Evidence for a Role of the Gut Hormone PYY in the Regulation of Intestinal Fatty Acid-binding Protein Transcripts in Differentiated Subpopulations of Intestinal Epithelial Cell Hybrids*

(Received for publication, December 17, 1996, and in revised form, February 12, 1997)

Gunnar Halléén and Gregory W. Aponte‡
From the Department of Nutritional Sciences, University of California, Berkeley, California 94720-3104

Peptide tyrosine tyrosine (PYY) is a gut hormone present in endocrine cells in the lower intestine that can be released by the presence of luminal free fatty acids (FFAs). The biological action of this peptide includes inhibition of gut motility and gastrointestinal and pancreatic secretions. Intestinal fatty acid-binding protein (I-FABP) binds FFA and may be involved in their cytosolic trafficking. Quantitative in situ hybridization on heterogeneous populations of small intestinal somatic cell hybrids selected for endogenous I-FABP expression (hBRIE 380i cells) demonstrated a 5-fold increase in I-FABP transcripts in response to PYY (within 6 h) that was confined to clusters of differentiated cells, whereas ribonuclease protection assays performed on heterogeneous populations of these cells showed no significant differences. High affinity PYY receptors, with an IC50 of 5–50 pM, were identified in both differentiated and non-differentiated cell populations, as determined by competitive binding assays and autoradiography. In situ hybridization of rat ileal tissue also revealed differing patterns of mRNA expression for liver fatty acid-binding protein (L-FABP) and I-FABP. Only I-FABP mRNA was detected in the villus tips. This localization correlated with the expression pattern of I-FABP mRNA in the hBRIE 380i cells where changes in transcripts were observed only in differentiated cells that did not incorporate bromodeoxyuridine. The sustained expression of I-FABP transcripts in the villar tips suggests (unlike L-FABP) that older terminally differentiated cell populations of the mucosa can still be PYY responsive. These studies demonstrate that physiological concentrations of PYY can regulate I-FABP and place this peptide in a key position as part of a feedback system that determines the processing of cytosolic FFA in the enterocyte. In addition, these studies suggest a mechanism whereby luminal agents can modulate expression of proteins in terminally differentiated cells in the gastrointestinal mucosa.

Peptide tyrosine tyrosine (PYY)¹ is member of a 36-amino acid regulatory peptide family that includes neuropeptide Y (NPY) and pancreatic polypeptide (PP). PYY has greater than 70% sequence identity with NPY and shares a common structural motif consisting of two antiparallel helices, an amino-terminal polyproline helix, and a long amphipathic helix connected by a β turn (1). PYY-secreting cells occur mainly in the distal small intestine and the large intestine, locations where dietary fatty acids can act as potent stimulants of PYY release into the circulation. PYY-induced effects on the gastrointestinal tract can be reproduced by infusions of the peptide at concentrations less than postprandial blood concentrations (2, 3). Specific receptors for NPY/PYY have been characterized in distinct gastrointestinal tissue, such as chief cells (4), and mucosa of the small and large intestine (5). Specific PYY receptors have also been reported in brain tissue (6, 7), spleen (8), vascular smooth muscle (9), and in several neuroendocrine cell lines (10). Such locations make it possible for PYY to act both as an endocrine and a paracrine agent. Many of the reported effects of PYY on the gut, such as the inhibition of intestinal secretion, motility, and gastric acid secretion, occur as interdigestive events coordinated with release of PYY after a meal. We and others have demonstrated that luminal oleic acid induces the release of PYY in the dog (11), rat (12), and in isolated primary cultured PYY cells from the canine mucosa (13). Although it has been proposed that NPY acts centrally to initiate feeding (14), PYY seems to modify digestive processes to ensure efficient utilization of ingested food. PYY acts to slow gastric emptying and intestinal transit, changes that increase the efficiency of nutrient digestion and absorption. In the central nervous system, PYY may act through specific receptors in the dorsal vagal complex to inhibit vagal tone (15). As a result, PYY establishes a negative feedback loop that could act centrally in the brain to inhibit neurally mediated pancreatic exocrine secretion. This negative feedback may serve as part of an “ileal brake” in response to excess dietary triglyceride. For example, triglyceride hydrolysis resulting in FFAs in the distal intestine would induce PYY secretion whenever the rate of triglyceride hydrolysis exceeded the rate of fatty acid absorption. This induction of PYY secretion would then inhibit intestinal motility, causing an increase in FFA absorption and a decrease in luminal FFAs. Therefore PYY, like other gut-regulatory peptides such as cholecystokinin and somatostatin, can act as both a hormone and a neuromodulator.

Because PYY secretion can occur in direct response to luminal long-chain FFAs, we chose to examine the possibility that this peptide may act on the expression of an intestinal cytosolic fatty acid-binding protein, the intestinal fatty acid-binding protein (I-FABP). Fatty acid binding-proteins (FABPs) are 14–15-kDa cytosolic proteins that bind fatty acids with affinities in the nanomolar range (16). An extensive number of cytosolic binding proteins have been grouped into the FABP gene family, including liver fatty acid-binding protein L-FABP (found in the
liver and intestine) (17), I-FABP (found only in the intestine) (17, 18), ileal gastrinotropin (19), cellular retinoic acid-binding proteins I and II (20, 21), cellular retinoic acid-binding proteins I and II (20), adipocyte and myelin FABP (22), and heart and epidermal FABP (23, 24). The intestine abundantly expresses both I- and L-FABP, each as 2–3% of total cytosolic protein (25).

The biological role of intestinal FABP is still largely speculative despite extensive knowledge about its binding properties, amino acid sequence, protein structure (26, 27), expression pattern during development (28–30), and localization of genetic elements that regulate regional and cell-specific patterns of expression within the gut epithelium (30). I-FABP is a likely regulator of intracellular fatty acid levels because of its involvement in the trafficking and/or metabolism of FFAs in the intestinal epithelia (31). I-FABP gene expression could therefore, be expected to be regulated by fatty acids and hormonal factors involved in fatty acid assimilation and metabolism. Because PYY is secreted in direct response to FFA PYY regulation of I-FABP gene expression could provide a mechanism of feedback regulation for the synthesis of I-FABP in response to the presence of luminal FFA. From a broader perspective, evidence of such a mechanism would expand the potential of other gut recepto-secrete cells as “transducers” or chemical receptors.

