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Ileal expression of gastrin and cholecystokinin

In search of a related hormone

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Abstract

Antibodies against the common active site of cholecystokinin (CCK) and gastrin stain three endocrine celltypes in the gut: G-cells (that synthesize gastrin) I-cells (that synthesize CCK), and TG-cells (whose product is unknown). In order to examine whether TG-cells either process progastrin or proCCK in a specific manner, or express a novel gastrin-CCK related hormone, we studied the distal porcine ileum, which have far more TG- than G- and I-cells. Ileal CCK and gastrin mRNA corresponded to those of the antroduodenal mucosa. Gel, ion-exchange and reversed-phase chromatography monitored with sequence-specific immunoassays showed that the ileal mucosa on average contain 0.3 and 7.6 pmol/g progastrin and proCCK, respectively; 1.1 and 13.5 pmol/g glycine-extended intermediates; and 1.1 and 24.8 pmol/g of bioactive carboxyamidated gastrin and CCK, respectively. Gastrin was present only as non-sulfated gastrin-34, whereas CCK occurred in forms similar to those of the proximal intestine. Although ileal progastrin processing is tissue specific, the amounts of gastrin and CCK are too small to explain the TG-cells. Moreover, since the ileal extracts were without traces of other peptides having the C-terminus common to gastrin and CCK, the results suggest that TG-cells produce a peptide, which is only weakly related to gastrin and CCK.

Key words: Cholecystokinin; Gastrin; Ileum; Prohormone processing

1. Introduction

Gastrin and cholecystokinin (CCK) have the same bioactive C-terminus, Trp-Met-Asp-Phe-NH_2. The relative affinity to gastrin and CCK receptors is determined by the structure of the N-terminal extensions of this C-terminus. The gastrin gene encodes an 80 and the CCK gene a 94 amino acid prohormone. Progastrin matures to gastrin-71, -34, -17, -14, and -6 [1]. Correspondingly, proCCK is processed to CCK-83, -58, -39, -33, -22, -8, and -5 [2].

In the gut gastrin is synthesized in antroduodenal G-cells, whereas CCK is synthesized in small intestinal I-cells and few neurons [3–5]. In addition the gut contains TG ('tetragastrin')-cells, which also stain with antibodies against the common C-terminus [4–7]. TG-cells, however, do not stain with antibodies for mid- and NH_2-terminal epitopes in gastrin or CCK peptides [4]. Moreover, TG-cells are ultrastructurally different from G- and I-cells [4,6]. Accordingly, TG-cells may express either a new gastrin-CCK related hormone, or they process progastrin or proCCK in a unique manner by which only the C-terminal epitope is exposed [4].

The distal ileum contains many TG-, few I-, and no G-cells [4]. It is therefore suited for the examination of the TG-cell hypothesis. Using sequence-specific immunoassays [8,9], we have now examined the expression of CCK and gastrin, as well as the possible occurrence of a related new hormone in the distal porcine ileum. In this study we consider three classes of preprohormone products: prohormones defined as products extended beyond the glycine residue at the C-terminus; glycine-extended intermediates; and α-amidated gastrins and CCKs in which the C-terminal phenylalanine is amidated using glycine as donor.

2. Materials and methods

2.1. Peptide extraction

Ileal mucosa (0.5 m from the caecum) was removed from adult pigs and immediately placed in liquid nitrogen. The frozen tissue was cut in pieces of a few milligrams, boiled in water (10 ml/g tissue) for 20 min, homogenized and centrifuged at $10,000 \times g$ for 30 min at 4°C. The supernatant was decanted (the neutral extract) and the pellet was redissolved in ice-cold acetic acid (10 ml/g) and left at room temperature for 20 min and centrifuged as above (the acidic extract). In order to concentrate the peptides further, 100 ml of neutral extract in 0.2% trifluoroacetic acid was applied on a Sep-Pak cartridge (Waters Ass, MA, USA), washed with 10 ml H_2O, 10 ml 10% CH_3CN, 10 ml 50% CH_3CN and 10 ml 80% CH_3CN all in 0.2% trifluoracetic acid. The extract was then dried in a vacuum centrifuge and resuspended in 10 ml H_2O.

