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Apelin, a New Enteric Peptide: Localization in the Gastrointestinal Tract, Ontogeny, and Stimulation of Gastric Cell Proliferation and of Cholecystokinin Secretion

GUIYUN WANG, YOUNES ANINI, WEI WEI, XIANG QI, ANNE-MARIE O'CARROLL, TOHRU MOCHIZUKI, HUI-QUIN WANG, MARK R. HELLMICH, ELLA W. ENGLANDER, AND GEORGE H. GREELEY, JR.

Departments of Surgery (G.W., W.W., X.Q., M.R.H., E.W.E., G.H.G.) and Human Biological Chemistry and Genetics (H.-Q.W.), The University of Texas Medical Branch, Galveston, Texas 77555; Department of Physiology (Y.A.), University of Toronto, Toronto, Ontario, Canada M5S 1A1; Shizuoka Cancer Center Hospital and Research Institute (T.M.), Shizuoka 420-0853, Japan; Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology (A.-M.O.), University of Bristol, Bristol, United Kingdom BS8 1TH

Apelin is a recently discovered peptide that is the endogenous ligand for the APJ receptor. The aim of this study was to characterize apelin expression (mRNA levels) in the rat gastrointestinal tract and pancreas, to localize distribution of apelin peptide-containing cells in the stomach by immunohistochemistry, and to characterize the ontogeny of gastric apelin expression and peptide and the influence of apelin on gastric cell proliferation *in vitro*. Additionally, the effect of apelin on cholecystokinin (CCK) secretion and the involvement of MAPK, protein kinase C, and changes in intracellular Ca^{2+} in apelin-induced CCK secretion *in vitro* were examined. Northern analysis showed a maximal apelin expression in the stomach with a lower expression level in the intestine. Apelin expression was not detected in the pancreas. Immunohistochemistry revealed abundant apelin-positive cells in the glandular epithelium of the stomach. The ontogeny study showed a higher apelin expression in the fetal and postnatal rat stomachs when compared with the adult stomach. In contrast to apelin expression, apelin peptide was not detected in the rat stomach until 20 d of age and then increased progressively with age. Apelin was shown to stimulate gastric cell proliferation *in vitro*. Apelin also stimulated CCK secretion from a murine enteroendocrine cell line (STC-1); apelin-stimulated CCK secretion is mediated through MAPK but not by intracellular Ca^{2+} signaling. Together, these data indicate that apelin is an important new stomach peptide with a potential physiological role in the gastrointestinal tract. (*Endocrinology* 145: 1342–1348, 2004)

APELIN-36, THE ENDOGENOUS ligand for the APJ receptor, is a recently characterized peptide from the bovine stomach (1–3). Before the isolation of apelin, the APJ receptor was referred to as an orphan receptor because its endogenous ligand was unidentified. Apelin was discovered by monitoring extracellular acidification and inhibition of cAMP formation in response to various tissue extracts in a Chinese hamster ovary cell line transfected with the APJ cDNA (2). Rat, mouse, cow, and human apelin cDNAs have been characterized (2, 4). These cDNAs encode a 77-amino-acid preproprotein and a 36-amino-acid variant of apelin appears to be the parent peptide. In all species cloned to date, a sequence of 23 amino acids in the C-terminal region is conserved, implying that the C-terminal region is critical for biological activity. Based upon the presence of multiple proteolytic cleavage/processing enzyme sites (*i.e.* arginine and lysine residues) in the apelin proform, a number of apelin variants are expected. However, the processing of proapelin to smaller apelin variants and characterization of involved prohormone convertases has not been described. Apelin variants isolated from tissue extracts include apelin-36, -17, -16, and -13 (2, 4).

The purpose of the present study was to characterize apelin expression in the rat gastrointestinal (GI) tract, to localize apelin peptide in the rat and human stomachs by immunohistochemistry (IHC), to characterize the ontogeny of apelin expression and peptide in the rat stomach, and to assess the effect of apelin on gastric cell proliferation. Additionally, the influence of apelin on cholecystokinin (CCK) secretion, and the involvement of MAPK, protein kinase C (PKC), and intracellular Ca^{2+} signaling in apelin-induced CCK secretion *in vitro* were tested.

Abbreviations: $[Ca^{2+}]_i$, Intracellular calcium concentration; CCK, cholecystokinin; C_T, cyclin threshold; F16, fetal d 16; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; IHC, immunohistochemistry; P1, postnatal d 1; PKC, protein kinase C; poly $(A)^+$, polyadenylated $(A)^+$.

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Materials and Methods

Animals

Adult timed-pregnant, nursing female and male Sprague Dawley rats were maintained in an air-conditioned (24 ± 2 C) and light-regulated room (lights on, 0600–1800 h). All animal experiments were conducted in accordance with mandated standards of humane care and were approved by the Institutional Animal Care and Use Committee.