To investigate how PYY may act to regulate I-FABP expression, we have used the Berkeley Rat Intestinal Epithelial hybrid cells (hBRIE 386i cells) as a model. The generation and characterization of these small intestinally derived cell lines have been described previously (32). These cells retain many characteristics of the enterocyte in situ such as cell polarity, apical microvilli, tight junctions, and others. Similar to the intestinal mucosa, these cells also exhibit a replicating cell population and a nonreplicating population. The nonreplicating cells can further be divided into nondifferentiated and differentiated phenotypes. The differentiated cells endogenously express I-FABP (33) and likely contain its entire genome. In the present study, we report that these cells also express PYY receptors. In light of previous observations that oleate is a major ligand for I-FABP and that luminal oleate also directly induces secretion of PYY, we present data to support the hypothesis that PYY may be part of a feedback regulation system for FFA processing in the intestine.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions—Subclones of the rat intestinal hybrid cell line (hBRIE 386i cells) that express I-FABP, hBRIE 386i cells (32, 33), were used in the present study. The hBRIE 386i cells were maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% bovine calf serum (BCS) (HyClone Labs, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). The cells were grown in multiwell dishes or T25 flasks (Corning, Corning, NY) and kept in an atmosphere of 5% CO2 and 95% air at 37 °C. Cells were grown on soft collagen type I gels, prepared from rat tails as described previously (34), or grown directly on tissue culture-treated plastic. Cells were seeded at high densities, 1 × 104 cells/T25 and 2 × 105 cells/well in 24-well dishes, unless otherwise indicated. Cells grown on tissue culture-treated plastic were harvested by trypsinization with 0.05% trypsin-EDTA (Life Technologies, Inc.) at 37 °C. Cells grown on collagen gels were harvested by treatment with 0.1% collagenase type I (Sigma) at 37 °C.

To study the regulation of expression of I-FABP, hBRIE 386i cells were grown on the collagen gels to confluency in the presence of Iscove’s modified Dulbecco’s medium containing 10% BCS. Experimental conditions were initiated on day 7 of confluency (unless otherwise indicated) by replacing the culture medium with limiting medium (Iscove’s modified Dulbecco’s medium containing 0.1% BCS and 4 μg/ml transferrin) with or without the factors to be tested, or with regular 10% BCS medium, as described previously (33). During the experimental conditions, one-half of the medium was replaced every day. Test factors added to the cells were insulin (Sigma) at 10 nM, human PYY (American Peptide Co., Sunnyvale, CA) at 10 nM to 1 μM, somatostatin-28 (Bachem, Torrance, CA) at 100 nM, and glucagon-29 (Bachem) at 100 nM.

Generation of Antiserum—Antisera were generated in guinea pigs using full-length recombinant I-FABP corresponding to the previously published amino acid sequence 1–132 (26), with an extension of two additional amino acids (Gly-Ser) at the C-terminal end. An S-ter-rase fusion protein was prepared by cloning of the I-FABP cDNA, generated by amplification of reverse-transcribed mRNA isolated from rat intestinal epithelial cells, into a pGEX-2T expression vector (Pharmacia Biotech Inc.). The fusion protein was isolated from bacterial lysates by glutathione-Sepharose 4B affinity chromatography, eluted, and cleaved by treatment with 2900 units/mg (Sigma) according to methods of the manufacturer. Isolated I-FABP was coupled to keyhole limpet hemocyanin (Sigma) using 1-ethyl-3(3-dimethylaminopropyl)carbo- midi (Sigma) according to the procedure described previously (12). Three guinea pigs were injected subcutaneously with the protein conjugate, corresponding to 40 μg of protein per injection by methods described previously (33). The response to immunization in each animal was determined by immunoblotting. A cytosolic preparation of rat intes-tinal epithelial cells was used as a reference for I-FABP immunore-activity. Soluble fractions of rat liver, glutathione S-transf erase-I- FABP, and glutathione S-transferase-expressing Escherichia coli BL 21 were prepared and used for the screening to determine antibody specificity. Animals that responded with high titers were anesthetized and bled from the heart usually three times (5 ml) 10 days apart.

Immunoblot Analysis—Cells were lysed in the culture flasks by the addition of 0.5% (v/v) Nonidet P-40 in TETN 250 (250 mM NaCl, 5 mM EDTA, and 25 mM Tris-Cl, pH 7.5), 1 mM T25 flask, containing 50 μM phenylmethylsulfonyl fluoride (Sigma) for 15 min at 4 °C. The lysates were collected and centrifuged at 15,000 × g for 3 min. Total soluble protein was determined according to the Bradford method (35) using the Bio-Rad protein assay reagent. Equal amounts of cytosolic protein were separated using 15% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad), according to standard methods (36, 37). Blotting and incubation with a polyclonal guinea pig antibody against rat I-FABP (gp 5111) antisera were as described previously (33). A peroxidase-coupled goat-anti-guinea pig IgG (Jackson Immunochemicals, West Grove, PA) was used as second antibody and detected by utilizing the enhanced chemiluminescence system (DuPont NEN). For the peroxidase-catalyzed reaction, dilutions of the cytosolic protein fractions were tested to determine that the amount of protein applied to the gel was in the linear range of the reaction. Films from the immunoblotting experiments were scanned using a GS-700 Imaging Densitometer (Bio-Rad) and quantified as described previously (33).

