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Research Article

Cholecystokinin (CCK) is a gastrointestinal hormone produced by discrete endocrine cells in the upper small intestine and released after ingestion of a meal. The present study was designed to determine if enhanced CCK secretion is associated with increases in intestinal CCK mRNA levels. Rats, prepared with indwelling intraduodenal cannulae, were first fed an elemental diet that did not stimulate CCK release. Next, as a means of stimulating CCK secretion, soybean trypsin inhibitor was perfused for up to 24 h. Trypsin inhibitor administration increased plasma CCK levels from 0.9 +/- 0.1 to approximately 5 pmol/liter. RNA was prepared from the proximal small intestine at various times after trypsin inhibitor perfusion and mRNA levels analyzed by hybridization with a CCK cDNA probe. After 12 and 24 h of trypsin inhibitor treatment there were three- and fourfold increases, respectively, in CCK mRNA levels. In comparison, there was no change in beta-actin mRNA levels. To determine if regulation of CCK mRNA was at the level of CCK gene transcription, labeled transcripts from nuclear run-on incubations were hybridized to immobilized CCK cDNA. In trypsin inhibitor-treated rats, a two- to threefold increase in transcriptional activity was observed, whereas beta-actin gene transcription levels were unaltered. These studies indicate that stimulation of CCK secretion is associated with an increase in intestinal CCK mRNA content resulting from an increase [...]

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Dietary Regulation of Rat Intestinal Cholecystokinin Gene Expression

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Abstract

Cholecystokinin (CCK) is a gastrointestinal hormone produced by discrete endocrine cells in the upper small intestine and released after ingestion of a meal. The present study was designed to determine if enhanced CCK secretion is associated with increases in intestinal CCK mRNA levels. Rats, prepared with indwelling intraduodenal cannulae, were first fed an elemental diet that did not stimulate CCK release. Next, as a means of stimulating CCK secretion, soybean trypsin inhibitor was perfused for up to 24 h. Trypsin inhibitor administration increased plasma CCK levels from 0.9 ± 0.1 to ~ 5 pmol/liter. RNA was prepared from the proximal small intestine at various times after trypsin inhibitor perfusion and mRNA levels analyzed by hybridization with a CCK cDNA probe. After 12 and 24 h of trypsin inhibitor treatment there were three- and fourfold increases, respectively, in CCK mRNA levels. In comparison, there was no change in β -actin mRNA levels. To determine if regulation of CCK mRNA was at the level of CCK gene transcription, labeled transcripts from nuclear run-on incubations were hybridized to immobilized CCK cDNA. In trypsin inhibitor-treated rats, a two- to threefold increase in transcriptional activity was observed, whereas β -actin gene transcription levels were unaltered. These studies indicate that stimulation of CCK secretion is associated with an increase in intestinal CCK mRNA content resulting from an increase in CCK gene transcription.

Introduction

Cholecystokinin (CCK) is a classical gastrointestinal polypeptide hormone that was first identified in intestinal extracts by its ability to stimulate gallbladder contraction (1). It has since been recognized that CCK is also a major regulator of exocrine pancreatic secretion and gastric emptying, and has important effects on intestinal motility and perhaps satiety (2, 3). Multiple molecular forms of CCK, ranging in size from the tetrapeptide CCK-4 to forms as large as CCK-58, have been identified in the intestine, brain, and plasma (4–8). As a typical gut

hormone, CCK is secreted into the circulation after the ingestion of nutrients. With the development of improved assays for CCK, it has recently become possible to define many of the factors that regulate CCK secretion (9, 10). In the rat, unlike other species in which fat and amino acids stimulate CCK release, ingested protein is the major stimulant of CCK secretion (11).

CCK secretion in the rat is under negative feedback regulation by pancreatic secretion, whereby active proteases in the intestinal lumen inhibit CCK secretion; inactivation of intestinal proteases or binding of proteases by substrates such as protein or trypsin inhibitor results in stimulation of CCK secretion (12). We have demonstrated previously that the ability of protein to stimulate CCK release in the rat is proportional to its ability to serve as a substrate for trypsin (11). In light of these recent observations, we have developed a model in which rats are completely nourished by an elemental diet that does not stimulate CCK release. By adding small amounts of soybean trypsin inhibitor to the diet, we were then able to stimulate CCK secretion physiologically without significantly altering the nutritional status of the animals.

