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PYY-Tag Transgenic Mice Displaying Abnormal (H^+-K^+)ATPase Activity and Gastric Mucosal Barrier Impairment

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SUMMARY: The mechanism by which the gastrointestinal hormones peptide YY and glucagon inhibit gastric acid secretion is largely unknown. PYY-Tag transgenic mice develop endocrine tumors in the colon that are composed mainly of peptide YY/enteroglucagon-producing L type cells. Therefore we studied the functional activity of such tumors and the gastric functions of PYY-Tag mice. Fasting and fed PYY-Tag transgenic mice and CD1 controls were assayed for circulating levels of peptide YY, glucagon, insulin, and gastrin. The gastric pH was determined and gastric samples were examined for (a) histologic appearance; (b) K^+-stimulated p-nitrophenylphosphatase activity and [^{14}C]aminopyrine accumulation of apical and tubulovesicle membranes; (c) adherent mucus determination by Alcian blue recovery; and (d) DNA/RNA/protein epithelial content and in vivo incorporation of [^{3}H]thymidine into DNA. Transgenic mice showed high serum levels of peptide YY and glucagon, increased gastric pH, and a high incidence of gastric ulcers after fasting. p-Nitrophenylphosphatase activity, [^{14}C] aminopyrine accumulation, and proton pump redistribution from cytoplasmic tubulovesicles to apical membranes were significantly lower in the gastric mucosa of transgenic mice compared with the controls. In addition, the adherent mucus was thinner, and [^{3}H]thymidine incorporation into the DNA was decreased. The abnormal and unregulated levels of circulating peptide YY and glucagon led to gastric acid inhibition and an impairment of gastric barrier function as a result of a striking reduction in epithelial proliferation. (*Lab Invest 2003, 83:47–54*).

Gastric acid secretion depends on the activity of (H^+-K^+)ATPase (EC.3.6.1.36) in parietal cells. In humans, basal acid production accounts for 0 to 5 mEq HCl per hour (resting mucosa), whereas after a meal it reaches 6 to 40 mEq HCl per hour (stimulated mucosa). At rest (H^+-K^+)ATPase functions are limited by its intracytoplasmic position in vesicles, also called tubulovesicles, and by the reduced permeability of tubulovesicles to KCl (Urushidani and Forte, 1997). The activation of parietal cells by secretagogues induces tubulovesicle fusion with the apical plasma membrane (membrane trafficking) and an increase in permeability to KCl. The regulation of parietal cell activity is directly or indirectly achieved by the intervention of neural, endocrine, and paracrine mechanisms (Urushidani and Forte, 1997; Zeng et al, 1997).

Peptide YY (PYY) and enteroglucagon are synthesized by L-type endocrine cells in the distal ileum and colon and have been shown to inhibit several gut functions including acid secretion (Adrian et al, 1985; Guo et al, 1987a; Holst, 1997; Pappas et al, 1986; Sheikh, 1991; Walsh, 1994). Considering the location of L cells in the distal gut, PYY and enteroglucagon may participate in the modulation of transit times, gastric and pancreatic secretion, and nutrient absorption (Goodlad et al, 1991). Both peptides are released into the circulation in response to meal ingestion, with special reference to fats, and are widely considered as potential enterogastrones. Indeed, at physiologic doses, PYY and enteroglucagon may inhibit gastric acid secretion induced by pentagastrin, meal, and vagal stimulation (Adrian et al, 1985; Anini et al, 1999; Guo et al, 1987b; Holst, 1997; Sheikh, 1991; Walsh, 1994; Wettergren et al, 1994; Yang, 2002; Zeng et al, 1997). Although PYY and enteroglucagon colocalize in the same endocrine cell type and are usually released simultaneously, under certain conditions, only one

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peptide may be selectively released (Anini et al, 1999; Brubaker, 1991; Plaisancié et al, 1996).

Recently, transgenic mice expressing the simian virus 40 large T antigen under the control of the rat PYY gene promoter (PYY-Tag) (Upchurch et al, 1994, 1996) were generated to examine the ontogeny of PYY-producing cells in the developing mouse pancreas and colon. PYY-Tag transgenic mice develop well-differentiated endocrine tumors of the proximal colon mainly composed of L type cells.

Our objective was to investigate the functional activity of such tumors, gastric acid secretion, and the mucosal barrier in the gastric mucosa of both fasting and fed PYY-Tag mice. A preliminary partial account of these results has already been published in an abstract form (Gastaldi et al, 2000; Laforenza et al, 2000).