2.2. Enzymatic treatment

In order to measure the gastrin and cholecystokinin precursors ex-

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tended beyond glycine in the amidation site (-Gly-Arg-Arg-), the extracts were incubated with TPCK trypsin (LS 0003741), Worthington, USA) and carboxypeptidase B (103 233, Boehringer Mannheim, Germany) [9]. Selected extracts were also incubated with arylsulfatase (2–400 μg Sigma type VIII from Abalone gut in 0.2 M sodium acetate, pH 5.0, 2 h, 37°C) in order to determine the degree of O-sulfation.

2.3. Radioimmunoassays

The following library of antisera was used: Ab. 2609 is directed against the common C-terminus of gastrin and CCK. Ab. 2609 binds both hormones irrespective of the degree of sulfation [8]. Ab. 2604 and Ab. 2605 are specific for gastrin peptides. While Ab. 2604 measures sulfated and non-sulfated gastrins equally, Ab. 2605 react less than 5% with sulfated gastrins [10,11]. Ab. 2605 is consequently used together with Ab. 2604 to assess the degree of sulfation. Ab. 1295 (a gift from J.H. Walsh, UCLA) is directed against the N-terminal sequence of gastrin-17. Thus, the antiserum will bind peptides with an epitope similar to the N-terminus of porcine gastrin-17 [12]. Ab. 3208 and Ab. 5284 are directed against the glycine-extended C-terminus of gastrin. Ab. 3208 measures both glycine-extended gastrins and CCKs, whereas Ab. 5284 recognizes only non-sulfated glycine-extended gastrins [9]. Ab. G-160 is specific for sulfated and α-amidated CCK-peptides. Consequently, it recognizes neither non-sulfated CCK nor gastrins [13]. Ab. 1564 is directed against sequence 74–79 of porcine proCCK. Consequently, it recognizes porcine CCK-58, CCK-39, and CCK-33 [14] as well as other fragments of proCCK containing sequence 74–79.

2.4. Chromatography

Extracts were applied to 10 × 1000 mm columns of Sephadex G-50 superfine (Pharmacia, Sweden) and eluted with 0.02 M veronal buffer (pH 8.4) at a rate of 4 ml/h. Fractions of 1.3 ml were assayed. The columns were calibrated with [^{125}I]albumin (void volume) and ^{22}NaCl (total volume) as well as synthetic CCK and gastrin peptides. In order to determine the sulfation of CCK peptides, anion-exchange chromatography was performed using a 10 × 150 mm DE-32 cellulose column (Whatman, UK) using a gradient from 0.05 M to 0.5 M NH_4HCO_3. The flow rate was 30 ml/h. Fractions of 2.5 ml were assayed. In order to determine the sulfation of gastrin peptides, anion exchange chromatography was carried out on 10 × 150 mm AE-41 cellulose (Whatman, UK) columns using a discontinuous gradient from 0.05 (6 h) to 0.2 M NH_4HCO_3 (pH 8.3 (300 ml)) [11]. The flow rate was 33 ml/h. Fractions of 2.8 ml were assayed. The identity of the peptides was further ensured