Apelin expression in the adult rat GI tract and IHC localization of apelin peptide in the adult rat and human stomach

Apelin mRNA levels in the adult rat GI tract were characterized by Northern analysis. *Ad libitum*-fed adult male Sprague Dawley rats were killed, and the full thickness of the stomach fundus, duodenum, jejunum, ileum, and colon were extirpated, rinsed with cold saline, and extracted for total cellular RNA. Total cellular RNA samples were then processed for isolation of polyadenylated $(A)^+$ [poly $(A)^+$] RNA (5, 6). Apelin expression was also analyzed in extracts of the rat pancreas using rat pancreas poly $(A)^+$ RNA that was prepared according to a previously described method (7). Apelin peptide in the adult rat and human stomachs was localized by means of IHC. Human stomach specimens were obtained from Brain and Tissue Banks for Developmental Disorders, University of Maryland (Baltimore, MD), and Birth Defects Research Laboratory, University of Washington (Seattle, WA).

Ontogeny of stomach apelin expression and peptide

Maximal apelin expression was found in the stomach fundus (*i.e.* oxyntic mucosa); therefore, the ontogeny of apelin expression was examined in the stomach. Fetal rat stomachs of both sexes at different stages of development [fetal d 16 (F16), F20, and F21] from timed-pregnant rats, stomachs of rat pups of both sexes at different ages [postnatal d 1 (P1), P3, P5, P7, P10, P16, and P24], and adult rat stomachs were harvested and extracted for RNA. In all dissections, care was taken not to include the pancreas. The entire fetal stomach was homogenized in a RNA extraction solution. For collection of pup and adult rat stomachs, the fundus was separated from the rumen and antrum, and the full-thickness fundus was homogenized in a RNA extraction solution. Total cellular RNA and then poly $(A)^+$ RNA were prepared. Litters were born at approximately 22 d gestation and were kept with their mothers until 21 d postpartum. For fetal and some early postnatal samples the stomach specimens from three to four littermates were pooled to constitute one sample. For IHC localization of apelin peptide, stomach fundus specimens (P1, P6, P13, P20, P40, and P60) were fixed in buffered formalin and then processed for identification of apelin-containing cells.

Chemicals and peptides

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise. Synthetic peptides were purchased from Bachem (Torrance, CA) and Phoenix (Belmont, CA) or synthesized by T. Mochizuki. The sequence of apelin-36 is LVQPRGPRSGPGPWQG-GRRKFRRQRPRLSHKGPMPF. Pharmacological inhibitors of MAPK (PD98059) and PKC (GF109203X) were purchased from Calbiochem Novabiochem (San Diego, CA). Oligo dT cellulose was obtained from Stratagene (San Diego, CA). Media and calf and horse sera were purchased from Mediatech (Herndon, VA), Hyclone (Logan, UT), and Invitrogen (Carlsbad, CA), respectively.

RNA purification and Northern blotting analysis

All tissue samples were removed quickly after animals were killed and immediately homogenized in 4 M guanidinium isothiocyanate containing 25 mM sodium citrate (pH 7.0), 0.5% sodium lauroylsarcosine, and 0.1 M β-mercaptoethanol. Extracts were frozen at −80 C until purification by ultracentrifugation over a cesium chloride cushion (2 ml, 5.7 M) (5, 6). Total cellular RNA samples were then extracted for poly $(A)^+$ RNA using oligo dT chromography. Poly $(A)^+$ RNA samples were separated on a 1% agarose gel (~10 μg/lane) in a 20 mM 3-[*N*-morpholino] propanesulfonic acid running buffer system (5, 6) and then transferred to a nylon membrane and subjected to Northern hybridization. Membranes were then stripped and rehybridized with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a reference mRNA species. ^{32}P-labeled probes prepared from Strip-EZ RNA kits (catalog no. 1366, Ambion Inc., Austin, TX) were used for Northern hybridizations. The cDNA probe for rat apelin was supplied by Lee *et al.* (8). Expression levels of apelin were quantitated by phosphoimaging.

Real-time RT-PCR analysis of apelin expression

Real time RT-PCR assays were done as described (9). Real time RT-PCR assays were used to determine relative gene expression on an Applied Biosystems (Foster City, CA) 7000 sequence detection system. Applied Biosystems Assays-By-Design containing a 20× assay mix of primers and TaqMan MGB probes (6-FAM dye-labeled probe) were used for the target gene, rat apelin (accession no. AF179679), and a predeveloped rat 18S rRNA (VIC dye-labeled probe) TaqMan assay reagent (P/N 4319413E) was used for internal control. Primers were designed to span exon-exon junctions so as not to detect genomic DNA. Primers and probe sequence were searched against the Celera database. The probe and primer sequences were as follows: probe, TGGCACTC-CACACACG; forward primer, GCTGCTCTGGCTCTCCTTGA; and reverse primer, CCATCTGGAGGCAGCATCA.