Results

PYY-Tag Mice Showed High PYY and Glucagon Serum Levels

Serum PYY levels were significantly higher in fed and fasting transgenic mice and corresponded to ~10- and ~50-fold, respectively, compared with controls (Table 1). Glucagon levels were also significantly higher (~2-fold) in transgenic mice compared with controls. PYY, but not glucagon, levels were increased in fasting transgenic mice compared with fed transgenic mice. Gastrin was significantly higher in fed transgenic mice compared with both controls and fasting mice (Table 1). No change was observed in insulin serum levels among any of the animal groups considered.

Gastric Endocrine Cells Are Unchanged in PYY-Tag Mice

Gastric endocrine cells of PYY-Tag mice were investigated by Grimelius' silver and vesicular monoamine transporter 2 (VMAT2) immunohistochemistry to assess histamine-producing enterochromaffin-like cells and by immunohistochemistry with specific antisera to determine ghrelin, gastrin, somatostatin, and the rare PYY cells. The qualitative assessment of size, shape, and number of argyrophil cells and of VMAT2-, ghrelin-, gastrin-, somatostatin-, and PYY-immunoreactive cells showed no apparent change as compared with nontransgenic CD1 mice, except in correspondence of mucosal ulceration (not shown). This observation suggests that high levels of circulating PYY and glucagon do not interfere with normal gastric endocrine cell differentiation.

Gastric Acid Secretion Is Reduced in PYY-Tag Mice

Gastric acid secretion analysis revealed that the fasting gastric pH was significantly higher in PYY-Tag transgenic mice than in the controls (3.85 ± 0.5 vs 2.53 ± 0.14, respectively [mean ± SEM], $n = 5$; $p \leq 0.02$, Student's unpaired *t* test). Under the fed condition, the gastric pH was also significantly higher in PYY-Tag transgenic mice than in controls (4.52 ± 0.37 vs 2.53 ± 0.46 respectively [mean ± SEM], $n = 5$; $p \leq 0.01$, Student's unpaired *t* test).

When assaying the gastric vesicles used for K^+-stimulated p-nitrophenylphosphatase (pNPPase) activity and aminopyrine uptake measurements (see following paragraphs), electron microscopic examination revealed that P1 was multilamellar and multivesicular with a mean diameter larger than for P3 (P1, 760 ± 32 nm; P3, 173 ± 7.2 nm [mean ± SEM], $n = 50$ from three different samples). Their appearance and morphometry were similar to those previously described (Forte et al, 1981; Reenstra and Forte, 1990).

The K^+-stimulated pNPPase activity in both PYY-Tag and control mice was measured with increasing concentrations of KCl and showed a hyperbolic course up to a concentration of 10 mM KCl (not shown). In PYY-Tag and control mice, pNPPase activity gradually decreased at concentrations of 10 to 50 mM KCl. V_{max} values were significantly lower in the homogenate and P1 fractions of fed transgenic mice compared with controls. In fasting transgenic mice, V_{max} value was significantly reduced only in the P1 fraction (Fig. 1). K_{max} values remained unchanged (not shown).

The meal-induced proton pump redistribution from cytoplasmic tubulovesicles to the apical plasma membrane was investigated by analyzing the ratio of the total K^+-stimulated pNPPase activity in P1 to the total activity in P3 (P1/P3 ratio) according to Reenstra and Forte (1990). The total pNPPase activity in P1 and P3 was obtained by multiplying the pNPPase specific activity (μmol/mg protein · hour) per milligram of total protein recovered in each fraction. The P1/P3 ratio was similar in fasting mice but significantly lower in fed transgenic mice (Fig. 2). The time course of [^{14}C]

Table 1. Serum Gastrointestinal Hormone (PYY, Glucagon, Insulin, and Gastrin) Concentrations in Normal (CD1) and PYY-Tag Transgenic Mice Under Fasting and Fed Conditions

	Fasting CD1	Fasting Transgenic	Fed CD1	Fed Transgenic
PYY (pg/ml)	42 ± 7	2130[a] ± 235	250[b] ± 94	2699[a,c] ± 285
Glucagon (pg/ml)	1102 ± 219	1870[a] ± 105	1015 ± 224	2396[a] ± 254
Insulin (μUI/ml)	1.87 ± 0.1	1.78 ± 0.04	1.96 ± 0.1	2.07 ± 0.2
Gastrin (pg/ml)	124 ± 30	118 ± 7	111 ± 34	264[d] ± 40

Values are mean ± SEM of at least eight different blood samples each from a different mouse.
[a] $p \leq 0.05$ (ANOVA followed by Newman-Keuls's *Q* test) vs CD1 mice.
[b] $p \leq 0.05$ vs fasting CD1 mice.
[c] $p \leq 0.05$ vs fasting transgenic mice.
[d] $p \leq 0.05$ vs fasting and fed CD1 mice and fasting transgenic mice.