The intracellular mechanisms regulating CCK synthesis have not been examined previously. In particular, the factors regulating gastrointestinal hormone gene expression have not been studied. With the recent cloning of a cDNA for rat CCK (13), however, it is now possible to study the regulation of CCK mRNA levels and gene transcription. By stimulating CCK secretion with trypsin inhibitor, we examined the relationship of CCK secretion to intestinal CCK gene expression.

Methods

Surgical preparation of rats. Male Sprague-Dawley rats weighing 330–350 g were prepared with indwelling intraduodenal and intraperitoneal cannulae as previously described (14). While the rats were under pentobarbital anesthesia, through a midline intraabdominal incision, a 0.9-mm silastic catheter was sewn into the proximal duodenum, 2 mm distal to the pylorus. A second cannula was sewn along the anterior abdominal wall for the intraperitoneal administration of antibiotic after surgery. Both cannulae were tunneled subcutaneously, exiting from an incision on the back. Rats were maintained in modified Bollman cages for a total period of 4 d (14). For the first three postoperative days, rats were given 5 mg/kg ampicillin and 10 mg/kg chloramphenicol to prevent wound infection. No medications were given within 24 h of beginning the experiments.

Feeding of rats. Animals had free access to water. Starting 12 h after surgery, rats were fed an elemental diet of Vivonex (Norwich Eaton Pharmaceuticals, Norwich, NY) by continuous perfusion into the duodenal cannula at a rate of 3.5 ml/h. This provided a daily intake of 84 cal consisting of 0.27 g nitrogen, 19.4 g carbohydrate, and 0.12 g fat. Feeding was maintained throughout the entire study period. 3 d after surgery, experimental animals had soybean trypsin inhibitor

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added to their diet so that they received 50 mg/h by continuous perfusion intraduodenally, along with the normal amount of Vivonex. Control animals remained on Vivonex alone. Duodenal perfusion of nutrients continued until the time rats were killed.

Collection of plasma and bioassay of CCK. Animals were decapitated and trunk blood was collected into iced, heparinized tubes. Blood was immediately centrifuged at 2,000 g for 10 min and plasma was extracted and concentrated by adsorption onto octadecylsilylsilica (C-18 Sep-Pak; Waters Associates, Millipore Corp., Milford, MA) cartridges. Plasma CCK concentrations were measured by a highly specific and sensitive bioassay (9, 10). Validation of this assay, which is based on the ability of CCK to stimulate amylase release from isolated rat pancreatic acini, has been previously described (9, 10).

Preparation of RNA from intestine. Within 1 min of killing, either the proximal 15 cm of small intestine, initiating at the pylorus, or a 15-cm segment of distal small intestine (extending 15 cm proximally from the colon) was removed and the luminal contents rinsed with 20 ml iced PBS. The intestine was then incised and the mucosa scraped off and immediately placed into guanidinium isothiocyanate and homogenized with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ). Total RNA was prepared by the guanidinium-cesium chloride method of Chirgwin et al. (15). RNA concentrations were estimated using absorbance at 260 nm ($1.0 \text{ OD} = 40 \mu\text{g}$).

Slot blots and Northern transfers. Poly (A)⁺ RNA was obtained by oligo (dt) cellulose chromatography (16). For Northern transfers, poly (A)⁺ RNA was denatured in formaldehyde, subjected to electrophoresis in 1% agarose, and transferred to nitrocellulose paper (17). For slot blots RNA was denatured as described by Thomas (18), and various dilutions of RNA were immobilized to nitrocellulose using a slot blot apparatus (Schleicher & Schuell, Inc., Keene, NH).