The efficiency of target gene amplification was validated using a reference amplification reaction. The absolute value of the slope of log input RNA amount *vs.* Δ cyclin threshold (C_T) was 0.06. One-step RT-PCR was performed with 40 ng total cellular RNA for both target gene and endogenous control. The TaqMan one-step RT-PCR master mix reagent kit (P/N 4309169) was used. The cycling parameters for one-step RT-PCR were as follows: RT at 48 C for 30 min, AmpliTaq activation at 95 C for 10 min, denaturation at 95 C for 15 sec, and annealing/extension at 60 C for 1 min for 40 cycles. Duplicate C_T values were analyzed in Microsoft Excel using the comparative $C_T(\Delta\Delta C_T)$ method as described by the manufacturer (Applied Biosystems).

IHC

Buffered formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized and rehydrated by passage through xylene and graded ethanol solutions. After deparaffinization, slides were treated with 1% H_2O_2 in PBS for 15 min, followed by microwave antigen retrieval at 100 C for 10 min in Dako target retrieval solution (Dako Corp., Carpinteria, CA) in an H2800 microwave processor (Energy Beam Sciences, Inc., Agawam, MA). After sequential 15-min incubations with 0.1% avidin and 0.01% biotin (Vector Laboratories, Inc., Burlingame, CA) to block endogenous avidin and biotin, slides were then incubated in 0.05% casein (Sigma) in 0.05% Tween 20 (Dako) in PBS for 30 min to block nonspecific protein binding. Primary rabbit antisera for apelin-36 (Phoenix) were applied to sections at a 1:300 dilution for 60 min. This apelin antibody recognizes all carboxyl-terminal fragments. Rabbit serum Ready-to-Use (InnoGenex, San Ramon, CA) was applied as a negative control. Biotinylated $F(ab')_2$ fragment of swine antirabbit Igs (Dako) served as the secondary antibody and was detected by streptavidin-horseradish peroxidase and colorized by diaminobenzidine (Dako). All antibody incubations and detection procedures were performed on a Dako Autostainer.

Slides were counterstained with Mayer's modified hematoxylin (Poly Scientific, Bay Shore, NY) before mounting and viewed under a Nikon Eclipse E600 microscope. Images were captured with a Nikon DXM1200 digital camera and ACT-1 (version 2.00) program.

Cell culture

A murine intestinal enteroendocrine cell line (STC-1) (10) that expresses and secretes CCK was grown in DMEM containing 2.5% fetal bovine serum, 15% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 C in 5% CO_2, and media were changed every 3–4 d. Cells were trypsinized, plated in six-well culture plates, and allowed to reach 60–80% confluence. On the day of the experiment, cells were washed twice with media (without serum) and then incubated with test agents in media. A human gastric epithelial cell line (SIIA) was used to study the influence of apelin on cell proliferation. SIIA cells were cultured in HAM's F-10 media with 5% fetal bovine serum. To examine the influence of apelin on cell proliferation, SIIA cells were cultured with synthetic apelin-36 or apelin-13 (10^{-8} M). Cells were plated in six-well plates initially at a density of 15×10^3 cells per well. Apelin was added daily starting 24 h after plating. Cells were counted daily.

RIAs

Media levels of CCK were measured using a standard RIA protocol (11) and an antiserum that detects CCK (antibody 5135, gift of J. Walsh,

UCLA, Los Angeles, CA). The intra- and interassay coefficients of variance are 5–7 and 10–12%, respectively.

Calcium imaging

Real-time recording of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was done in single STC-1 cells using a method reported previously (12, 13). STC-1 cells were plated on glass coverslips (25 mm) at a density of approximately $1.5–3 \times 10^5$ cells per coverslip, cultured for 48 h, washed with KRH (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, and 25 mM HEPES, pH 7.4) and loaded with 2 μM fura-2 AM (Molecular Probes, Eugene, OR) for 50 min at 25 C. Loaded cells were washed three times with KRH and incubated in KRH plus 0.1% BSA for 60 min at 25 C in the dark. Cells were then exposed to apelin or bombesin and imaged using a Nikon Diaphot inverted microscope (Garden City, NJ). The microscope is coupled to a dual monochromator system via a fiber optic cable (Photon Technology International, South Brunswick, NJ). Fluorescence was detected using an intensified charged coupled device camera (Dage-MTI Inc., Michigan City, IN), and images were processed using ImageMaster software (Photon Technology).