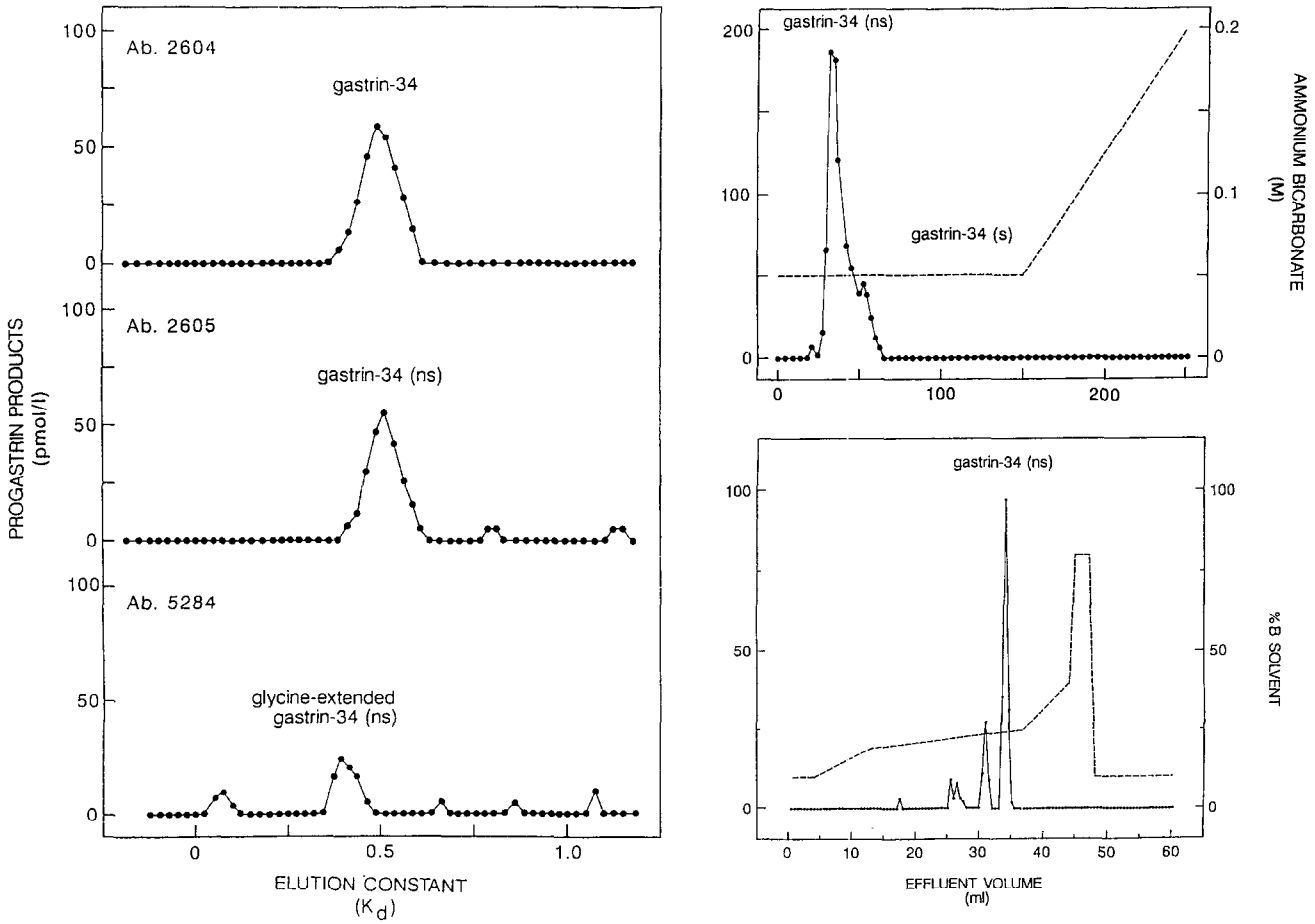

Fig. 1. Left panel: gel chromatography of the neutral ileal extracts monitored with antibodies that recognize the C-terminus of gastrin. Five ml ileal neutral extract was freeze-dried, redissolved in 1 ml H_2O, applied to a Sephadex G-50 superfine column (10 × 1000 mm) and eluted at 4°C with 0.02 M veronal buffer (pH 8.4) at a rate of 4 ml/h. Upper panel, Ab. 2604, which measures sulfated and non-sulfated gastrins equally; Middle panel, Ab. 2605, which measures sulfated gastrins with low potency; Lower panel, Ab. 5284 directed against non-sulfated glycine-extended gastrins. Upper right panel: anion-exchange chromatography of ileal extracts. Of this extract 100 ml extract was subjected to a Sep-Pak C-18 and eluted with 10 ml 50% CH_3CN in 0.2% trifluoracetic acid, and redissolved to 10 ml with H_2O, applied to an AE-41 cellulose column (10 × 150 mm) and eluted at 20°C with NH_4HCO_3 in a discontinous gradient as shown in the figure (– – –). Fraction of 2.8 ml were collected at a rate of 33 ml/h. The elution was monitored using Ab. 2604. Lower right panel: neutral extract was purified on a Sep-Pak C-18 Column. Reverse-phase HPLC of ileal neutral extract. 1.2 ml of this was applied to a Vydac C_4 silica column (250 mm), which was eluted with the indicated gradient of CH_3CH with 10 mM NH_4CH_3COOH (solvent B) at 50°C and a flow of 1.0 ml/min (– – –). Fractions of 0.5 ml were assayed with the gastrin specific antiserum No. 2604.