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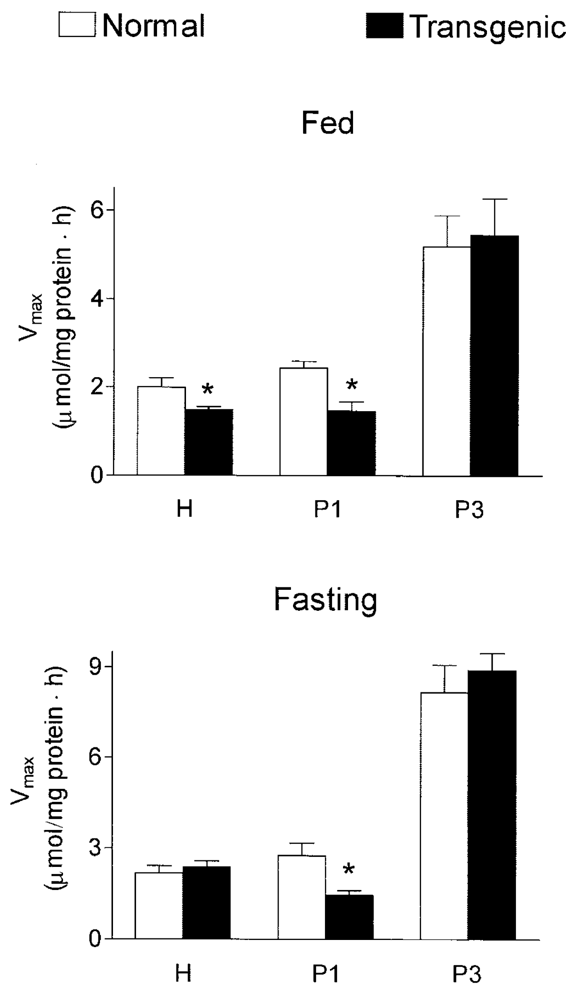

Figure 1.
The K^+-stimulated p-nitrophenylphosphatase (pNPPase) activity of homogenate, apical membrane vesicles, and tubulovesicles from gastric mucosa of normal and transgenic mice under fasting and fed conditions. The pNPPase activity was expressed as V_{max} values obtained from a least square curve fitting as described in "Materials and Methods." *H* = homogenate; *P1* = apical membrane vesicles; *P3* = tubulovesicles; $*p \leq 0.05$ vs normal mice (Student's unpaired *t* test). Values are mean ± SEM of at least five different experiments.

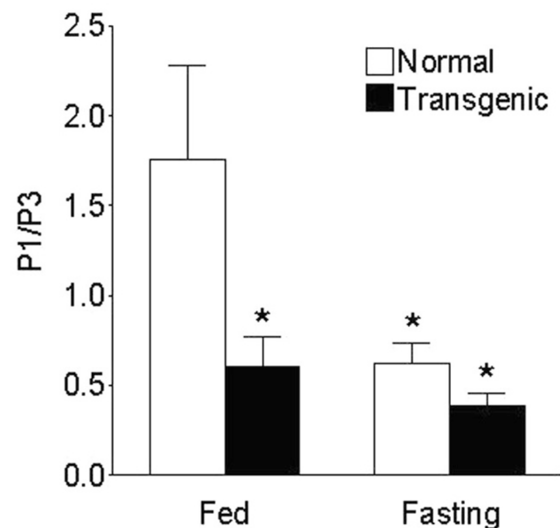

Figure 2.
Membrane distribution of total pNPPase activity (P1/P3 ratio) in gastric mucosa of normal and transgenic mice under fasting and fed conditions. *P1* = apical membrane fraction; *P3* = tubulovesicle fraction. Values are mean ± SEM of at least five different experiments. $*p \leq 0.05$ vs fed normal mice (ANOVA followed by Newman-Keuls's *Q* test).

K^+-stimulated aminopyrine uptake showed a transient peak at 1 minute of incubation in the P1 and P3 vesicles of both fasting and fed mice. The H^+ uptake of P1 vesicles was significantly reduced in fed and fasting transgenic mice compared with controls at 0.5 to 5 minutes of incubation (Fig. 3). P3 vesicles displayed no differences between the groups (not shown). Overall, the above data indicate abnormal functioning of the major mechanisms of gastric acid production and secretion.