Preparation of Probes and Ribonuclease Protection Assays (RPAs) —Total RNA was isolated from the cells by a method described previously (38). Antisense and sense RNA probes for rat I-FABP, L-FABP, and β-actin were prepared for RPAs and for in situ hybridization. Plasmids containing rat cDNAs for I-FABP (pCRII-I-FABP, generated from reverse-transcribed mRNA isolated from rat small intestine), L-FABP (pGLF-1) (a gift from Dr. J. J. Gordon, Washington University School of Medicine) and a plasmid containing the cDNA for rat β-actin (a gift from Dr. S. H. Mellon, University of California, San Francisco, CA) were used to synthesize antisense and sense probes for the hybridization reactions. The plasmids were linearized and then transcribed by T7 and SP6 polymerases using the Ambion Maxiscript in vitro transcription kit (Ambion, Austin, TX) in the presence of [α-32P]UTP (800 Ci/mmol) or [γ-32P]UTP (3000 Ci/mmol) (DuPont NEN) as described previ-ously (33). The transcription mixtures were separated on 5% polyacrylamide/8 M urea gels, and the bands corresponding to labeled full-length transcripts were isolated. The size of the labeled RNA transcripts were as follows: I-FABP antisense, 474 nt; L-FABP sense, 448 nt; L-FABP antisense, 368 nt; L-FABP sense 378 nt; and β-actin antisense, 250 nt. After hybridization of the probes to their respective mRNAs followed by RNase treatment, the protected fragments corresponded to 414 nt for I-FABP antisense, 333 nt for L-FABP antisense, and 150 nt for β-actin antisense.

In a typical protection assay, the labeled RNA probes for I-FABP and β-actin and total RNA (5–20 μg/sample) were mixed and incubated according to the method described for the Ambion RPA procedure. The protected fragments were separated on 5% polyacrylamide/8 M urea gels and visualized by autoradiography as described previously (38). For quantitative studies, the amount of RNA used in each reaction was predetermined to be in the linear range of the assay by adding increasing amounts of sample RNA to the reaction mixture. Autoradiograms from the RPAs were scanned using a GS-700 Imaging Densitometer (Bio-Rad) and quantified as described previously (33).

Tissue and Cell Preparation for In Situ Hybridization—
was prepared by hypotonic lysis in 5 mM HEPES (Sigma), pH 7.4, and incubated for 16–22 h at 4 °C. The sucrose-equilibrated sections were transferred to molds containing the optimum cutting temperature compound (OCT; Miles, Inc., Elkhart, IN) and frozen on dry ice. Embdedded frozen tissue was sectioned, 10 μm/slide, at −20 °C using a cryostat and mounted on RNase-free glass slides, either the Probe On Plus slides (Fisher, Santa Clara, CA) or slides subbed in gelatin (300 Bloom; Sigma) and poly-l-lysine (M₄ > 300,000; Sigma). Slides with mounted sections (3–4 sections/slide) were then stored at −80 °C until used.

Culture flasks containing cells grown on soft collagen gels were rinsed with PBS, placed on ice, and fixed for 3 h at 4 °C by the addition of 5 ml fixative per T25 flask. The cell layers (cells fixed to the collagen gel) were removed, rinsed in ice-cold PBS, placed in 30% sucrose in PBS, and equilibrated for 16–22 h at 4 °C. The equilibrated cell layers were cut in 2–3-cm² pieces that were rolled and transferred to molds containing the OCT compound (OCT; Miles, Inc., Elkhart, IN) and frozen on dry ice. The tissue sections were incubated with 100 μg/ml bacitracin (Sigma) and 1 mM phenylmethylsulfonyl fluoride for 15 min at 4 °C, followed by homogenization using a loose-fitting glass pestle. The homogenates were centrifuged at 15,000 × g for 3 min at 4 °C, and pellets were washed with PBS and stored at −80 °C until used. The radioisotope assay was performed using the method described previously (13). The crude membrane pellets were resuspended in receptor binding buffer consisting of 20 mM N-morpholinoethanesulfonic acid, pH 6.8, 120 mM NaCl, 5 mM MgCl₂, 4.7 mM KCl, 1 mM EGTA, 0.5% (w/v) bovine serum albumin, 1 mg/ml bacitracin, and 0.01 mg/ml leupeptin (Sigma).

In a typical binding experiment, the crude membrane preparation (100 μg protein) was mixed with [125I]-labeled PYY in a total volume of 100 μl in receptor binding buffer for 16 h at 4 °C. Competitive displacement of [125I]-labeled PYY was determined in the presence of 5 pM to 100 nM PYY, and nonspecific binding was determined by the addition of 1 μM PYY. Bound and free [125I]-labeled PYY was separated by centrifugation at 15,000 × g for 3 min at 4 °C, and the pellets were washed in ice-cold PBS, re-centrifuged, and counted using a gamma counter (Beckman Instruments). Triplicates of each sample were tested in every assay, and the results were expressed as a percentage of bound label over total bound label after subtracting nonspecific binding. Each binding assay was repeated at least twice.

For receptor autoradiography, the cells were grown on the soft collagen gels in 24-well dishes as described above. On days 3–7 past initial confluence (cells 2 days after seeding in log phase), the medium was changed to Iscove’s modified Dulbecco’s medium containing 0.5% (w/v) bovine serum albumin, 0.1 mg/ml bacitracin, 50 μM phenylmethylsulfonyl fluoride, and [125I]-labeled PYY in a total volume of 0.5 ml. The cells were incubated with label for 30 min at 37 °C, and nonspecific binding was determined in the presence of 1 μM PYY. The binding reaction was stopped by transferring the cell layers (cells attached to the gels) to ice-cold PBS, pH 7.4, for 10 min and then incubated in fixative (4% paraformaldehyde in PBS, pH 7.4) for 3 h at 4 °C. The fixed cell layers were washed in ice-cold PBS for 20 min and heat-mounted on microscope slides precoated with Mayer albumin fixative (41). The mounted cell layers were dried at 37 °C for 16 h, coated with nuclear emission NTB2 (Kodak), and developed after different exposure times (2–3 weeks later) to determine that exposure was in the linear range.