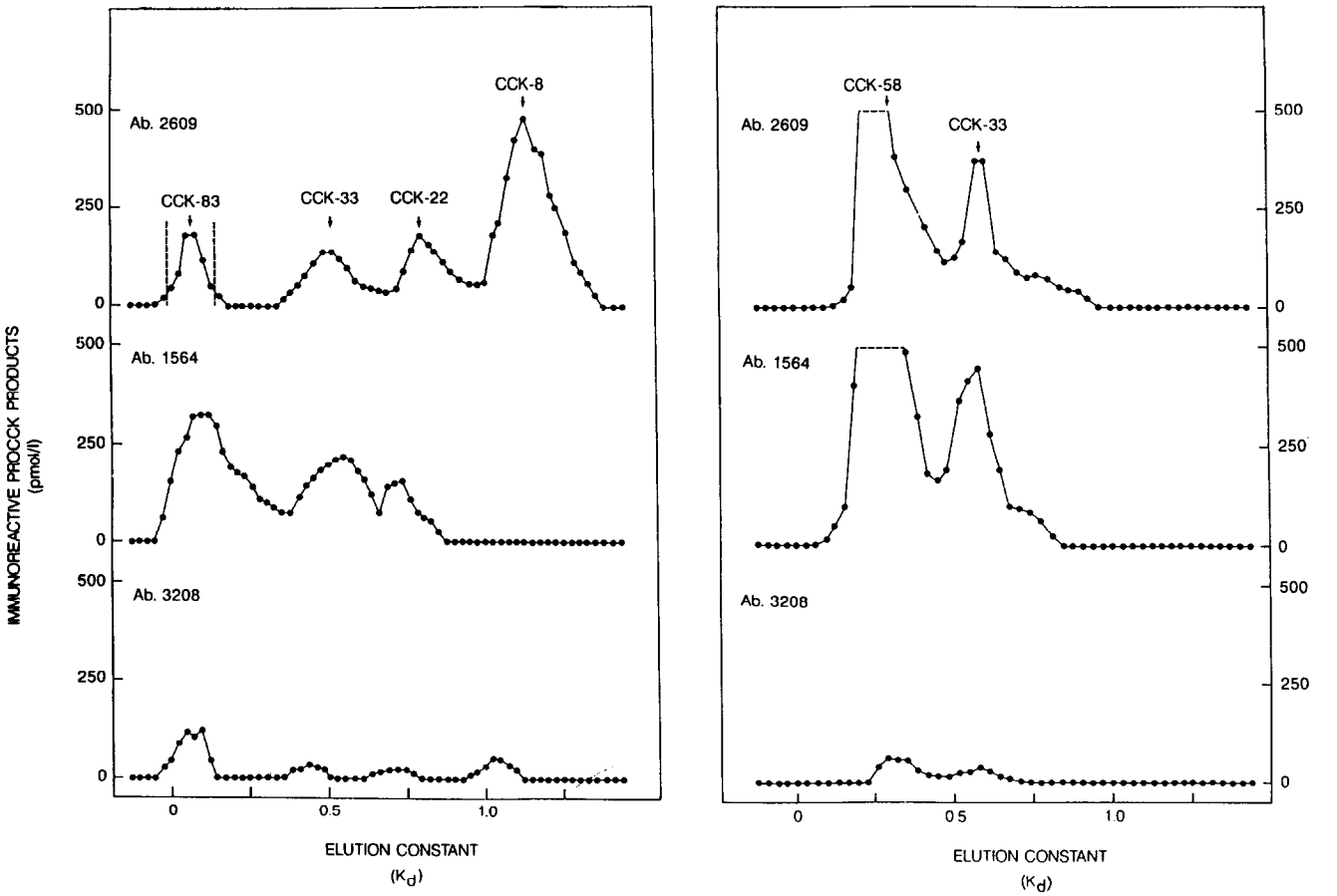

Fig. 2. Gel chromatography of neutral (left panel) and acidic (right panel) ileal extracts monitored radioimmunochemically using Ab. 2609 that recognize the common C-terminus of gastrin and CCK (upper panel), Ab. 1564 that specifically recognizes CCK (middle panel), and Ab. 3208 that binds glycine-extended gastrin and CCK (lower panel). The fractions between the bars were pooled. Five ml ileal neutral extract was freeze-dried and redissolved in 1 ml H_2O, then applied to a Sephadex G-50 superfine column (10 × 1000 mm) and eluted at 4°C with 0.02 M veronal buffer (pH 8.4) at a rate of 4 ml/h.