PYY-Tag Mice Developed Gastric Peptic Ulcers and Displayed an Abnormal Gastric Barrier

Fasting PYY-Tag transgenic mice showed a high incidence of reddish band-like lesions in the glandular part of the stomach, more frequently in males (70% of examined mice, $n = 20$) than in females (50% of examined mice, $n = 24$). At histologic examination, the lesions consisted of peptic ulcers of varying extent and depth (Fig. 4). No lesion was observed in the gastric fundum, antrum, or proximal part of the duodenum. In contrast, the stomachs of fed transgenic mice and of fasting and fed CD1 mice (controls) displayed normal macroscopic and microscopic features. Inflammatory processes appeared in the proximity of gastric lesions, whereas they were absent in the adjacent mucosa.

The analysis of the different parameters involved in the gastric barrier revealed that in vivo [^{3}H]thymidine DNA incorporation was significantly lower in the gastric mucosa of both fasting (~65% reduction) and fed (~85% reduction) transgenic mice compared with the controls (Fig. 5A). However, despite the reduction in the DNA synthesis rate, the total DNA as well as the RNA and protein contents of the gastric mucosa were all similar in both normal and transgenic mice under both fasting and fed conditions (Fig. 5, B to D).

Finally, Alcian blue recovery from the gastric-bound mucus was significantly reduced in fasting transgenic mice compared with controls (73.6 ± 0.7 µg/gm body weight [mean ± SEM], $n = 5$, vs 147.1 ± 19.2 µg/gm body weight, $n = 5$; $p \leq 0.05$ Student's unpaired *t* test). Altogether the above data reveal an unbalanced functioning of the gastric barrier in PYY-Tag mice.

Discussion

The results of this study indicate that PYY-Tag mice carry functioning colonic endocrine tumors, with high levels of circulating PYY and glucagon, and display reduced gastric acid and mucus secretion associated with a striking impairment in gastric epithelial proliferation. The reduced mucous secretory function and epithelial renewal of the gastric mucosa in PYY-Tag

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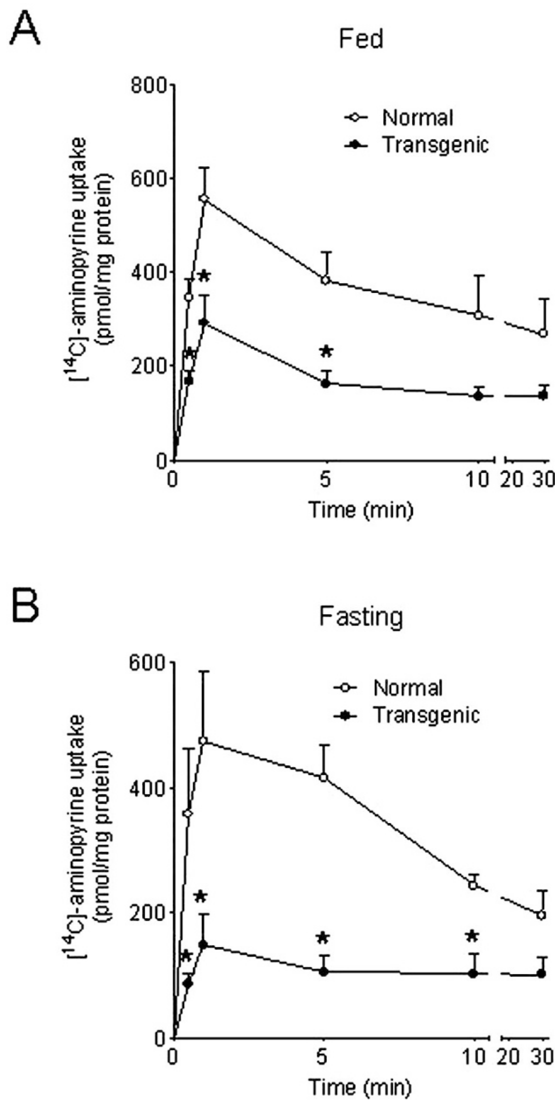

Figure 3.
K^+-dependent [^{14}C]aminopyrine uptake by gastric mucosal apical membrane vesicles from normal and transgenic mice under fed (A) and fasting (B) conditions. K^+-dependent [^{14}C]aminopyrine uptake was obtained by subtracting uptake values measured in the presence of KCl from those in its absence. Symbols represent mean ± SEM of triplicate determinations from each of five different experiments. *$p \leq 0.05$ vs normal mice (Student's unpaired *t* test).