Labeling with Bromodeoxyuridine (BrdUrd) and Immunocytochemistry—hBRIE 3801 cells were grown on collagen type I gels in T25 tissue culture flasks (Corning) as described. At 7 days after initial confluence, the cells were incubated with 100 μM bromodeoxyuridine (BrdUrd; Sigma) for 40 min and harvested with collagenase and trypsinized as described above. Dispersed cells were resuspended in PBS to a final concentration of 1 × 10⁶ cells/ml and placed on slides by cytopinning (Shandon Southern Products, Cheshire, United Kingdom). 1.5 × 10³ cells/slide. The cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 1 h, rinsed in PBS, dehydrated in 70% ethanol, and immersed in 1% (v/v) HCl for 1 h at 37 °C. Slides were rinsed in 0.1 M borate buffer, pH 8.5, and nonspecific binding was blocked by incubation in blocking buffer consisting of normal goat sera dilution 1:50 in PBS for 5 min. Sections were then stained for I-FABP and anti-BrdUrd using the methods described previously (33) or double immunostained using an antisera mixture containing mouse anti-BrdUrd (Dako, Carpenteria, CA) at a dilution of 1:40 and anti-I-FABP gp 1100 at 1:50 in blocking buffer, incubated for 30 min at 24 °C, and rinsed with PBS. The secondary antibody mixture was added containing fluorescein isothiocyanate–coupled goat anti-mouse and rhodamine-coupled goat anti-guinea pig (Jackson ImmunoResearch, West Grove, PA). Each solution was added at the final dilution of 1:50 in blocking buffer and the sections were incubated for 30 min at 4 °C. Slides were rinsed in PBS and mounted. Staining was detected using Nikon Optiphot (Nikon Corp., Tokyo, Japan), and images were captured using a Sony DK5000 CCD camera (Sony Corp., Tokyo, Japan).

Quantitation of In Situ Hybridization, Cytochemistry, and Autoradiography—Cell or tissue sections were viewed under a Nikon Optiphot microscope using a ×20 objective lens with differential interference contrast optics and condenser. Each image was captured and recorded twice using a digital charged coupled device (CCD) camera (Sony DK5000) and stored as 1100/1500 pixel files. The first recorded image of the cell or tissue section was captured utilizing darkfield illumination. Under this condition, only the autoradiographic grains would appear, and the tissue was not visible. The second image of the same field of objective was recorded by differential interference contrast, which gave maximum resolution of tissue and diminished contrast of the autoradiographic grains. Quantitation of the radiographic grain distribution was performed only on the darkfield images. Therefore, the analysis of the distribution of autoradiographic grains was performed in the ab-
RNA was isolated and hybridized to antisense probes for I-FABP and hBRIE 380i cell population, cells were incubated from 6 h to 3 days in either limiting (A; 0.1% BCS) or normal (B; 10% BCS) media. Total RNA was isolated and hybridized to antisense probes for I-FABP and β-actin using RPAs. The protected fragments (I-FABP, 414 nt, and rat β-actin, 150 nt), corresponding to 10 μg of total RNA, were separated and quantitated using 5% polyacrylamide/8 M urea gels as described under “Experimental Procedures.” The highest level of induction was observed after 6 h of incubation with PYY in the normal media, and this increase gradually returned back to control levels after 3 days of treatment (B). A similar trend was observed in the limiting media, although the magnitude of the induction was smaller (A). The ratio of the protected fragments for I-FABP and β-actin in each sample was compared with that of control cells not treated with PYY (Ctl). RNA from both control and PYY-treated cells were analyzed under identical conditions. In the representative autoradiogram shown, the 6-h controls were included from a separate gel in the same experiment. No changes in I-FABP mRNA levels were detected between the 6-h and 1-day controls under any conditions. A mixture of yeast tRNA and the two probes digested with RNase was used as a control for hybridization specificity (probe ctrl).

sence of the tissue image. After the darkfield images were quantified, they were layered and merged with the differential interference contrast images. A photographic montage (from each new image) was then constructed, and the autoradiographic grain distribution pattern was correlated with the corresponding cell populations or tissue regions.

The quantitation of the autoradiographic grains was accomplished using IP Labs Spectrum (Signal Analytics Corp., Vienna, VA) image analysis system. For each micrograph, the number of autoradiographic grains as well as their percentage of occupation over a uniform rectangular region of interest, (chosen as 225 μm²) was determined. As part of the analysis, it was established that there were no difference in the average number of labeled cells between the test and control samples. It was, therefore, assumed that the changes in average labeling per cell area reflected the change in mRNA levels induced by the test conditions. Because the grain count was subject to variation due to image threshold and segmentation, we chose to base our calculation on the percentage of developed emulsion over a constant area within the region of interest at a fixed threshold. Background measurements were obtained from sections processed in parallel with the sense probe. For the autoradiographic data derived from the in situ hybridization studies, both the background and the values obtained from the sense strand hybridization (which in all cases was equal to the background) was subtracted from the values obtained from the antisense hybridizations. The values obtained for the percentage of occupied areas of each region of interest was analyzed by a one-way analysis of variance using Dun-can’s test to determine sample means that significantly differed. All values used were absolute and not normalized between samples. In this way, the values represented the most conservative estimate of the differences between sample groups.

RESULTS

Effect of PYY on the Expression of I-FABP as Determined by Immunoblotting and RPAs—To determine if PYY could have an effect on I-FABP expression in the total heterogeneous hBRIE 380i cell population, cells were incubated from 6 h to 3 days in the presence of physiological concentrations of the hormone. In preliminary studies, a 2.0-fold induction of I-FABP message levels was observed in the presence of 100 nM PYY for 6 h in normal culture medium (10% BCS) (Fig. 1B). The elevated mRNA levels decreased to 1.2-fold of control values after 24 h and returned to control levels after 3 days of continuous treatment with PYY (Fig. 1B). To minimize potential effects on gene expression by other factors in the calf serum, we used limiting media (0.1% BCS), which was sufficient to maintain the hBRIE 380i cells for at least 7 days (33). In the presence of 100 nM PYY, cells incubated in the limiting media showed a time-dependent effect on I-FABP mRNA levels similar to that found with the normal media, although the magnitude of induction was less, 1.4-fold after 6 h and back to control levels already after 24 h (Fig. 1A). When cells were incubated with increasing concentrations of PYY (0.1 nM-1 μM) for 3–6 h, no significant effect of dose on message levels in the total cell population was observed. These preliminary data demonstrated a maximal increase in mRNA of 1.35 ± 1.23-fold (n = 3) when the cells were incubated with 100 nM PYY for 6 h (Fig. 1B and Table I), a dose within the physiological range (12). Although the changes in mRNA levels were found to be consistently elevated after 6 h treatment, this increase in transcript expression level was too small when compared with control values to be of statistical significance.