by reversed-phase chromatography. Extracts concentrated on C-18 Sep-Pak cartridges were applied to a Vydac (Hesperia, CA, USA) C_4 silica column (250 mm), which was eluted in a CH_3CN gradient with 10 mM NH_4 acetate at 50°C at a rate of 1 ml/min. Fractions of 0.5 ml were assayed. The column was calibrated with porcine gastrin-34 in sulfated and non-sulfated forms.

2.5. cRNA probes and oligoDNA primers

A [^{32}P]UTP-labelled rat CCK cRNA probe was transcribed by T7 RNA polymerase from cDNA inserted into a pBluescript vector (Stratagene, La Jolla, CA, USA). The CCK cDNA extends from position 163 to 543 of the rat CCK sequence [15]. The human gastrin cRNA probe was transcribed with SP6 RNA polymerase from a 261 base pair (bp) *Hin*dIII fragment of the human cDNA [16] subcloned into a pGEM3 vector (Promega, Madison, WI, USA) [17]. The oligonucleotide probes correspond to the nucleotides 10–30 (sense) and nucleotides 374–394 (antisense) of the porcine gastrin gene [18]. For cloning purposes *Bam*HI and *Eco*RI restriction enzyme recognition sites were included at the 5′ end of the sense and antisense primers, respectively.

Table 1
Porcine ileal gastrin and CCK immunoreactivity (pmol/g wet weight (mean and range (n = 6))

	Gastrin	CCK
Prohormone	0.3 (0.02–0.4)	7.6 (4.5–12.0)
Glycine-extended intermediates	1.1 (0.4–1.4)	13.5 (8.7–24.3)
Carboxyamidated peptides	1.1 (0.7–1.4)	24.8 (10.2–35.8)

2.6. RNA extraction and hybridization

Total cellular RNA was isolated [19] and poly (A)$^+$ RNA was obtained by oligo(dT)-cellulose chromatography [20]. RNA was fractioned on 1% agarose-formaldehyde gels, transferred to Hybond-N nylon membrane (Amersham, UK) and UV cross-linked. Hybridization was performed in 50% formamide, 600 mM NaCl, 8 mM Na EDTA 120 mM Tris-HCl with 1% SDS and 1% non-fat dried milk powder at pH 7.4 for 18 h at 65°C. The filters were washed for 3 × 20 min in 0.1 SSC (sodium, sodium citrate) with 0.1% SDS at 65°C, and exposed for 3–14 days at −80°C.

2.7. Reverse transcription and polymerase chain reaction

Reverse transcription and polymerase chain reaction were performed using a GeneAmp RNA PCR kit according to the manufacturers instructions (Perkin-Elmer Cetus, Norwalk, CT, USA). Briefly, 1 μg of total cellular RNA was reverse transcribed using an oligo $dT_{(16)}$ primer followed by 30 cycles of amplification (1 min at 94°C, 1 min at 62°C, 2 min at 72°C, and finally 7 min at 72°C). The amplified products were separated by gel electrophoresis on a 3% Nusieve 3:1 agarose gel (FMC Bioproducts, Rockland, NY, USA) in 1 × TAE buffer and visualised by ethidium bromide staining. For control, the product was cleaved with *Bal*I and *Pvu*II restriction enzymes, which yielded the correct sizes of the products (data not shown).