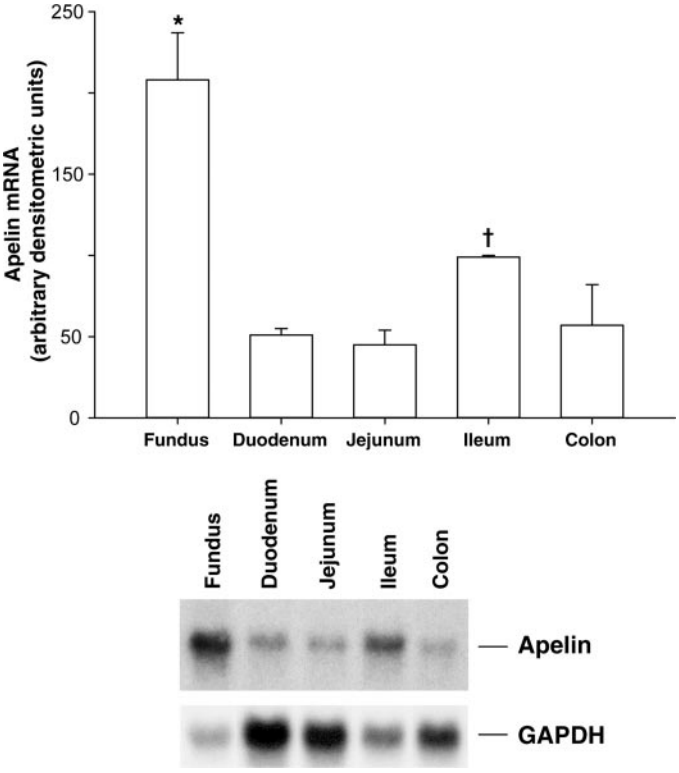

FIG. 1. *Top*, Apelin expression (mRNA levels) in poly $(A)^+$ RNA extracts of various regions of the adult rat GI tract. The highest apelin expression is measured in the stomach fundus. The mean ± SEM of three rats is shown. Apelin expression levels are not shown as a ratio (*i.e.* apelin mRNA/GAPDH mRNA) because GAPDH levels vary across tissue sites. *Bottom*, Northern hybridization showing the profile of apelin expression in the rat GI tract. The profile of GAPDH expression is shown as a reference gene. *, $P < 0.05$ *vs.* small and large intestine; †, $P < 0.05$ *vs.* duodenum, jejunum, and colon.

Statistics

Results are shown as means ± SE. Data were analyzed by a one-way or two-way ANOVA followed by the Newman-Keuls test where pertinent. Differences with a value of $P < 0.05$ were considered significant.

Results

GI distribution of apelin expression and IHC analysis of apelin peptide in the stomach

Northern analysis showed maximal apelin expression in the stomach with lower expression levels in the intestine (Fig. 1). Apelin mRNA levels were significantly ($P < 0.05$) higher in the ileum when compared with apelin mRNA levels in the duodenum and colon. Apelin is not expressed in the rat pancreas (data not shown). IHC examination of the adult rat stomach showed abundant apelin peptide in the cytoplasm of epithelial cells in the oxyntic mucosa (Fig. 2). A high-power magnification indicates that apelin peptide is stored in vesicle-like structures and that apelin staining is localized adjacent to the lumen of the gastric glands (Fig. 3). IHC examination of the adult human stomach showed apelin staining in the gastric epithelium (Fig. 2). IHC examination of the rat duodenum failed to identify apelin-containing cells (data not shown).

Ontogeny of stomach apelin expression and peptide

Northern analysis and real-time RT-PCR showed that apelin mRNA levels were significantly higher in the fetal and postnatal rat stomach when compared with the adult stomach (Fig. 4). Apelin mRNA levels in fetal and postnatal stomach samples did not differ significantly. IHC did not detect apelin peptide in stomachs harvested from pups at 2, 7, and

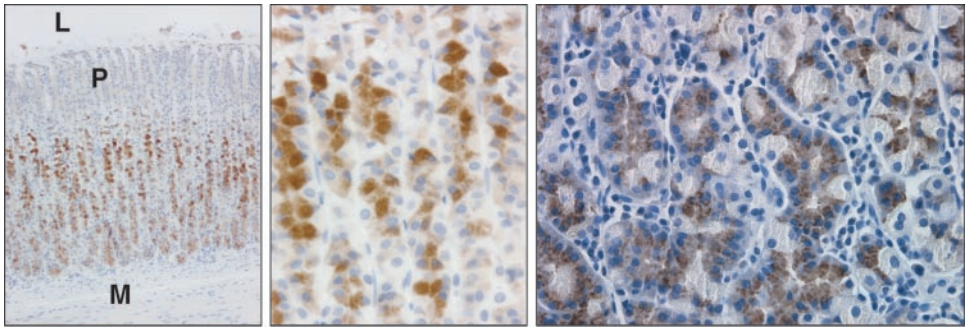

FIG. 2. *Left*, IHC localization of apelin-containing cells in the oxyntic mucosa of the adult rat stomach. Strong apelin staining (*red-brown*) is observed in the deep glandular epithelium of the fundus. *Middle*, A high-power magnification of stomach apelin-containing cells. *Right*, IHC localization of apelin-containing cells in the adult human stomach. This tissue was devoid of any pathophysiology. Apelin staining (*brown*) is observed in the glandular epithelium. No staining was observed with use of preimmune serum (not shown). Micrographs were captured with a Nikon Eclipse E600 microscope with Plan Fluor. Magnification, ×100, ×200, ×200, respectively. L, Stomach lumen; P, region of gastric pits; M, muscle layer of stomach.

13 d of age (data not shown). Apelin-containing cells were observed initially at 20 d of age in the rat stomach (Fig. 5), and apelin staining intensity (*i.e.* number of apelin-containing cells) in the stomach increased progressively until adulthood. Apelin peptide was not detectable by IHC in the human fetal stomach (data not shown).

