postfeeding decline (by 67%) in GIP concentration (Fig. 4). Neurotensin concentrations declined significantly ($P < 0.02$) in all assayed tissues within 24 h after feeding and remained significantly lowered for 3 d (Fig. 4).

Gel-Permeation Chromatography of Python Hormones and Neuropeptides. We found the major molecular forms of CCK (from small intestine), GIP (pancreas), glucagon (pancreas), neurotensin (stomach), somatostatin (pancreas and stomach), substance P (small intestine), and VIP (stomach) to elute in the same positions as those of their synthetic mammalian counterparts (Fig. 5). A higher molecular weight GIP was also detected, corresponding to the unprocessed pro-GIP known for mammalian intestine (17). In addition to the peak of intestinal CCK-like immunoreactivity eluting in the same position as mammalian CCK-33, we detected higher molecular weight material, which probably represents an N-terminally extended form of python CCK; such forms (CCK-58 and CCK-83) are known in mammals.

Discussion

Pythons experience postprandially matched increases in plasma and decreases in tissue concentrations of GI peptides, synthesize GI peptides in similar locations as mammals, and possess pep-

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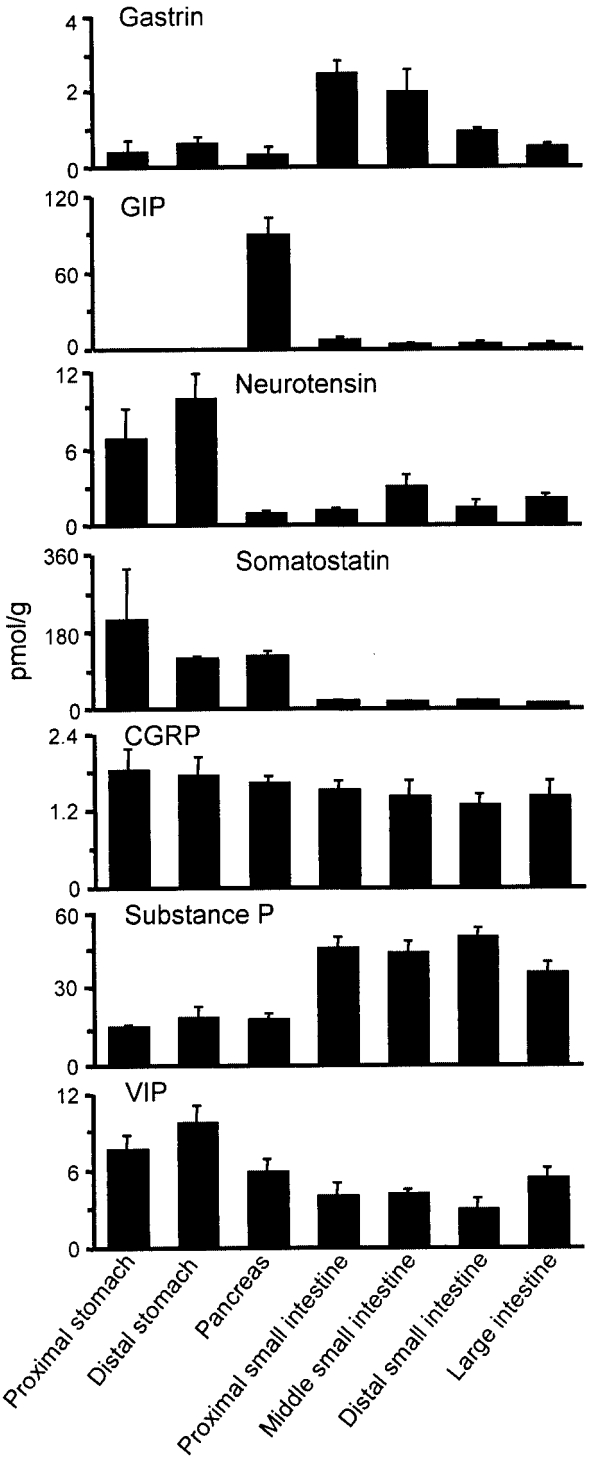

Fig. 3. Concentrations (pmol/g wet mass) of seven hormones and neuropeptides in the proximal and distal halves of the stomach, pancreas, proximal and middle and distal thirds of the small intestine, and large intestine of three pythons 1 d after they had consumed meals equivalent to 65.7% of their body mass. Note that gastrin, GIP, neurotensin, somatostatin, substance P, and VIP immunoreactivity are highest in the small intestine, pancreas, stomach, stomach and pancreas, intestines, and stomach, respectively, whereas CGRP is apparently not localized.