3. Results

3.1. Progastrin and its products

The ileum contained 0.3 pmol/g of progastrin, 1.1 pmol/g glycine-extended, and 1.1 pmol/l amidated gastrin (Table 1). The amidated gastrin eluted as non-sulfated gastrin-34 (Fig. 1). The identity of gastrin-34 was corroborated by rechromatography after incubation with trypsin, using antibodies directed the C- and the N-terminus of gastrin-17. Likewise, glycine-extended and further C-terminally extended precursors eluted after cleavages in one peak corresponding to glycine-extended gastrin-17 (data not shown). The lack of sulfation was demonstrated by the similar binding to Ab. 2604 and 2605 (Fig. 1), and further by elution in a position identical to that of non-sulfated porcine gastrin-34 in three chromatographic systems (Fig. 1). Measurements of glycine-extended gastrins and progastrins using antiserum 5284 before and after arylsulfatase treatment yielded identical results (data not shown). Consequently, also the gastrin precursors were non-sulfated.

3.2. ProCCK and its products

The ileum contained 7.6 pmol/g proCCK, 13.5 pmol/g glycine-extended, and 24.8 pmol/g amidated CCK (Table 1). Chromatography showed that amidated and glycine-extended CCK were present in several molecular forms of which the predominant were CCK-58 and 8 (Fig. 2). The CCK peak near the void volume eluted after cleavage with trypsin and carboxypeptidase B, in two peaks corresponding to glycine-extended and amidated CCK-8. Chromatography monitored by the assay specific for sulfated CCK showed that ileal CCK was completely sulfated (Fig. 3).

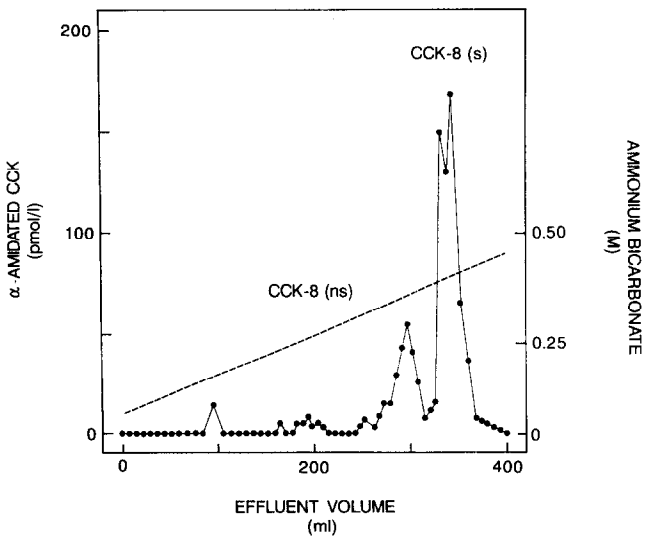

Fig. 3. Anion-exchange of 5 ml of neutral ilial extract was loaded on a DEAE-32 cellulose (10 × 150 mm) and eluted at 20°C with NH_4HCO_3 (33 ml/h) a continuous gradient as shown in the figure (- - - -). Fractions of 2.8 ml were monitored radioimmunochemically using Ab. 2609.

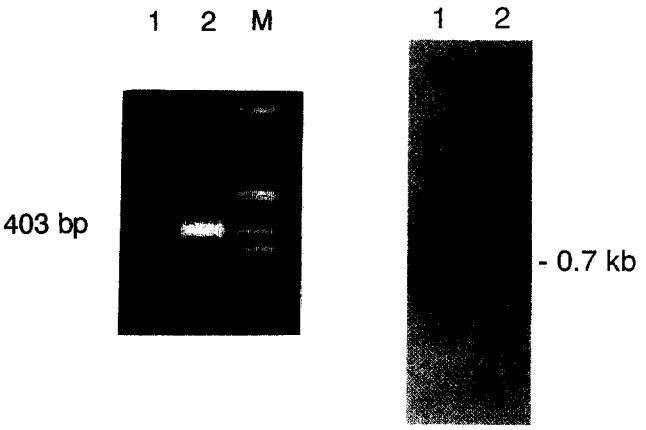

Fig. 4. Left panel: one μg of total RNA was reverse transcribed and amplified using a polymerase chain reaction with primers for porcine gastrin. 20 μg of sample was loaded and electrophoresed at 40 V for 6 h. Lane 1, ileal extract; lane 2, antral extract, and lane 3, 1 kb DNA ladder (Gibco/BRL). Right panel: Northern analysis of CCK mRNA in extract of porcine ileum. Total RNA: lane 1, 5 μg from duodenum; lane 2, 23 μg from ileum.