To determine whether the PYY-induced changes in mRNA levels paralleled alterations in protein expression, relative changes in I-FABP expression in the total heterogeneous cell population were determined. Contrary to the observed induction of message after 6 h, an induction of protein was detected only after 3 days of PYY treatment. During this 3-day period, protein levels were increased 2-fold above the control values in the presence of both 10 and 100 nM PYY (Fig. 2). With the addition of 1 μM PYY, I-FABP mRNA levels plateaued at 2–3-fold above control levels (Fig. 2). hBRIE 380i cells were also cultured in the presence of insulin, glucagon, and somatostatin to test if these major metabolic peptide hormones could alter the PYY-induced effect on I-FABP expression in the heterogeneous cell population. No significant changes in message levels could be detected in cells that were incubated with insulin (10 nM), glucagon (100 nM), or somatostatin (100 nM) together with PYY for 6 h compared with cells treated with PYY alone (Table I).

Distribution of I-FABP and Its Transcripts in Whole Tissue and in hBRIE 380i Cells—To determine if the I-FABP response to PYY occurred in either the differentiated fat-polarizing cluster cells or the surrounding less mature dividing sublayer cells, we first determined whether the distribution patterns of I-FABP mRNA and protein in ileal tissue were analogous to those observed in the hBRIE 380i cells. In these studies, we measured I-FABP immunoreactivity by utilizing a polyclonal guinea pig anti-I-FABP antisera (gp1100). This antibody, which was generated to recombinant I-FABP, demonstrated no cross-reactivity with L-FABP either by immunoblot analysis or

### Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mRNA levels</th>
<th>% of control, n = 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture media alone</td>
<td></td>
<td>100.4 ± 0.1</td>
</tr>
<tr>
<td>PYY 100 nM</td>
<td></td>
<td>135.1 ± 1.2</td>
</tr>
<tr>
<td>PYY 100 nM + Som* 100 nM</td>
<td></td>
<td>74.4 ± 4.9</td>
</tr>
<tr>
<td>PYY 100 nM + Ins 10 nM</td>
<td></td>
<td>93.4 ± 5.2</td>
</tr>
<tr>
<td>PYY 100 nM + Gluc 100 nM</td>
<td></td>
<td>99.0 ± 18.0</td>
</tr>
</tbody>
</table>

* Som, somatostatin-28; Ins, insulin; Gluc, glucagon-29.
by immunocytochemistry. In tissue, I-FABP immunoreactivity was found from the lower three-fourths of the villus, starting from a region proximal to the proliferative zone of the crypts, and extending to the villus tips. These findings agree with previous observations by others (42) (Fig. 3). As in the intestine, I-FABP was only expressed in nonreplicating hBRIE 380i cells (Fig. 4). Cells examined 7 days after confluence expressed I-FABP in populations that were BrdUrd-negative (Fig. 4, A and B). I-FABP examined in cells that were subconfluent or in log phase also did not express I-FABP (Fig. 4, C and D).

Although the distribution of both mRNA and protein has been reported for L-FABP in the intestine (42–45), there has been no previous reports for the distribution pattern of I-FABP in the intestine (42–45). As shown in Fig. 6, I-FABP immunoreactivity was confined to an area from mid-villus to the villar tips (Fig. 5, A and B). I-FABP was also found to be expressed in a subpopulation of nondividing cells. To further confirm that only the nondividing cell populations were expressing I-FABP, hBRIE 380i cells were harvested at log phase and then cytospun, fixed, and double immunostained as above (C and D). No immunoreactive I-FABP was detected in the dividing cells (D). Bar, 50 μm.

FIG. 3. Montage of light micrographs of the immunocytochemical localization of I-FABP in the rat ileum. Indirect immunofluorescence was performed on 8-μm frozen longitudinal cross-sections of tissue using the specific anti-I-FABP guinea pig antibody, gp 1100, at a final dilution of 1:200 as the primary antiserum and visualized by use of a secondary antibody, goat anti-guinea pig (1/100), coupled to fluorescein isothiocyanate. No staining was observed when the primary antisera were replaced with preimmune guinea pig sera or when gp 1100 was preabsorbed with I-FABP for specificity control (data not shown). I-FABP immunoreactivity was confined to an area from mid-villus extending to the villar tips. Bar, 100 μm.

FIG. 4. Light micrographs of hBRIE cells treated with BrdUrd. In A and B, hBRIE 380i cells were grown to 7 days past confluence, incubated with 100 μM BrdUrd, and then dispersed, cytospun onto microscope slides, fixed, and double immunostained for I-FABP and BrdUrd as described under “Experimental Procedures.” The arrows indicate examples of cells that were immunopositive for I-FABP (as depicted by the red rhodamine immunofluorescence). There was no overlap in cells that were dividing, as depicted by the uptake of BrdUrd and green fluorescein isothiocyanate fluorescence (A), with those synthesizing I-FABP (B). I-FABP was also found to be expressed in a subpopulation of nondividing cells. To further confirm that only the nondividing cell populations were expressing I-FABP, hBRIE 380i cells were harvested at log phase and then cytospun, fixed, and double immunostained as above (C and D). No immunoreactive I-FABP was detected in the dividing cells (D). Bar, 50 μm.