cDNA labeling and hybridization of filters. These studies used a full-length CCK cDNA probe containing 33 nucleotides in the 5'-noncoding region, the complete coding region, and 199 nucleotides in the 3'-noncoding region (13). This cDNA cloned into the plasmid pBR322 was kindly provided by Dr. Jack Dixon (Purdue University, West Lafayette, IN) (13). A cDNA probe for β -actin also cloned into pBR322 (19) was used as a control. These cDNA probes were nick-translated (labeling kit; Bethesda Research Laboratories, Gaithersburg, MD) to specific activities of 1×10^9 cpm/ μg . Filters containing RNA were prehybridized and hybridized with 1×10^7 cpm/ml CCK cDNA or β -actin cDNA and washed as described (17). Filters were exposed to Kodak XAR-5 film in the presence of two intensifying screens at -70°C for 8 h for β -actin and 24 h for CCK.

Isolation of nuclei and run-on assays. Nuclei were prepared from 1×10^8 cells of intestinal epithelium by washing twice in buffered saline followed by resuspension in lysis buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 5 mM MgCl₂, and 0.5% NP-40 (20). Cells were lysed in a Dounce homogenizer (Kontes Glass Co.) with eight strokes of the loose pestle and a nuclear pellet was obtained by centrifugation at 800 g for 5 min. Visual inspection under a phase contrast microscope revealed no intact cells. Nuclei were resuspended in storage buffer (40% glycerol, 50 mM Tris [pH 8.3], 5 mM MgCl₂, and 0.1 mM EDTA) and either used immediately or stored at -70°C .

Transcription assays were performed using 5×10^7 nuclei in each reaction as described by Thompson et al. (20) as modified by McDonald and Goldfine (21). RNA products were purified using DNase, proteinase K in the presence of SDS, and salt precipitation (20, 21). Five μg of either the control plasmid pBR322, the plasmid containing β -actin cDNA, or the plasmid containing CCK cDNA were denatured by heating to 65°C for 1 h in 0.2 M NaOH, neutralized with an equal volume of cold 3 M sodium acetate, and applied to nitrocellulose using a slot-blot manifold. The slots were washed with 500 μl of $10\times$ standard saline citrate (SSC = 0.3 M NaCl and 0.03 M sodium citrate) and the filters baked under vacuum at 80°C for 2 h. The filters were then prehybridized and hybridized in 2 ml of buffer at 60°C for 40 h as described (21) except that hybridizations were performed in 6×5 ml tissue culture plates. After hybridization, filters were washed sequentially in 100-ml vol of $2\times$ SSC at 65°C for 1 h, $2\times$ SSC with 10

$\mu\text{g/ml}$ ribonuclease A at 37°C for 30 min, and $0.5\times$ SSC with 0.1% SDS for 2 h at 65°C . Filters were exposed to film with two intensifying screens at -70°C for 48 h (β -actin) or 3–7 d (CCK and pBR322).

Statistical analysis. Values are expressed as the mean \pm 1 SEM. Comparison of responses were made by analysis of variance (22). Posthoc analysis of the difference between points was carried out by means of the Newman-Keuls test. Differences with a *P* value of < 0.05 were considered significant.

Results

CCK secretion. Rats were maintained on an elemental diet by continuous duodenal perfusion of Vivonex. This supplement consists primarily of free amino acids and carbohydrate and contains little fat. Plasma CCK levels achieved by this feeding are shown in Fig. 1. After 3 d of feeding (time 0) animals were either continued on Vivonex (controls) or had soybean trypsin inhibitor added to the duodenal perfusion at a rate of 50 mg/h. Animals were killed after 4, 12, or 24 h. Plasma CCK levels increased from basal levels of 0.9 ± 0.1 pmol/liter (mean \pm SEM, $n = 4$) to 4.8 ± 0.5 pmol/liter by 4 h after starting the trypsin inhibitor perfusion, and levels remained elevated for the duration of the study.

mRNA levels. Poly (A)⁺ RNA was prepared from the proximal 15 cm of intestine and the distal 15 cm of small intestine of rats fed ad lib. and was chromatographed and analyzed by Northern transfer (Fig. 2). With the CCK cDNA probe autoradiographs of these transfers displayed a single band of ~ 750 bp. With the β -actin probe, a single band of $\sim 2,000$ bp was seen. These electrophoretic patterns are consistent with previously reported mRNA sizes for these proteins (12, 18). The relative abundance of actin mRNA was comparable in both the proximal and distal intestine. CCK mRNA was $460 \pm 70\%$ (mean \pm SEM, $n = 3$) more abundant, however, in the proximal small intestine. This finding is consistent with the greater concentration of CCK and CCK-producing endocrine cells in the proximal intestine as compared with the distal small intestine (23).