3.4. Gastrin and CCK mRNA

Northern analysis of RNA from the duodenal and ileal mucosa demonstrated a specific 0.7 kb CCK mRNA in both tissues (Fig. 4, left panel), whereas the gastrin mRNA in the ileum was below the detection limit. Reverse transcription PCR revealed, however, gastrin mRNA in the ileum (Fig. 4, right panel) in low concentrations.

4. Discussion

The present study has shown that both CCK and gastrin genes are expressed in the distal ileum. Ileal progastrin is processed in a unique tissue-specific manner, whereas ileal proCCK is processed as in the proximal small intestine. We did not find other peptides having the characteristic C-terminus, Trp-Met-Asp-Phe-NH_2. Therefore, the results suggest that TG-cells produce a peptide with a C-terminus resembling, but still different from that of CCK and gastrin.

Expression of the CCK gene was expected, since the distal ileum contains a few I-cells [4,5]. Accordingly, ileal proCCK processing as in proximal parts of the small intestine [21] was to be expected. More surprising is gastrin in the distal ileum. Previous studies have confined gastrin synthesis in the digestive tract to G-cells in the antroduodenal mucosa and - sporadically - in the proximal jejunum [22,23]. Antral G-cells synthesize five bioactive gastrins, of which gastrin-6 is completely sulfated, whereas gastrin-17, -34, and -71 are partly sulfated (for review, see [1]1). Consequently, the ileal processing to a single carboxyamidated form, non-sulfated gastrin-34, is unique. It bears some resemblance to the processing in pituitary corticotrophs, where non-sulfated gastrin-34

constitutes a majority of the α-amidated gastrin [24]. But corticotrophs contain in addition gastrin-71, and besides less than 2% of the pituitary progastrin mature to α-amidated gastrins [25]. It is noteworthy that the proteolytic processing to gastrin-34 requires cleavage only at two -Arg-Arg- sites, whereas mono-Arg and the -Lys-Lys- site N-terminal to the gastrin-17 sequence are unprocessed. Thus, ileal gastrin producing cells express a smaller number of processing enzymes than G-cells. Accordingly, our results argue that complete progastrin processing requires at least three different endoproteases, one for -Arg-Arg- cleavage, one for -Lys-Lys- cleavage, and one for mono-Arg cleavages.

The major question of this study was whether the gastrin or CCK in the ileum is compatible with the abundant TG-cells. Comparison with other gastrin or CCK producing endocrine tissues makes it unlikely that the immunoreactivity of ileal TG-cells can be accounted for by the low concentrations of CCK and gastrin. On the contrary, the ileal CCK is easily explained by the small ileal population of I-cells, and the ileal concentration of gastrin-34 is anyway too low to be identified by immunocytochemistry.

An explanation for the lack of TG-cell products in this study might be poor efficiency of the extraction techniques. However, since all known fragments of progastrin and proCCK are well extracted from other mucosal tissue by the general techniques used in this study [26], we find such explanation unlikely. Rather, our results suggest that the peptide(s) in the secretory granules of the TG-cells are neither proCCK nor progastrin products. The results also exclude the possibility that TG-cells express a proper third member of the gastrin-CCK family. In order to qualify as such, an intact C-terminal -Trp-Met-Asp-Phe-NH_2 sequence is required. If ileal extracts contained peptides with such C-terminus (but otherwise different from gastrin and CCK) they would inevitably have been measured by antiserum No. 2609. The results therefore indicate that TG-cells synthesize a peptide, which has a limited similarity with gastrin or CCK. Such peptide(s) is not recognized in the highly specific radioimmunoassays, although they may bind antibodies by the less specific and competitive immunocytochemical techniques. Recent examples of such less specific staining are the lymnaDFamide peptide in snail neurons [27], and the CGRP-containing neurons in the spinal cord [28]. So far, however, CGRP in the gut has been found only in neurons [29,30]. Consequently, the molecular nature of the TG-cell product still remains to be identified. Similarity with invertebrate lymnaDFamides [27] might be a starting point for further search.

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