Established as differentiated nonreplicative clusters of cells (which were 1–2 cells thick composed of elongated cuboidal epithelial-like cells) as noted between arrows a and b of Fig. 7. In contrast, the autoradiographic grain density, indicative of message for I-FABP, was not significantly above background in the nondifferentiated replicative (single-cell thick) region of the cell monolayer (between arrows b and c). Effect of PYY on the Expression of I-FABP in hBRIE 380i Cells as Determined by Quantitative In Situ Hybridization—To test if PYY might be inducing changes in expression of I-FABP message only in the corresponding subpopulation of hBRIE 380i cells with the highest abundance of transcripts (the differentiated cluster cells as shown in Fig. 7), in situ hybridization was performed on cells that had been treated with PYY for 6 h. Analysis of the distribution density and percentage of area of label over a uniform region of interest (as described under “Experimental Procedures”) revealed that a 6-h treatment with
FIG. 6. Demonstration of specificity for probes and distribution of mRNAs for I-FABP and β-actin in the hBRIE 380i cells and rat ileum. Probes for antisense rat I-FABP (474 nt) and β-actin (290 nt) and a sense probe for I-FABP (448 nt) were labeled with 32P-labeled and hybridized to cryostat sections (10 μm) of rat ileum and the hBRIE 380i cells as described under “Experimental Procedures.” Hybridization with the I-FABP antisense probe was only observed in the villus area in ileal sections (upper left panel) and in clusters of hBRIE 380i cells rather than monolayer cells (lower left panel). Hybridization with the β-actin antisense probe demonstrated an even distribution of message in both tissue (upper right panel) and the hBRIE 380i cells (lower right panel). When ileal tissue (upper middle panel) and hBRIE 380i cells (lower middle panel) were hybridized with the I-FABP sense probe, no autoradiographic grains were observed either in the tissue or the cells.

FIG. 7. Light micrographic montage demonstrating the localization of I-FABP mRNA to the differentiated clusters in the hBRIE 380i cells. Cells, grown to confluency for 7 days on collagen type I gels, were fixed, embedded for longitudinal cryostat sectioning, and hybridized to a [32P]UTP-labeled antisense I-FABP probe (474 nt) under RNase-free conditions as described under “Experimental Procedures.” Nondifferentiating differentiated cluster cells (between arrows a and b) displayed the highest density of autoradiographic silver grains compared with the less mature proliferating monolayer cells (between arrows b and c). For each slide, the grain densities in the respective cell populations were analyzed and compared as percentages of a series of identical areas of interest (of 219–μm2 areas), over the entire objective field as described under “Experimental Procedures.” I-FABP mRNA abundance in the differentiated clusters was found to be from 3–5-fold higher than in the monolayer cells. The digital images were processed as in Fig. 5 and as described in the text. Bar, 50 μm.

100 nm PYY induced a greater than 5-fold increase in I-FABP expression in the differentiated cell clusters (Fig. 8 and Table II), which was significant. The nondifferentiated proliferative cell region of the monolayers displayed no significant differences between PYY-treated and untreated cells, although there appeared to be a small increase in cells treated with PYY (Table II). Because of the limits of sensitivity of the technique, it cannot be concluded that I-FABP message was totally lacking in the proliferative cells. However, in our hands, I-FABP transcripts were confined to the differentiated cell populations of the cell clusters (n = 10 whole monolayer preparations of hBRIE 380i cells).

Identification and Distribution of PYY Receptors on the hBRIE 380i Cells—To determine the presence of cell membrane
receptors for PYY in the hBRIE 380i cells, we used a radioreceptor assay specific for PYY binding sites. Iodinated PYY was purified by gel filtration chromatography, and fractions were screened for receptor binding using a membrane preparation of the hBRIE 380i cells. A number of different membrane preparations of the hBRIE 380i cells were tested to establish a binding assay of maximum sensitivity. We found that a crude membrane preparation was sufficient in most experiments and that the relatively high levels of nonspecific binding could be eliminated by further purification of \(^{125}\)I-labeled PYY. The specific activity of \(^{125}\)I-labeled PYY was estimated to be \(500 \text{ C/mmol (assuming 60% incorporation). The number of receptors in the hBRIE 380i cells was estimated to be in the range of 450–900 receptors/cell. This range agrees with other studies described above with membranes prepared from dividing cells grown on plastic for 2 days. Displacement of label with unlabeled PYY resulted in specific binding of 73.2 ± 0.2% for 5 pm, 66.7 ± 0.3% for 10 pm, 45.2 ± 0.8% for 50 pm, 14.1 ± 0.7% for 100 pm, 45.5 ± 0.3% for 1 nm, 0 ± 0.4% for 10 nm, 6.0 ± 0.5% for 100 nm, and 6.3 ± 0.3% for 1 \(\mu\)m \((n = 3). Total binding, without unlabeled PYY, was used as a reference at 100% and constituted 7.2 ± 0.9% of total added \(^{125}\)I-labeled PYY. Based on these displacement studies, the half-maximal inhibition concentration (IC\(_{50}\)) was in the pm range (5–50 pm), with no significant difference between proliferating and more mature cells (days 7–11 after initial confluency).

The results from the in situ hybridization studies, indicating that I-FABP mRNA was mostly present in the differentiated cell clusters, suggested that receptor binding might be higher in a specific subpopulation of cells. Such a possibility could explain the small changes observed in mRNA levels in response to PYY in the total heterogeneous cell population and the selective induction of I-FABP in the differentiated cell clusters. To test for such a subpopulation, we performed receptor autoradiography of \(^{125}\)I-labeled PYY bound to the intact unsectioned cell layers grown on collagen gels (Fig. 9) and examined these preparations as whole mounts. Results from these studies clearly demonstrated that PYY-binding sites were equally present both in the more differentiated cluster cells and the surrounding less mature dividing monolayer cells (Fig. 9, B and C, and Table III). In addition, receptor autoradiography also revealed the presence of PYY-binding sites in preconfluent hBRIE 380i cells, which were mainly dividing cells not yet in the cluster-forming stage (Fig. 9A and Table III). No statistical differences in receptor density could be observed in the two subpopulations of cells.