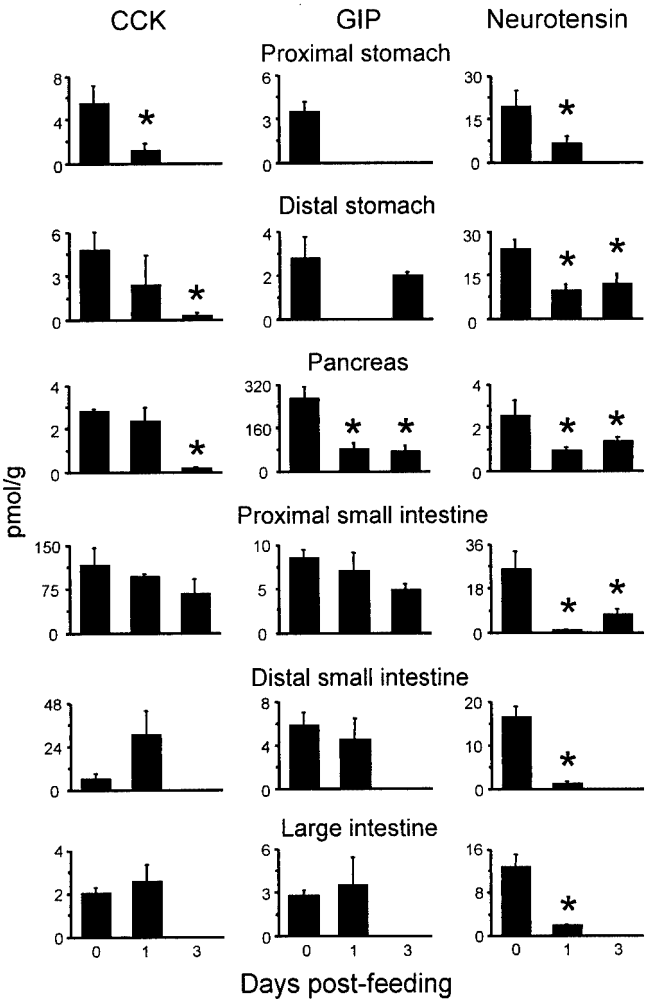

Fig. 4. Concentrations (pmol/g wet mass) of CCK, GIP, and neurotensin in the proximal and distal halves of the stomach, pancreas, proximal and distal thirds of the small intestine, and large intestine during fasting (day 0) and at 1 and 3 d after pythons ($n = 3$ for each time period) had consumed meals equivalent to 65.7% of their body mass. Fed values significantly lower ($P < 0.05$) than fasted values are noted (*).

tides that have chromatographic similarity or identity to their mammalian analogues. These findings suggest the regulatory role of these GI peptides in the pythons' postfeeding responses, which include acidifying stomach contents, release of pancreatic enzymes and bile, a doubling of intestinal mass, and 10- to 40-fold increases in intestinal nutrient transport. Each of the 11 peptides that we studied is believed to regulate digestive function and/or morphology in mammals. We shall now consider each peptide individually, before proposing four further projects to study GI peptides in the python model.

GI Peptide Comparisons Between Pythons and Mammals. *CCK.* In pythons as in mammals, CCK is concentrated in the small intestine and occurs as multiple forms differing in molecular weight (Fig. 5). The postfeeding increase in plasma CCK is much greater in pythons (25-fold) than in humans (3- to 4-fold) (18, 19). In mammals, CCK is proposed to stimulate pancreatic enzyme secretion and gallbladder contraction, inhibit gastric emptying and acid secretion, and mediate the satiety response (3). In pythons, CCK may also trigger observed increases in pancreatic enzyme activity and decreases in gallbladder volume (4, 5).