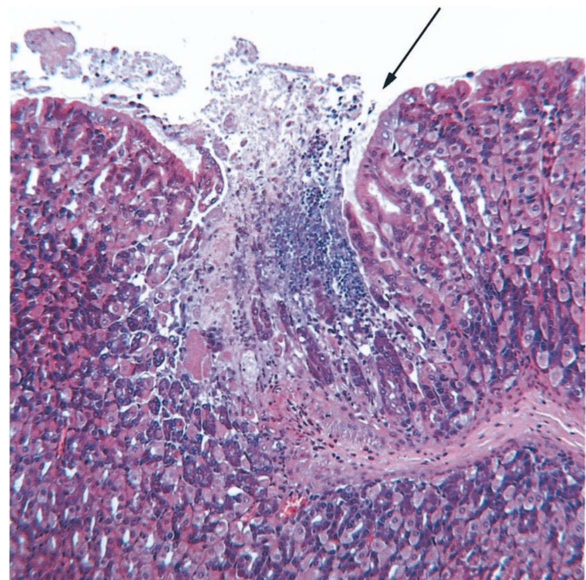

Figure 4.
Gastric mucosa, glandular portion, of fasting transgenic mice. Note the distribution of the surface epithelium, with loss of specialized cells and inflammatory infiltration. The ulcer affects the mucosa and the initial submucosa. The arrow indicates a solution of continuity of the mucosa with the fibrinoid clot and the inflammatory infiltration. Paraffin-embedded section of gastric mucosa was examined by hematoxylin-eosin staining (×50, original magnification).

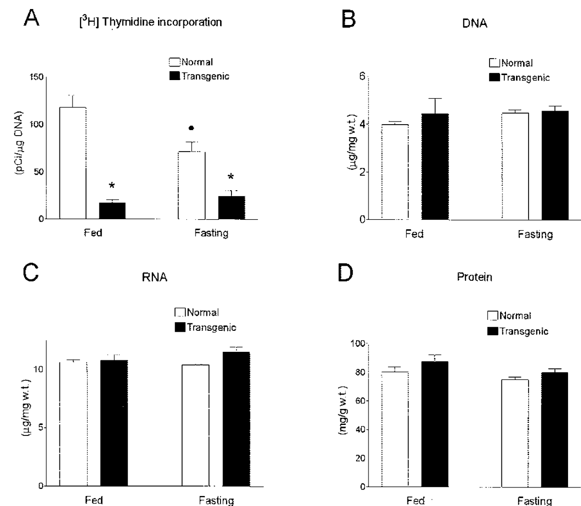

Figure 5.
[^{3}H]Thymidine incorporation into DNA (A), total DNA (B), RNA (C), and protein (D) content in gastric mucosa of normal and transgenic mice under fasting and fed conditions. Values are mean ± SEM of at least five different experiments. *$p \leq 0.05$ vs fasting and fed normal mice; ●$p \leq 0.05$ vs fed normal mice (ANOVA followed by Newman-Keuls's *Q* test).

mice resulted in gastric peptic ulcer formation under fasting stimulus. The abnormal gastric acid secretory function of PYY-Tag mice proved to be a result of reduced gastric (H^+-K^+)ATPase activity and inhibited meal-stimulated "trafficking " of (H^+-K^+)ATPase-rich tubulovesicles. Overall, these data would suggest that ongoing mucosal renewal is required for epithelial maintenance and secretory function.

PYY-Tag mice develop well-differentiated endocrine tumors of the large intestine mainly composed of L-type endocrine cells thus coexpressing both PYY and enteroglucagon (Upchurch et al, 1996). Not surprisingly, we show here that such tumors determined high blood levels of both PYY and glucagon (Table 1). PYY levels were ~10- and ~50-fold higher in fed and fasting transgenic mice, respectively, whereas glucagon levels only doubled. Notably, similar glucagon levels were observed both in transgenic mice expressing the large T antigen in glucagon-producing cells of the gut and pancreas (GLUTag-Y) and mice developing L cell carcinomas of the large intestine (Brubaker et al, 1992). These results suggested that a similar proglucagon processing mechanism existed in tumor cells in both transgenic strains. In addition, we show

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that the insulin serum levels of PYY-Tag mice were normal, which is consistent with the previously reported rarity of pancreatic insulin-producing tumors in PYY-Tag adult mice (Upchurch et al, 1994). Finally, circulating gastrin levels were significantly higher in the fed condition, although normal in fasting PYY-Tag mice (Table 1). This finding indicates that the elevated gastrin levels noted were attained under the physiologic meal stimulus, thus reflecting the reduced acid secretion capacity [reduced (H^+-K^+)ATPase activity] of PYY-Tag mice gastric mucosa. The lack of intraluminal H^+ ions is a potent physiologic stimulus for antral gastrin production. Incidentally, these data confirm previous observations that gastrin is not produced by colonic endocrine tumors (Upchurch et al, 1996). Moreover, the unchanged gastrin levels observed in fed versus fasting control mice can be explained with the prompt restoration of the acidic intraluminal pH (see "Results") together with the rapid G17 disappearance half-time (Walsh, 1994).