**DISCUSSION**

The results in the present study demonstrate that physiological concentrations of PYY can induce I-FABP mRNA expression in a time- and differentiation-dependent manner. Examining the effects of PYY on I-FABP mRNA expression by cell population (utilizing quantitative measurements from autoradiograms generated by in situ hybridization) over a 5-fold increase in transcripts was observed only in cells that were differentiated and grouped in clusters. No change in message was detected in the cells comprising monolayers outside of the cell clusters. Because 75 and 80% of the cells in culture were in non-I-FABP-expressing monolayers, the measurement of I-FABP and its message by Western blots and RTPCs was not sufficiently sensitive to detect significant changes in small groups of cells. The BrdUrd and immunocytochemical studies established that both the dividing cells as well as the nondividing (less mature) subpopulation of cells did not express I-FABP and that only the more differentiated nondividing cluster cells expressed I-FABP. The results from mRNA in situ hybridization in the hBRIE 380i cells confirmed that the
mRNA was also predominantly localized to this subpopulation of nondividing mature cluster cells. This was likely the reason the induction of mRNA in response to PYY, as measured in the total heterogeneous hBRIE 380i cell population, was relatively small (1.4-fold) and not statistically significant. However, these results were similar to earlier published in vivo studies of Bass (47), who reported a maximal 1.4-fold induction of I-FABP in the ileal mucosa from rats fed a high fat diet.

The pattern of I-FABP gene expression has been investigated previously as a model for determining mechanisms involved in intestinal epithelial cell proliferation, differentiation, and development. In these studies, I-FABP promoter-growth hormone transgenes were used (48, 49), as well as intestinal isografts (50). One conclusion derived from using these techniques was that correct temporal and spatial I-FABP gene expression in the intestinal mucosa was independent of extracellular factors. It has, however, been questioned if I-FABP expression, in addition to its differentiation-dependent regulation, could also be modulated by luminal and/or circulating factors. The proposed “programming” of I-FABP expression at the level of the progenitor cell has been based on data derived from transgenic mice. In these studies, the −1178 to 28 nt of the rat I-FABP gene was used to identify specific patterns of gene expression in the gut (51). A number of elements within this region have been identified as potential targets for regulation by both hormonal and unidentified intestine-specific nuclear factors (51–53). One of these sequences was a potential CCAAT/enhancer-binding protein element, and specific isoforms of CCAAT/enhancer-binding protein have been proposed to play a role in the differentiation of enterocytes (54). Another potential site of hormonal gene regulation is a 14-nt sequence that is present in three copies in the rat I-FABP promoter (27), which has a high degree of homology with elements known to bind members of the steroid hormone receptor family such as the chicken ovalbumin upstream promoter transcription factor (55). Receptors for retinoic acid, thyroid hormone, vitamin D₃, and the peroxisome proliferator activator receptor also bind to sequences similar to the 14-nt repeat (56–58), as well as other members of the steroid hormone receptor family, such as hepatic nuclear factor 4 and apolipoprotein regulatory protein 1 (59). It remains to be determined whether the elements necessary for tissue-specific expression of I-FABP could also be activated by extracellular factors.

Other studies, using the intact animal, have demonstrated that I-FABP expression could be both stimulated and inhibited in a region-specific manner by high and low fat diets, respectively (47). It was observed that I-FABP induction in intact rats occurred only in the ileum and not in the jejunum and was extend to the villar tips, in agreement with earlier in situ hybridization studies in rat small intestine (43, 44). The axial position of I-FABP immunoreactivity and mRNA would indicate that the expression of the protein is maintained in the nonreplicating, terminally differentiated cells up to the point of their exfoliation. These observations indicate that the two proteins, despite similarities in distribution, could be controlled by different regulatory systems.

The distribution pattern for I-FABP and I-FABP mRNA in the rat ileum, with whole-mounts on glass slides, then fixed and incubated with radiolabeled PYY. Slides were immersed in photographic emulsion, incubated, and developed as described under “Experimental Procedures.” The autoradiographs were examined, and darkfield images were captured using Sony DR5000 digital camera and quantitated using IP Labs Spectrum image analysis software. 10 to 20 areas of 225 μm² (regions of interest) were quantitated for silver grains for each monolayer. Each value is the mean ± S.E. for six monolayers. PYY binding was found to be independent of the cell population or the state of replication.

![Image](http://www.jbc.org/)
hypothesized that under conditions of increased dietary fat intake, both I-FABP and L-FABP in the ileum could be specifically induced to compensate for the elevated fatty acid levels. It was also proposed that the lack of change of I-FABP in the jejunum was due to it being maximally expressed, because this region of the gut was responsible for most of the fat absorption under normal conditions. More recently, it has also been reported that bezafibrate, a plasma lipid-lowering agent, only produced slight increases of 1.6–2-fold in I-FABP expression levels in both the intact rat small intestine and in intestinal explants (60, 61). Our observation that the I-FABP message was maintained in the older differentiated cell population in both tissues and cell lines and that PYY can modulate this message in vitro suggests strongly that I-FABP transcripts can be regulated by extracellular factors well after the cells have migrated out of the proliferative cell crypt region. Because the hBRIE 380i cells were derived from the hybridization of an isolated terminally differentiated small intestinal enterocyte with a spontaneously transformed small intestinal mucosal epithelial cell, it is likely that the entire I-FABP gene has remained intact. Elements upstream of −1178 that might be important in regulating I-FABP expression might not be necessary for its constitutive expression or “positional address.” It can be speculated that PYY or other gastrointestinal peptides might activate factors that stimulate binding of nuclear proteins to any of the characterized elements or to other undescribed promoter sequences.