To determine the relationship between CCK secretion and CCK mRNA levels, intestine from the same animals in which CCK was measured was harvested for preparation of RNA. At

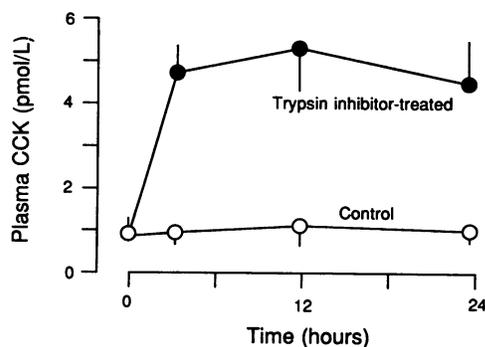


Figure 1. Effect of dietary changes on plasma CCK levels. Rats prepared with indwelling duodenal canulae were fed by continuous perfusion either an elemental diet alone (\circ) or an elemental diet plus trypsin inhibitor (\bullet). At the times indicated, rats were killed and blood was collected for measurements of CCK. Each point is the mean \pm SEM of four animals. There was a statistically significant increase in plasma CCK levels in animals treated with trypsin inhibitor ($P < 0.05$).

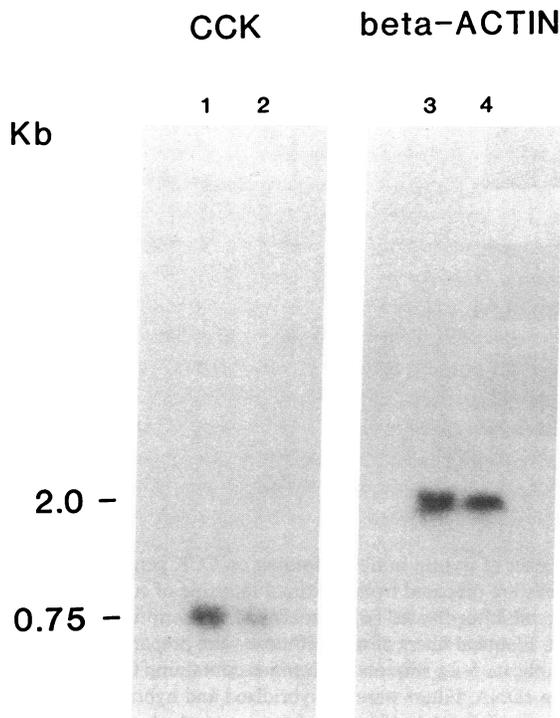


Figure 2. Hybridization analysis of poly (A)⁺ RNA from proximal and distal intestine. 20 μ g poly (A)⁺ RNA from either proximal (lanes 1 and 3) or distal (lanes 2 and 4) rat small intestine was electrophoresed on an agarose gel. After transfer to nitrocellulose, the RNA was hybridized with CCK or β -actin cDNA probes. Sizes are indicated on the left in kilobases (kb).

the times indicated, total RNA was prepared from the proximal intestine, slotted onto nitrocellulose, and hybridized with labeled cDNA probes (Fig. 3). The relative abundance of β -actin RNA did not change with trypsin inhibitor feeding. CCK RNA levels increased $330 \pm 50\%$ ($n = 4$), however, within 12 h and $400 \pm 80\%$ after 24 h of treatment.