Although in PYY-Tag transgenic mice high levels of circulating PYY and glucagon associate with severe impairment of both secretory and barrier functions of the gastric mucosa, and despite the reduction of DNA synthesis rate observed, we did not find any notable change in gastric endocrine cells at qualitative assessment. This observation indicates that the differentiation of gastric endocrine cells is preserved in PYY-Tag transgenic mice. In addition, although only for gastrin cells, their secretory properties in response to physiologic stimuli seem to be intact, because the elevated gastrin levels released under meal stimulus by PYY-Tag mice have been observed (see previous paragraph). Finally, no PYY cell proliferation/transformation was observed in the stomach of PYY-tag mice, indicating the lack of transgene expression in this tissue.

A number of published works have demonstrated that PYY and proglucagon-derived peptides may inhibit gastric acid and pepsin secretion through both neural and non-neural pathways (Adrian et al, 1985; Holst, 1997; Sheikh, 1991; Walsh, 1994; Wettergren et al, 1994). PYY may modulate gastric acid secretion induced by vagal stimulation, through Y_2 receptors located in the area postrema and in the dorsal vagal complex (Lloyd et al, 1996; Yang, 2002). Moreover, PYY does not interact directly with the parietal cells but may inhibit gastric acid secretion by Y_1 receptor activation on enterochromaffin-like cells, with the consequent blockage of histamine release (Zeng et al, 1997). Proglucagon peptides were shown to directly or indirectly inhibit gastric acid secretion by various mechanisms for the different species investigated (Beales and Calam, 1996; Gros et al, 1995; Holst, 1997; Kirkegaard et al, 1982; Wϕjdemann et al, 1999). Finally, the simultaneous co-release of PYY and proglucagon-derived peptides by L cells (Brubaker, 1991; Plaisancié et al, 1996) may have had a cumulative effect on the inhibition of gastric acid secretion (Anini et al, 1999; Wettergren et al, 1997).

Our data on PYY-Tag mice confirm the negative effects of these hormones on gastric acid secretion, providing an explanation for the mechanisms behind such inhibition. The unregulated high levels of circulating PYY and glucagon in PYY-Tag mice were associated with reduced proton pump expression. This was a result of a decrease in the V_{max} pNPPase (decreased proton pump number) and reduced H^+ transport by gastric apical membranes (Figs. 1 and 3) both under fasting and fed conditions. This hypothesized reduction of (H^+-K^+)ATPase in PYY-Tag mice, inferred from the decrease in the V_{max} pNPPase, has been recently confirmed by immunohistochemical and Western blot experiments (Carmosino et al, 2001). The reduced ability to secrete protons is reflected in the macroscopic increase of gastric pH observed under both fasting and in fed conditions in transgenic mice. Moreover, meal-induced gastric acid stimulation resulted in elevated levels of circulating gastrin, which, however, failed to restore gastric acid output to normal in transgenic mice (Figs. 1 and 3). The P1/P3 ratio reduction indicates that the gastric mucosa of transgenic mice under a meal stimulus also display the functional characteristics of the fasting mice (Fig. 2). Altogether, these data indicate that persistently elevated levels of PYY and glucagon render the gastric mucosa relatively insensitive to physiologic secretory stimuli and suggest that PYY and glucagon play some role in the rapid blockage of acid secretion by inhibiting proton pump translocation from the cytoplasmic tubulovesicles to the apical membrane of the gastric mucosa.