In the present studies, an induction of I-FABP mRNA was demonstrated in hBRIE 380i cells in the presence of PYY for 6 h, although no significant induction of protein expression could be detected in the heterogeneous cell population before 3 days. This finding parallels our observation that insulin-induced inhibition of I-FABP expression was only detectable after 2 days of hormone treatment, despite an early decrease in mRNA levels (6 and 24 h, data not shown). In our previous studies, however, changes in individual cell populations were not examined. Although the protein turnover rate for I-FABP has not yet been determined, it is possible that the delayed increase in protein is a reflection of a relatively slow protein turnover rate. The half-life for I-FABP in the liver has been estimated to be 3.1 days (62). The half-lives for both L-FABP and I-FABP in intestinal epithelial cells are still unknown. If the turnover rate for I-FABP in the gut proves similar to that of L-FABP, it is possible that maximum protein concentration is reached when the differentiated enterocytes have migrated to the area of the villus tip, although increases in message levels occur at an earlier time. The differentiation pattern for hBRIE 380i cells in culture has been reported previously, and in several aspects, these cells differentiate in a pattern similar to the intestinal mucosa, i.e., there is a replicative nondifferentiated population of cells that develops into a differentiated nonproliferating population with tight junctions, polarity, and apically expressed micr PV (32, 33). If I-FABP expression in hBRIE 380i cells is similar to intact cells in situ, then maximal expression of protein in hBRIE 380i cells would occur when the cells that were previously exposed to PYY would have reached the more differentiated state. Therefore, a rapid change in I-FABP transcription rate and/or mRNA stability might be detectable at the level of protein only days after the initial incubation with PYY. In the present study, it was also observed that PYY-induced mRNA levels returned to control levels after 3 days of continuous treatment with PYY. This decrease in message after prolonged hormone exposure might indicate a down-regulation of receptor-mediated cellular signaling, a typical response to elevated levels of receptor agonists. It is also possible that maintenance of higher expression levels requires a higher concentration of factors, such as metabolic hormones and nutrients, than were present under our incubation conditions.

In the intact rat small intestinal epithelium, PYY receptors have been reported to be of the Y2-prefering subtype (63), whereas the Y1 subtype predominates in both human and rabbit colonic mucosa as well as in HT29 cells (46, 64). Competive binding studies demonstrated that the hBRIE 380i cells expressed high affinity PYY-binding sites, with displacement of labeled peptide in the picomolar range. This agrees with other studies, demonstrating IC50 values of 0.5 nM for NPY/PYY receptors in HT29 cells (46), 0.3 nM in PYSV-PCT cells (renal proximal tubule cell line) (65), 18–30 pm in neuroblastoma cell lines (66), and an IC50 of 31 pm in pancreatic vascular smooth muscle cells (9). Dissociation constants (Kd) of 0.4 nM (5) and 0.05–0.1 nM (67) have been estimated for NPY/PYY receptors on epithelial cells in the rat small intestine. A number of NPY/PYY/P receptor subtypes have also been identified in other tissues. All PYY/NPY receptors have been determined to belong to the G-protein-coupled superfamily of receptors (68), although specific G-protein subunits and signal transduction pathways have not been identified for every receptor type. Displaceable high affinity PYY-binding sites were present in equal amounts in both the differentiated and less mature subpopulations of the hBRIE 380i cells, whereas PYY induction of I-FABP mRNA occurred only in the differentiated cell population. It is possible that different receptor subtypes are present in the two-cell populations and/or that different G-proteins and signal transduction cascades are used. This could be one reason that only a subpopulation of the differentiated hBRIE 380i cells were measurably responsive to PYY induction of I-FABP transcripts. It is also likely that other intracellular factors present only in the mature differentiated cells are necessary for PYY responsiveness. It remains to be determined if PYY binding is to the same receptor (Y1- or Y2-prefering) in different cell populations or if the hBRIE 380i cells express another, as of yet unidentified, subtype of the NPY/PYY/P receptor family. Therefore, the action of PYY in the intestine may be a result of the integration of signaling events initiated by activation of more than one receptor subtype.

Several established mechanisms could be involved in the PYY mode of action on the gastrointestinal mucosa or the hBRIE 380i cells. For example, PYY could initiate a signal transduction cascade leading to direct activation (or deactivation of inhibitors) of transcription factors regulating I-FABP gene transcription analogous to activation of the MAP kinase cascade and nuclear factors in response to insulin receptor binding. Because we did not observe a significant increase in I-FABP mRNA during the shorter incubation period of 3 h, it is likely that changes in message levels at 6 h may be due to indirect mechanisms, such as mRNA stabilization or induction of transcription factors, rather than direct transcriptional effects on the I-FABP gene. It has been demonstrated previously that both olate and elevated intracellular levels of cAMP can induce mRNA expression for the closely related cytosolic adipocyte-FABP through mechanisms involving message stabilization and transcriptional activation, respectively (69, 70).

I-FABP binds long-chain fatty acids (≥C18:1) with nanomolar affinities (16) but has little or no affinity for medium-chain fatty acids (C8–C12) (71). Recent studies have strengthened previous proposals that L-FABP is directly involved in the trafficking of olate and other FFA (31). It is thus likely that I-FABP is placed at a potentially key point in determining the fate of FFA, either as metabolites and/or as enzyme activators. We have demonstrated previously that luminal FFA, such as...
oleate, directly stimulates PYY release. These observations lead to the possibility that in the intact animal, the concentration and position of a dietary FFA in the intestine can be monitored by receptor-secretory cells, such as PYY cells, and that these cells can respond by releasing PYY, which in turn alters the fate of the FFA in the cytosol of the enterocyte.

In the present studies, we have established that PYY induces I-FABP mRNA expression in intact cells, both in a concentration and time range, similar to that observed in whole animals given luminal FFA or fed a high fat diet. Although these studies did not determine if the induction of I-FABP is specific to PYY or a result of a more generalized response of the enterocyte to PYY, the data from both the mucosal tissue and the fate of the FFA in the cytosol of the enterocyte.

From a broader perspective, PYY modulation of I-FABP expression also brings to light a mechanism whereby luminal signals could modulate the expression of other proteins or products of differentiation in the intestinal epithelium through the release of intestinal regulatory peptides.

Acknowledgment—We thank Drs. M. A. Williams and H. Sui for insightful comments and suggestions during the preparation of the manuscript.

References

Evidence for a Role of the Gut Hormone PYY in the Regulation of Intestinal Fatty Acid-binding Protein Transcripts in Differentiated Subpopulations of Intestinal Epithelial Cell Hybrids

Gunnel Halldén and Gregory W. Aponte

doi: 10.1074/jbc.272.19.12591

Access the most updated version of this article at http://www.jbc.org/content/272/19/12591

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 69 references, 36 of which can be accessed free at http://www.jbc.org/content/272/19/12591.full.html#ref-list-1