Run-on assays. To determine whether the observed changes in CCK RNA levels that occurred with trypsin inhibitor treatment were transcriptional, nuclear run-on assays were performed. In nuclear run-on transcription assays, transcripts that are initiated in the whole cell are elongated in isolated nuclei and reflect the ongoing rate of transcription (24). To examine the transcription system, 5 μ g of CCK cDNA plasmid, pBR322 (the cloning vector used for CCK), and β -actin cDNA plasmids were immobilized on nitrocellulose and hybridized with the nuclear transcription run-on products (Fig. 4). Nuclei from trypsin inhibitor-treated intestine at 4 h increased the CCK hybridization signal $300 \pm 40\%$ ($n = 4$) over the corresponding unstimulated control signal. In comparison, the β -actin signal remained unchanged, and no significant signal was seen with pBR322. Transcription signals remained elevated relative to β -actin at 12 and 24 h of trypsin inhibitor treatment. The addition of 2 μ g/ml α -amanitin to the nuclear incubation (a concentration that preferentially inhibits RNA polymerase II [24]), markedly reduced the signal obtained. This finding indicated that the CCK transcription signal was produced by RNA polymerase II, and therefore, was either mRNA or its precursor.

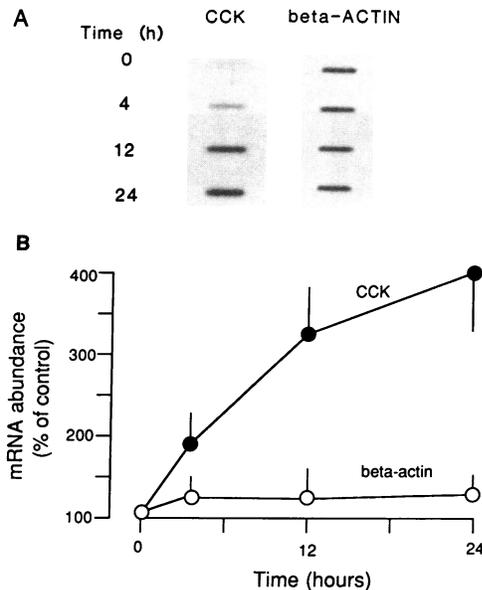


Figure 3. Effect of trypsin inhibitor feeding on CCK mRNA levels. Coincident with measurements of plasma CCK, RNA was prepared from the proximal small intestine from the rats depicted in Fig. 1. (A) Total RNA was slotted onto nitrocellulose and filters were hybridized with either the CCK or β -actin cDNA probes. The times indicated on the left represent the duration of trypsin inhibitor perfusion. The data shown are representative of four experiments. (B) Densitometry scanning of slot blots probed with either β -actin (\circ) or CCK (\bullet) cDNA are shown in relation to duration of trypsin inhibitor administration. Each point represents the mean \pm SEM of four animals.

Discussion

In the current study, we have utilized an in vivo rat model for stimulating CCK secretion in an effort to study the regulation of intestinal CCK gene expression. Previously we and others have shown that CCK secretion is stimulated by either the presence of intact protein (11) or the inhibition of intraluminal trypsin activity (12). In the current study, we introduced trypsin inhibitor via an indwelling intraduodenal cannula and demonstrated that high plasma CCK levels are associated with increased intestinal CCK mRNA levels. This dietary influence on CCK mRNA levels was specific as no parallel change in β -actin mRNA levels could be detected.

The relationship between stimulation of hormone secretion and hormone synthesis has not been previously documented for any gastrointestinal hormone. In other endocrine systems the relationship between stimulation of hormone secretion and hormone synthesis has been confusing. Growth hormone (GH)¹ mRNA levels have been demonstrated to be regulated by thyroid and glucocorticoid hormones (25). These elevators of GH mRNA levels, however, have no effect on GH secretion (25). In contrast, studies have revealed that stimulation of GH secretion by growth hormone releasing factor is independent of GH gene transcription (26). The present study provides in vivo evidence that secretion of the intestinal hor-