GIP. As for CCK, the postfeeding increase in plasma GIP is larger in pythons (6-fold, apparently released from the pancreas;

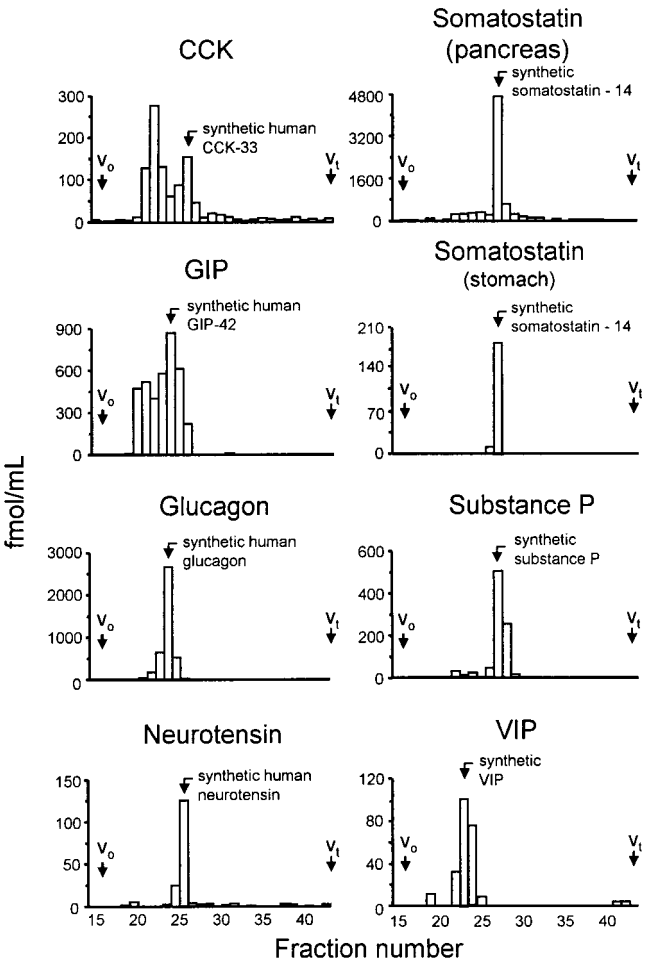

Fig. 5. Gel permeation chromatograms showing the elution patterns of python CCK (from small intestine), GIP (pancreas), glucagon (pancreas), neurotensin (stomach), somatostatin (stomach and pancreas), substance P (small intestine), and VIP (stomach). For each peptide, we obtained two chromatograms that proved to be extremely similar; therefore, we show only one of the two chromatograms. The positions of synthetic mammalian standards are indicated by arrows, as are the void (V_0 = elution position of bovine albumin) and total (V_t = elution position of NaI) column volumes. Note the match in elution position between the python peptide and synthetic mammalian peptide in each case, plus additional higher molecular weight forms of CCK and GIP (at lower fraction numbers).

Fig. 4) than in mammals (2- to 3-fold) (18–20). Surprisingly, whereas mammalian GIP is produced in the small intestine, where it is thought to function as an incretin (an intestinal signal stimulating insulin secretion triggered by luminal carbohydrates), that function seems unlikely in pythons, which synthesizes GIP instead in the pancreas.

Glucagon. Glucagon occurs in the pancreas of both pythons and mammals. Surprisingly, whereas plasma insulin rises and glucagon falls in fed mammals, fed pythons exhibit a paradoxical 6-fold increase in plasma glucagon. A possible explanation is that pythons consume meals much higher in protein than do mammals, and amino acids are known to stimulate pancreatic glucagon secretion in mammals (21). Glucagon's triggering of gluconeogenesis, glycogenolysis, and lipolysis may provide a hormonal mechanism for pythons' postfeeding increases in plasma glucose and lipids, both of which may serve to fuel the up-regulation of python's gut after feeding (4, 5). Interestingly, two mid- to N-terminally directed antibodies that cross react with mammalian enteric glicentin (an elongated proglucagon) failed to detect significant immunoreactivity in python stomach or small intestine. Either the proglucagon gene is not expressed in python gut or its python products are so structurally distinct that they cannot be detected by mammalian antisera. The former explanation seems more likely because python pancreatic glucagon differs by only one amino acid from human glucagon (8).

Neurotensin. The postfeeding increase in plasma neurotensin is greater in pythons (3.3-fold, apparently originating from the stomach and intestine; Fig. 4) than in humans (2-fold) (18, 19). Python neurotensin is identical in structure to that of alligators and chickens but differs from mammalian neurotensin by three amino acids (9). In mammals, administration of neurotensin only at nonphysiologically high concentrations stimulates gut motility and pancreatic and intestinal secretion, whereas no role of neurotensin has been found at normal circulating levels (3). Our observations (Figs. 1 and 4) do suggest a physiological role of neurotensin in pythons.