Proliferative action of apelin on stomach cells in culture

To examine the influence of apelin on stomach cell proliferation, human gastric epithelial cells (SIIA) were cultured with synthetic apelin-13 (10^{-8} M). Apelin-13 stimulated cell proliferation significantly when compared with control vehicle-treated cells (Table 1). The proliferative action of apelin-36, at 10^{-8} M, was also tested. The stimulatory action of apelin-36 (data not shown) was significantly less (105–108% of controls) when compared with apelin-13 (120–145% of controls).

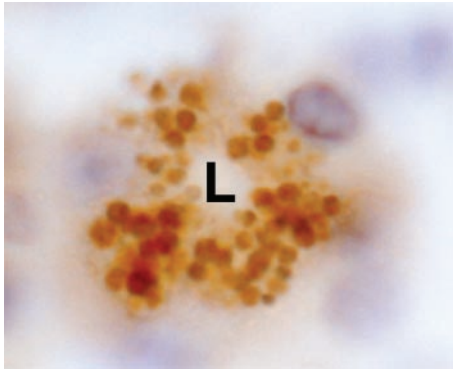

FIG. 3. High-power micrograph showing apelin storage vesicles in a gastric gland of rat stomach. Apelin-staining vesicles are packed adjacent to the lumen (L) of the gastric gland, suggesting release of apelin into the stomach lumen. Magnification, ×400.

Apelin stimulates CCK secretion from STC-1 cells

A pilot experiment indicated that apelin-36 did not stimulate CCK secretion (data not shown), whereas apelin-13 stimulated CCK secretion by STC-1 cells, a murine intestinal enteroendocrine cell line that expresses and secretes CCK. Apelin-13, therefore, was used in the CCK secretion experiments. Incubation of STC-1 cells with varying doses of apelin-13 caused a dose-related increase in CCK secretion (Fig. 6). Apelin at 10^{-7} M and 10^{-9} M caused 2-fold and 1-fold elevations in media CCK levels, respectively. Apelin at 10^{-9} M did not increase CCK levels when compared with control treatment. Apelin-19 and -12 also stimulated CCK secretion equipotently with apelin-13 (data not shown).

Apelin does not activate calcium signaling in STC-1 cells

Exposure of STC-1 cells to apelin failed to activate intracellular Ca^{2+} response (Fig. 7). In contrast, exposure of STC-1 cells to bombesin produced a calcium response characterized by an initial rapid elevation in $[Ca^{2+}]_i$ followed by a second sustained elevation in $[Ca^{2+}]_i$ that decreased to resting levels over 10–20 min. Neither a brief exposure of STC-1 cells to apelin before bombesin treatment nor exposure of cells to apelin with bombesin simultaneously altered the bombesin-induced elevation in $[Ca^{2+}]_i$ (data not shown).

Apelin-stimulated CCK secretion requires MAPK but not PKC activation

To examine whether MAPK and PKC are coupled to activation of the APJ receptor and CCK secretion, STC-1 cells