1. Abbreviations used in this paper: GH, growth hormone.

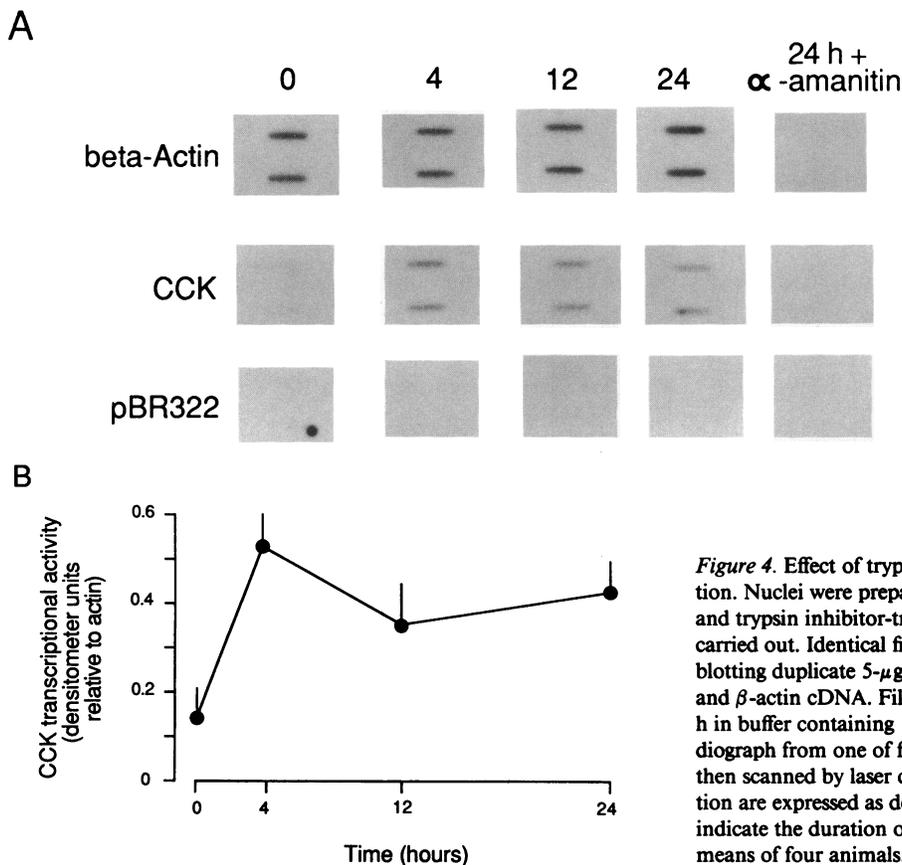


Figure 4. Effect of trypsin inhibitor feeding on CCK gene transcription. Nuclei were prepared from proximal intestine of control (○) and trypsin inhibitor-treated (●) animals and transcription assays carried out. Identical filters of nitrocellulose were prepared by slot-blotting duplicate 5- μ g aliquots of plasmids containing CCK cDNA and β -actin cDNA. Filters were prehybridized and hybridized for 48 h in buffer containing 1×10^7 cpm of run-on products. (A) Autoradiograph from one of four experiments. (B) Autoradiograms were then scanned by laser densitometry and changes in CCK transcription are expressed as densitometer units in relation to β -actin. Times indicate the duration of trypsin inhibitor perfusion. Results are the means of four animals.

more CCK was associated with changes in CCK mRNA levels.

The observed increase in CCK mRNA levels could result from two possible mechanisms. One possibility is that stabilization of existing mRNA occurs such that CCK mRNA is not degraded as rapidly, i.e., it has a longer $t_{1/2}$. A second possibility is that trypsin inhibitor stimulation of CCK secretion influenced CCK gene transcription. Because CCK is not an abundant gene product in intestinal mucosa, we used a highly sensitive run-on method previously developed to measure low abundant oncogenes (20).

Nuclear run-on assays indicated that increased CCK mRNA levels were accompanied by increases in CCK gene transcription. This effect was specific, as no increase in β -actin gene transcription was observed. We consider that the transcription assay displayed a high degree of fidelity since nonspecific hybridization as measured by hybridization to the pBR322 control plasmid was undetectable. In addition, the inclusion of α -amanitin eliminated both β -actin and CCK transcription signals. These data indicate that the increase in CCK mRNA is secondary to an increase in the transcription of the CCK gene. These observations provide the first evidence for transcriptional regulation of an intestinal polypeptide hormone gene.

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