Despite the chronic reduction in acid production, we also observed gastric ulcers in fasting transgenic mice (Fig. 4). The two components of the gastric mucosal barrier investigated here (the thickness of adherent mucus and epithelial proliferation) were both significantly decreased in transgenic mice (see "Results" and Fig. 5A). It could be speculated that elevated levels of PYY and/or glucagon may be associated with reduced vagal activity, the most powerful stimulant for mucus secretion (Robert, 1987). However, further investigation is necessary to fully support this hypothesis. Additionally, our data showed a marked decrease in [^{3}H]thymidine incorporation in the gastric mucosa of both fasting and fed transgenic mice (Fig. 5A), with unchanged mucosal cellular densities (unchanged total DNA content; Fig. 5B). This would indicate an overall reduction of cell renewal in transgenic mice stomach mucosa that does not allow tissue-maintaining homeostasis and repair after injury, even after the mild stress stimulus of 16-hour fasting. To our knowledge, the negative trophic effect of PYY and proglucagon-derived peptides on the gastric mucosa has not been reported until now. Both PYY and proglucagon-derived peptides display opposite effects on different gastroenteropancreatic tissues. PYY may stimulate DNA synthesis and cell growth in some cell and tissue models including the intestinal epithelium, while it has an inhibitory effect on pancreatic growth (Mannon, 2002; Tseng and Liu, 2002). Moreover, PYY treatment reduces growth in pancreatic and breast tumors (Tseng and Liu, 2002). Glucagon 1-21 significantly decreased both the weight and the crypt cell production rate in rat small intestine but not in the

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stomach (Goodlad et al, 1991). Finally GLP-2 was demonstrated as stimulating crypt cell proliferation and inhibiting epithelial apoptosis only in the intestine (Drucker et al, 1996, 2001; Tsai et al, 1997). Indeed, GLP-2 receptors are rare in the stomach and mostly localized in endocrine cells (Munroe et al, 1999; Yusta et al, 2000).

As observed in our study, an increased susceptibility of gastric mucosa to damage caused by stressful stimuli (fasting) with concurrent reduction of the gastric acid and mucus secretion has been reported in streptozotocin-diabetic rats (Igarashi et al, 2000; Korolkiewicz et al, 1999). Interestingly, high PYY levels have been found in several gastrointestinal disorders including diabetes gastroenteropathy (El-Salhy et al, 2002). It can be speculated that gastrointestinal disorders leading to high PYY levels may decrease the mucosal defensive mechanisms thus favoring the risk of ulcer onset.

In conclusion, in PYY-Tag transgenic mice, high levels of circulating PYY and glucagon inhibit gastric mucosa functions by reducing acid and mucous production associated with diminished mucosal cell turnover. Overall, our data support the hypothesis that PYY and glucagon have a general inhibitory effect on gastric mucosa and suggest that ongoing renewal is required for gastric epithelial maintenance and function.

Materials and Methods

Transgenic Mice and Controls

The generation of PYY-Tag mice has been previously described (Upchurch et al, 1994). Transgenic and CD1 mice were housed at the animal facility of the Institute of Human Physiology in Pavia and cared for according to current European law for animal practice. Tail DNA, sampled under anesthesia (Avertin), was checked by PCR for the large T antigen insert amplification, and positive transgenic mice were maintained in an outbred CD1 background. Controls and transgenic mice were divided into two groups: (a) with unrestricted access to food from 4 to 8 am to simulate the condition of physiologic meal stimulation; and (b) fasting for at least 16 hours with free access to water. Thus, the gastric mucosa we obtained from both fasting and fed mice should be considered different from those used by Forte and colleagues (1981) because the animals were not pharmacologically treated. Moreover, the mice were kept on a raised mesh-cage bottom to prevent coprophagy. Age- and sex-matched CD1 and PYY-Tag mice were killed at 8 am by cervical dislocation after light Avertin anesthesia and checked at autopsy for tumor burden (transgenic mice) or other macroscopic abnormalities.

Light Microscopy and Serum Assays

Gastric samples were removed, dissected along the greater curvature, rinsed with cold saline, fixed in buffered 10% formalin, and processed into paraffin. Serial paraffin sections (2–4 μm) were brought to water and stained with hematoxylin and eosin for conventional histologic examination or with Grimelius' silver impregnation (Grimelius, 1968) and sera specific for ghrelin, gastrin, somatostatin, PYY, and the VMAT2 as previously described (Rindi et al, 2000, 2002; Upchurch et al, 1996) for endocrine cell assessment.

Serum samples were obtained at autopsy, snap-frozen in liquid nitrogen, and stored at $-80°$ C for future use. Radioimmunoassay for PYY, pancreatic glucagon, insulin (Biochem Immunosystems, Italy), and gastrin (G17) (CIS BioInternational, France) were performed according to the manufacturers' instructions.

Gastric Secretion Studies

Measurement of Gastric pH. Gastric pH of PYY-Tag and control mice was assessed as previously described (Langhans et al, 1997). In brief, mice were anesthetized with Avertin and the stomach was exposed and excised after legation at the esophagus and pylorus extremities. The stomach was injected with 0.5 ml of saline into the lumen, the gastric fluid content was recovered, and the pH was measured with a Beckman Φ43 pH meter (Beckman Instruments, Irvine, California).