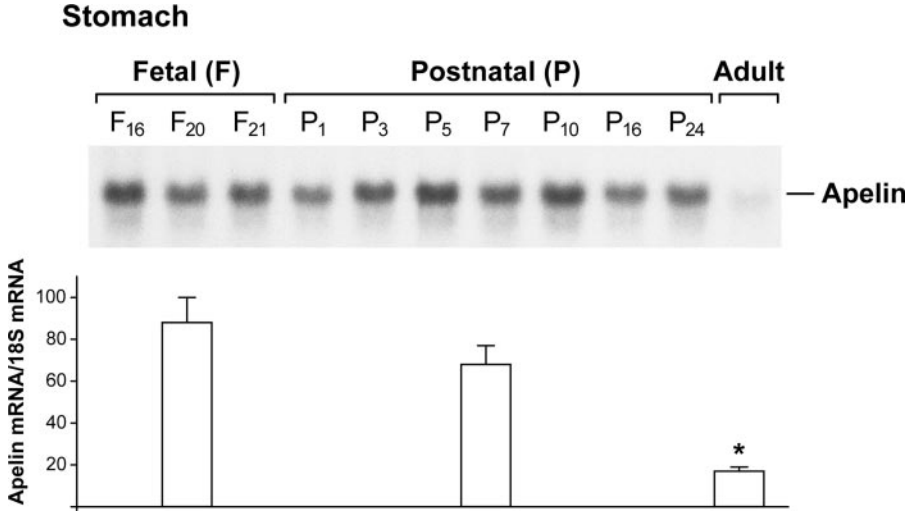

FIG. 4. *Top*, Ontogeny of apelin expression (mRNA levels) in the developing rat stomach. Northern hybridization shows the profile of apelin expression in the fetal and postnatal rat stomachs compared with the adult rat stomach. Notice that apelin expression is barely discernible in the adult stomach on this film exposure. *Bottom*, Real-time RT-PCR analysis of apelin expression in the fetal, postnatal, and adult rat stomach. Stomach apelin and ribosomal 18S mRNA levels were measured by real-time RT-PCR. Apelin expression was normalized to a stomach RNA specimen that contained nominal levels of apelin mRNA and then to stomach ribosomal 18S levels. For fetal and some postnatal stomach samples, total cellular RNA was pooled from two to five littermates to constitute a single sample. The mean apelin mRNA/18S mRNA values were calculated by pooling the readings of three to five separate samples or pools for the F16–F21, P1–P24, and adult samples. Real-time RT-PCRs show that stomach apelin expression is elevated significantly during the fetal and postnatal periods when compared with stomach apelin expression in the adult rat. *, $P < 0.05$ *vs.* fetal and postnatal stomach apelin mRNA levels.

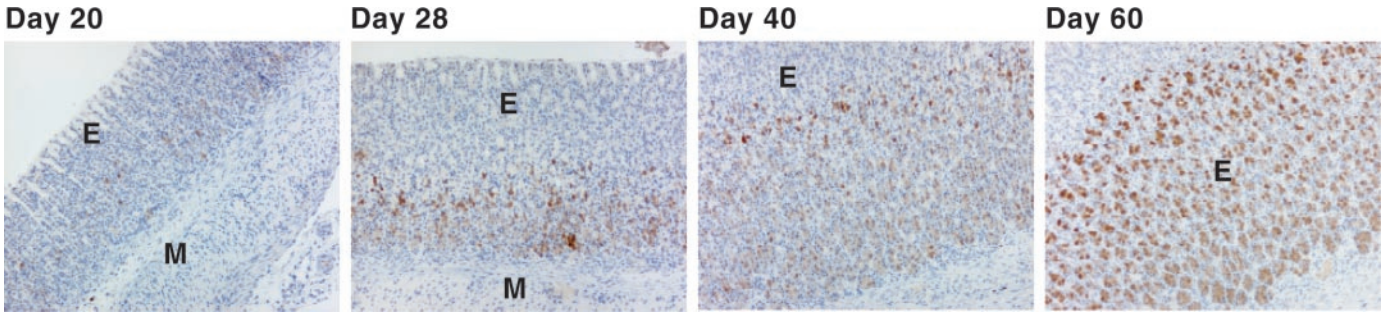

FIG. 5. Ontogeny of apelin peptide in the oxyntic mucosa of the developing rat stomach. Apelin-containing cells (*brown*) were observed initially in the rat stomach at 20 d of age by IHC. IHC failed to detect apelin peptide in stomach specimens harvested from pups at 2, 7, and 13 d of age (data not shown). The number of apelin-containing cells increased progressively with age. Photos were captured with a ×10 objective. M, Muscle layer of stomach; E, gastric mucosal epithelium.

TABLE 1. Apelin stimulates proliferation of gastric cells (SIIA) in culture

Days of treatment	Cell number	
	Control	Apelin-treated
1	$5.1 \times 10^4 \pm 1242$	$6.1 \times 10^4 \pm 772^a$
2	$9.4 \times 10^4 \pm 1574$	$1.2 \times 10^5 \pm 4387^a$
3	$1.6 \times 10^5 \pm 4991$	$2.3 \times 10^5 \pm 4678^a$

To examine the influence of apelin on stomach cell proliferation, SIIA cells were cultured with synthetic apelin-13 (10^{-8} M). Cells were plated in six-well plates initially at a density of 1.5×10^4 cells per well. Apelin was added daily starting 24 h after plating. Cells were counted daily.

$^a P < 0.05$ *vs.* control; n = 4 wells per treatment.

were pretreated with either a MAPK (PD98059, 3 μM) or a PKC inhibitor (GF109203X, 5 μM) for 30 min, rinsed, and then exposed to apelin (10^{-6} M) for 60 min. Pharmacological blockade of MAPK activity decreased apelin-stimulated CCK release, whereas inhibition of PKC activity in STC-1 cells did not affect apelin-induced CCK secretion (Fig. 8).

Discussion

Apelin is a recently characterized peptide that was isolated from bovine stomach extracts based upon its ability to increase the extracellular acidification rate in Chinese hamster ovary cells transfected with a G protein-linked orphan receptor called the APJ receptor (1–3). The APJ gene is one of the first genes encoding an orphan G protein-coupled receptor to be cloned (14). Apelin is named after APJ endogenous ligand. The APJ receptor is structurally similar to the angiotensin II receptor (~30% homology). Apelin has no sequence homology with angiotensin II, and angiotensin II does not have acidification-promoting activity in cells expressing the APJ receptor, indicating that the APJ receptor is not related functionally to the angiotensin II receptor (2).

Like many other regulatory peptides, pharmacological studies indicate that apelin has multiple biological activities. Reported actions for apelin include inhibition of proinflammatory cytokine production by mouse spleen cells (4), chemotactic activity on CHO-A10 cells (15), and lowering of blood pressure (8) and stimulation of drinking behavior in rats (16), and apelin is thought to function as a coreceptor with CD4 in the process of HIV infection (1, 8, 15, 17). The effects of apelin on the GI tract have not been investigated previously, although there is an abstract describing its CCK-releasing activity (18).