Somatostatin. Whereas most other GI peptides stimulate gut functions in mammals, somatostatin inhibits gastric and pancreatic secretion, gut motility, gallbladder contraction, and intestinal amino acid absorption upon the completion of digestion. Hence, python somatostatin, whose location (stomach and pancreas, Fig. 3) is the same as in mammals, may have a similar role.

Gastrin. Gastrin in mammals acts on the stomach by stimulating acid secretion and growth (3). Because feeding in pythons does stimulate acid secretion and stomach growth (5), we expected a similar role of python gastrin. Instead, we were surprised to be unable to detect gastrin in the python stomach by either a gastrin-specific antiserum (Gas179) or a nonspecific antiserum (O2) that fully cross reacts with all mammalian amidated gastrins and CCKs. The low gastrin immunoreactivity detected in python tissues (Fig. 3) likely represents cross reactivity of the mammalian gastrin antisera with python CCK. Nevertheless, we suspect that pythons do have gastrin but that its amino acid sequence makes it immunologically distinct from mammalian gastrin.

Motilin and PYY. Motilin and PYY were detected in python gut tissues at only low concentrations by both N- and C-terminally directed antisera to these mammalian hormones, suggesting poor cross reactivity and structural distinctness of the python analogues.

CGRP, substance P, and VIP. The neuropeptides CGRP, substance P, and VIP are produced by intrinsic gut neurons in mammals (1) and were found in all analyzed gut tissues of pythons (Table 1), as they are in mammals. Python substance P and VIP are chromatographically similar to their mammalian analogues (Fig. 5); python and human substance P differ by only a single amino acid (9), and VIP is structurally conserved among vertebrates from sharks to mammals. Hence, these neuropeptides may act in pythons as they do in mammals, where CGRP and VIP cause vasodilation, control bicarbonate secretion, inhibit gastric acid secretion, and relax gut smooth muscle, and where substance P contracts gut smooth muscle and controls intestinal secretion and blood flow.

Studies of Regulatory Peptides in the Python Model. Although pythons and humans are surely not identical in all features of their gut regulatory biology, pythons still offer several advantages as a model species. These advantages include very large regulatory spans, ease of captive maintenance, tolerance of surgical modifications of the gut, and dissociation between trophic and functional regulation of the intestine (4, 6). Contrary to what one might imagine, pythons are gentler and easier to maintain than rats. We suggest four further studies of GI peptides in the python model.

Proglucagon products. In mammals, the proglucagon gene has at least five products thought to differ in function: glucagon in

the pancreas (increases plasma glucose) and, in the intestine, glicentin, oxyntomodulin (an inhibitor of gastric acid secretion), GLP-1 (an acid secretion inhibitor and incretin), and GLP-2 (a stimulant of intestinal growth) (3). Whereas python glucagon is clearly expressed in the pancreas, there was no cross reaction of the glucagon antibodies with intestinal glicentin or oxyntomodulin. Warranting study is determining whether the proglucagon gene and therefore its products are entirely absent from the python intestine.

Gastrin. Because pythons swallow large prey completely intact, prey digestion requires gastric hypertrophy and massive acid production. In mammals, both of these functions are stimulated by gastrin, but antisera to mammalian gastrin was essentially unable to detect gastrin in the python's stomach. Do pythons have a structurally distinct gastrin, or does some other peptide regulate the responses of the python stomach?

Functional regulation of intestine. In surgically modified models of mammalian intestine designed to study intestinal regulation (e.g., resection and Thiry Vella loops), it is difficult to study functional regulation (i.e., up-regulation of transporters and enzymes) because it is dwarfed by trophic regulation (i.e., intestinal growth). In contrast, surgically isolated loops of python intestine exhibit a very large functional response to feeding (up to 10-fold up-regulation of nutrient transporter activities) but no trophic response (6). Hence, pythons would be a good model species to identify which peptides regulate intestinal functional responses.

Responses to synthetic peptides. Because eight GI peptides have now been isolated and sequenced from pythons (8, 9), those peptides could be synthesized and individually infused into fasted pythons to determine their role in the gut's response to feeding.

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