Vesicle Preparation and Electron Microscopy Analysis. At autopsy, the mouse stomach was rapidly removed, cut open, and washed with ice-cold saline. The glandular mucosa was scraped with a sterile scalpel (usually, 0.35–0.4 gm per preparation). Apical membrane vesicles (P1) and tubulovesicles (P3) were isolated from the total homogenate as described by Reenstra and Forte (1990). Morphologic appearance of P1 and P3 fractions from control mice were assessed by a conventional electron microscopy technique as described by Gastaldi et al (1992).

p-Nitrophenylphosphatase Activity. K^+-stimulated pNPPase activity was measured to evaluate (H^+-K^+)ATPase activity according to the method of Reenstra and Forte (1990) with or without increasing concentrations of KCl (0.5–50 mM). The best fit of each curve and the relative Michaelis-Menten constants, K_m and V_{max}, were calculated for 0.5 to 10 mM KCl concentrations by computerized least-square regression analysis (GraphPad Prism 2.01, San Diego, California).

Proton Transport. [^{14}C]Aminopyrine uptake was used to evaluate the extent of H^+ transport in P1 and P3 vesicles according to the method of Im et al (1984), with minor modifications. In brief, 12.5 μl of vesicles suspended in a medium containing (in mM) 300 D-mannitol, 2 $MgCl_2$, and 10 Hepes-NaOH (pH 6.8) were incubated at 25° C with 250 μl of solutions containing the following: 3 μM [dimethylamine ^{14}C]aminopyrine (specific activity: 4.03 GBq/mmol) (Amersham Pharmacia Biotech, Milan, Italy); 2 mM $MgCl_2$; 2 mM Na_2ATP; 10 mM Hepes-NaOH, pH 6.8, and valinomycin (5 μg/ml) in the presence of 150 mM KCl or 300 mM D-mannitol. Incubation was terminated by adding 3 ml of cold stop solution (150 mM NaCl and 1 mM Hepes-NaOH, pH 6.8); the amount of incorporated radioactivity was measured after vesicle separation by

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a rapid filtration procedure using cellulose nitrate microfilters with a 0.2-μm pore diameter (MFS Dublin, California). In each experiment, blanks were prepared to evaluate the unspecific radioactivity of [^{14}C]aminopyrine adsorbed by the microfilter, and the values were subtracted from the total radioactivity retained on the filter. Radiometric measurements were taken by using a Packard Tri-Carb model 2000 CA liquid scintillation counter (Packard Instruments Company, Inc., Downers Grove, Illinois).

Mucosal Barrier Studies

Determination of DNA Synthesis and DNA/RNA/Protein Content. The rate of DNA synthesis was estimated by measuring the in vivo incorporation of [methyl-^{3}H]thymidine into the DNA. Each mouse was given an intraperitoneal injection of [methyl-^{3}H]thymidine (specific activity, 185 GBq/mmol) (Amersham Pharmacia Biotech, Milan, Italy) in a dose of 37 KBq/gm body weight. After 1 hour, the mice were killed and their stomachs were removed and cleaned. The gastric mucosa was scraped with a razor blade and weighed; total DNA, RNA, and protein were extracted as previously described (Dembinski et al, 1982). The total DNA content was assayed according to the method of Giles and Myers (1965), the RNA content according to the method of Fleck and Begg (1954), and the protein content according to the method of Lowry et al (1951).

Determination of Adherent Mucus. Measurement of mucus levels adherent to the gastric epithelial surface was done only in fasted mice according to the method of Corne et al (1974). Briefly, the glandular portion of the stomachs was soaked for 2 hour at 25° C in a solution containing 0.1% Alcian blue 8GX, 160 mM sucrose, and 50 mM sodium acetate-HCl, pH 5.8. The unbound dye was removed by two washings of 15 and 45 minutes in 250 mM sucrose, and then the dye complexed with mucus was eluted by incubating the stomachs in 0.5 M $MgCl_2$ for 2 hours. The solution obtained was shaken with diethyl ether and the optical density of the aqueous phase read at 605 nm with a Uvikon 930 spectrophotometer (Kontron Instruments, Milan, Italy).

Statistical Analysis

The significance of the differences of the means under different experimental conditions was evaluated by using the ANOVA method followed by Newman Keuls's *Q* test and Student's unpaired *t* test (GraphPad Prism 2.01).

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