Apelin is produced in several tissues of the body, including the heart, brain, lung, pregnant and lactating breast, and GI tract (4, 19). In the present study, Northern analysis demonstrated the highest apelin expression in the stomach fundus with lower expression levels in the intestine. IHC shows abundant apelin peptide in the glandular epithelium of the rat and human stomachs. Although apelin peptide was not detected in the rat duodenum by IHC (data not shown), production of marginal levels of apelin peptide in the intestine may exceed the lower limit of sensitivity for IHC. The abundant pattern of apelin peptide staining in the stomach mucosa suggests that apelin is produced in multiple gastric mucosa cell types. Although we do not characterize the cell types in the gastric epithelium that produce apelin, preliminary findings show that chromogranin A, a marker of enteroendocrine cells (20) is colocalized with apelin in a portion of the gastric epithelial cells, indicating that some of the gastric apelin cells are endocrine in nature.

Northern analysis and real-time RT-PCR showed that stomach apelin expression was activated early during development because stomach apelin mRNA levels in the rat fetus were elevated at d 16 of gestation and remained elevated during the perinatal period. Despite the high expression levels of apelin in the fetal and postnatal stomachs, apelin peptide was detectable by IHC only at weaning and increased progressively with age. The finding that gastric stores of apelin peptide increased at the time of weaning suggests that apelin peptide synthesis is activated by the switch from milk to solid food. Interestingly, short-term primary cultures of dispersed stomach cells harvested from rat pups 5–17 d of age showed apelin peptide production and secretion within 2–3 d of culture (data not shown), suggesting that translation of stomach apelin is inhibited during the perinatal period. The inhibitory mechanism may involve apelin itself because nursing pups consume large amounts of apelin (~300–600 ng/ml) (Greeley, Jr., G. H., unreported data) contained in milk ingested from their lactating mothers. The observations that rat pups consume large amounts of apelin and that there is a developmental increase in apelin peptide in the stomach after weaning suggest that apelin has an important developmental role in the GI tract. Because the present study shows that apelin stimulates stomach cell proliferation *in vitro*, one obvious possibility is a tropic role in

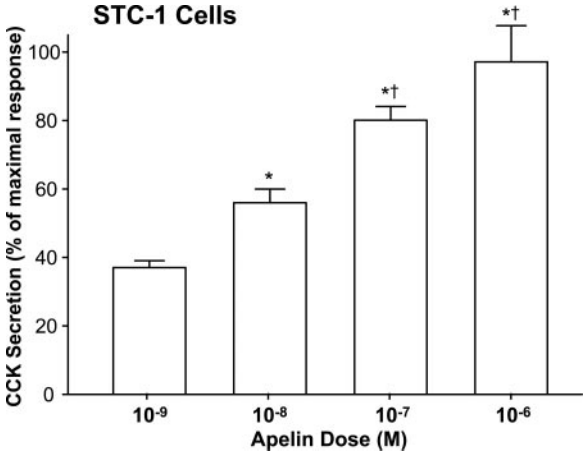

FIG. 6. Synthetic apelin-13 stimulates CCK secretion from STC-1 cells. CCK secretion was measured using a CCK RIA. †, $P < 0.05$ *vs.* controls or lower apelin dose; *, $P < 0.05$ *vs.* controls or 10^{-9} M apelin.

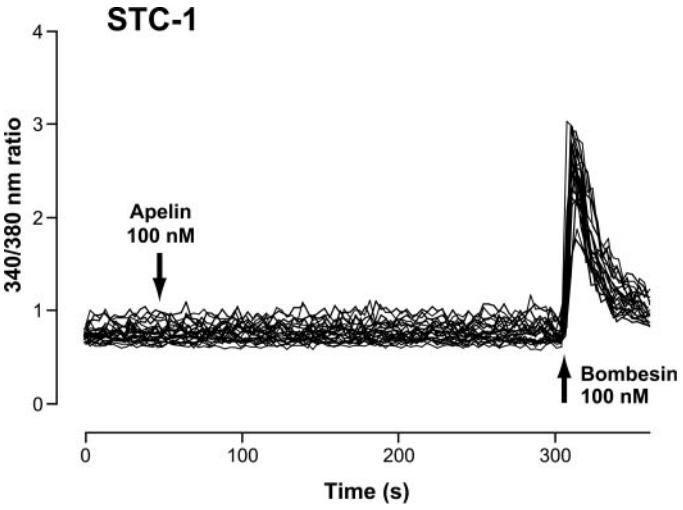

FIG. 7. Apelin fails to increase $[Ca^{2+}]_i$ in STC-1 cells, whereas bombesin increases intracellular calcium levels. The different lines represent recordings from multiple STC-1 cells done simultaneously.

regulation of GI epithelial proliferation. Interestingly, our findings show that both apelin-36 and apelin-13 stimulate cell growth; however, apelin-36, the parent peptide of apelin-13, is less potent.

Interestingly, the developmental appearance of stomach apelin peptide differs when compared with the developmental patterns of stomach ghrelin and gastrin and an ileocolonic hormone, peptide YY. In the rat, gastrin, ghrelin, and peptide YY peptides appear in late gestation initially and increase to maximal levels at approximately 18–21 d of age (5, 6, 21). Of further interest is the finding that apelin expression in the lung shows a developmental pattern opposite to that observed for the stomach (data not shown). In contrast to the relatively high expression levels of apelin in the fetal and postnatal stomachs, apelin expression is low and increases progressively with age after birth in the lung. Together, the findings that apelin expression is maximally activated in the fetal stomach and pregnant breast, whereas apelin expression in the lung is increased postnatally, indicates that transcriptional regulation of apelin is complex and tissue specific.

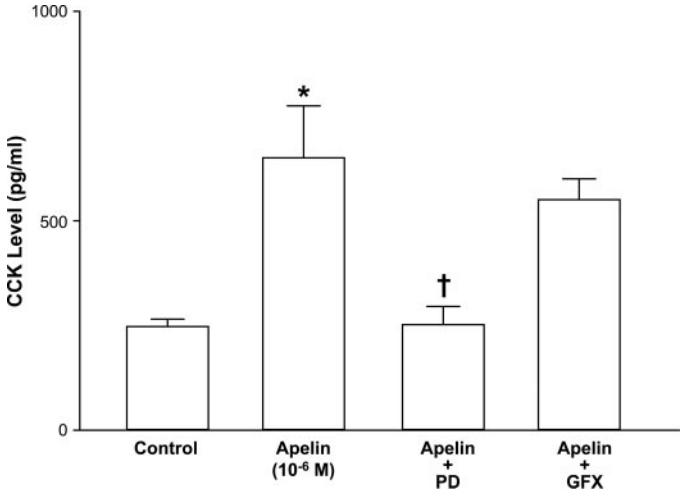

FIG. 8. Inhibition of apelin-induced CCK secretion from STC-1 cells by a MAPK inhibitor (PD98059; 3 μM) but not by a PKC inhibitor (GF109203X; 5 μM). PD98059 and GF109203X were added to the STC-1 cells 30 min before apelin was added. *, $P < 0.05$ *vs.* control; †, $P < 0.05$ *vs.* apelin alone.

The present findings show that apelin-13 stimulates CCK release from a murine small-intestinal cell line (STC-1) that expresses and secretes CCK. The finding that apelin stimulates CCK secretion from STC-1 cells indicates that apelin can act directly on CCK cells to stimulate CCK secretion. Although the apelin receptor, the APJ receptor, has not been localized to CCK cells, the present findings implicate that the APJ receptor is found on CCK cells. Pharmacological blockade of the MAPK pathway but not the PKC pathway inhibited apelin-13-induced CCK secretion from STC-1 cells, indicating that the MAPK pathway is involved in apelin-induced CCK secretion. It should be pointed out that the PKC inhibitor GF-109023X is known to inhibit both conventional and novel PKC isoforms (22), leaving the possibility that atypical PKC isoforms might participate in the apelin-induced CCK secretion from STC-1 cells. The present findings also indicate that apelin-induced CCK secretion is not dependent upon a concurrent elevation in $[Ca^{2+}]_i$ because intracellular calcium levels of STC-1 cells do not change when challenged by apelin exposure. This finding agrees with an earlier report showing lack of involvement of $[Ca^{2+}]_i$ in apelin activation of the APJ receptor (23). Apelin-13 was used in the CCK secretion studies because apelin-36 failed to activate CCK secretion *in vitro* from STC-1 cells. Although apelin processing has not been described, apelin-36 presumably represents a parent form of apelin-13. Earlier reports indicate that the smaller apelin variants such as apelin-17 and apelin-13 show a greater biological activity when compared with apelin-36 in the extracellular acidification assay (2, 15). We have also found that apelin-19 and apelin-12 can stimulate CCK secretion potently from STC-1 cells (Greeley, Jr., G. H., unreported data).

The findings that apelin stimulates CCK secretion and the measurement of apelin peptide in a luminal perfusate of the rat intestine (data not shown) suggests that apelin is a potential luminal factor, *i.e.* a lumone that stimulates CCK se-

cretion. Previously documented luminal CCK-releasing factors include monitor peptide, luminal CCK-releasing factor, and diazepam-binding inhibitor (24–26). The present IHC data indicate that apelin is not produced by the intestine; however, apelin may travel by a luminal route from the stomach to the intestine to stimulate CCK release. Other gastric peptides, including proxenopsin and leptin, have been shown to be secreted into the gastric lumen where they are either processed by pepsin or travel to the duodenal lumen to stimulate CCK secretion (27–30).

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Address all correspondence and requests for reprints to: George H. Greeley, Jr., Ph.D., Department of Surgery, The University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555-0725. E-mail: ggreeley@utmb.edu.

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