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Glycine-extended post-translational processing intermediates of gastrin and cholecystokinin in the gut

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Summary

Cholecystokinin (CCK) and gastrin are two polypeptide hormones of the gut that share complete structural homology in their carboxyl-terminal pentapeptide. Both peptides are biologically activated from their glycine-extended precursor forms by a carboxyl-terminal α-amidation reaction. In the present studies we used region specific antisera to characterize the carboxyl-terminally amidated and glycine-extended forms of gastrin and CCK in mammalian intestine. Multiple amidated molecular forms of gastrin and CCK and their corresponding glycine-extended forms were detected throughout the most of the small bowel. Although, we detected substantial amounts of glycine-extended CCK in the proximal rat duodenum, we detected none of the corresponding amidated molecular forms. In contrast, the proximal duodenum of dog and hog contained both glycine-extended and amidated CCK. These findings suggest that there may be peptide, tissue and species specific differences in expression and activity of the peptide α-amidating enzyme.

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Abbreviations: CCK, cholecystokinin; G34, gastrin tetratriacontapeptide; G17, gastrin heptadecapeptide; G-gly, glycine-extended gastrins; CCK-gly, glycine-extended cholecystokinins; G14, gastrin tetradecapeptide; CCK8, cholecystokinin octapeptide; CCK8ns, nonsulfated cholecystokinin octapeptide; CCK33, cholecystokinin tritriacontapeptide; G13-gly, glycine-extended gastrin tridecapeptide; G6-gly, glycine-extended hexagastrin; CCK8-gly, glycine-extended cholecystokinin octapeptide; RP-HPLC, reverse phase high performance liquid chromatography; G17II, sulfated gastrin heptadecapeptide; G34II, sulfated gastrin tetratriacontapeptide.

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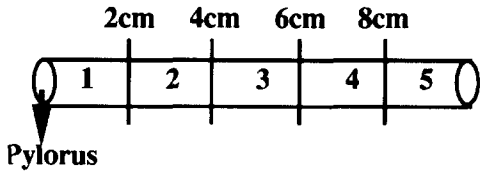
Introduction

The biologically active carboxyl-terminal pentapeptide of the hormone gastrin is identical to that of cholecystokinin (CCK). On the basis of the structures of their precursors, as derived from the nucleotide sequences of cDNA clones encoding them, both peptides appear to be synthesized as large translation products that are post-translationally processed to various extents [1,2]. This processing results in the formation of multiple molecular forms of CCK and two primary molecular forms of gastrin of 34 (G34) and 17 (G17) amino acids in length. An important feature of the biological activation of both peptides is the enzymatic formation of a carboxyl-terminal α-amide residue from a glycine-extended precursor which serves as the substrate for the reaction. In previous studies we have identified and characterized the glycine-extended forms of gastrin (G-gly) in gastrointestinal tissues and have demonstrated that G17 and G34 are amidated proportionately in antral tissue [3–5]. Although multiple forms of amidated CCK are present in the small bowel, its glycine-extended (CCK-gly) post-translational processing intermediates have not been characterized. The availability of region-specific antisera that recognize only G-gly or both G-gly and CCK-gly has permitted us to characterize the glycine-extended forms of CCK and gastrin present in mammalian small intestine.

Materials and Methods

Tissues and extraction

Male Sprague-Dawley rats (250–300 g) were given free access to food and water prior to the time of study. The animals were anesthetized with sodium pentobarbital 30 mg/kg i.p. and the small bowel was resected from the pylorus to the cecum through a midline abdominal incision. The small bowel was divided into two sections at a point 2.0 cm from the pylorus. The segments of bowel were then longitudinally incised, washed in 0.9% NaCl to remove food contents and debris and frozen immediately on dry ice. Because we found in preliminary studies that CCK and gastrin content in rat small intestine changed markedly within 2 cm from the pylorus we elected to evaluate the tissue from five segments of the rat duodenum 2 cm in length taken from pylorus to 10 cm distal. The segments were dissected and numbered as shown below.



Scheme I

Additionally, we elected to evaluate duodenal tissue taken from two other species, thus duodenal tissue was taken from three mongrel dogs and three hogs which were under general anesthesia administered by the Unit for Laboratory Animal Medicine (U.L.A.M.) at the University of Michigan. Circumferential sections of duodenum 2 cm in length were cut from just beyond the pylorus and 20 cm distal to that site. The tissue was rinsed in 0.9% NaCl to remove debris, then the mucosa from the hog and dog was carefully scraped from the muscularis and frozen on dry ice. Intestinal sections from rat, hog and dog were extracted in boiling water (10 ml/g tissue) for 10 min and centrifuged at 3000 rpm at 4 °C for 20 min. One half of the water extract supernatant was removed and frozen at −70 °C. An equal volume of 3 M acetic acid was added to the remaining supernatant and pellet and boiled for an additional 10 min. The samples were again centrifuged at 3000 rpm at 4 °C and the water/acetic acid supernatant stored at −70 °C. This water/acetic acid extraction method has been extensively by others [6–8] and greater than 70% recovery of the major molecular forms of gastrin and CCK from tissue was achieved.

For rat tissues a second extraction technique was applied to insure extraction of all CCK molecular forms [9]. Immediately after death the stomach and intestines were removed, rinsed with cold water and boiled for 2 min. The adipose tissue was then quickly removed, the tissue diluted 10-fold in 2% trifluouroacetic acid (TFA) and homogenized at 4°C for 2 min. Extracts were then immediately analyzed by reverse phase high performance liquid chromatography (RP-HPLC).

Peptides

The following peptides were synthesized by Peninsula Laboratories (Belmont, CA) and used in radioimmunoassays to verify the specificity of the region specific antisera and to serve as standards for calibration of gel filtration and ion-exchange columns: nonsulfated human gastrins 14 (G14), 17 and 34; nonsulfated rat gastrin 17; sulfated CCK octapeptide (CCK8) and unsulfated CCK octapeptide (CCK8ns), nonsulfated porcine CCK33; Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-Gly, glycine-extended human gastrin 13 (G13-gly); and Tyr-Gly-Trp-Met-Asp-Phe-Gly glycine-extended hexagastrin (G6-gly). The peptide Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-Gly, glycine-extended CCK8 (CCK8-gly), was a kind gift of Jens Rehfeld (Copenhagen, Denmark). Sulfated human gastrin 17 (G17II) was obtained from Sigma (St. Louis, MO). Additionally, we obtained rat gastrins from rat antral tissue which was extracted in the same manner as the duodenum. Amidated and glycine-extended gastrins were utilized to establish cross-reactivities of the antisera and for standardization of chromatography columns.

Radioimmunoassays

Gastrin, CCK and their respective glycine-extended processing intermediates were measured by radioimmunoassay. Three antisera were used to measure amidated gastrin and CCK. We recharacterized each with the purified and synthetic peptides described above and our cross-reactivity curves confirmed those previously reported (Table I) [9–14].

TABLE I

Cross-reactivity of antisera for amidated and glycine-extended gastrin and CCK peptides

Peptide	Antisera % cross-reactivity				
	1611	5135	2605	7207	8237
Human G14[a]	>95%	>95%	100%	<0.1%	<1.0%
Human G17	100%	100%	100%	<0.1%	<1.0%
Human G34	>95%	>95%	>95%	<0.1%	<1.0%
Rat G17	100%	100%	100%	<0.1%	<1.0%
Human G17II[b]	>95%	>95%	<10%	<0.1%	<1.0%
CCK8[c]	<2.0%	>95%		<0.1%	<1.0%
CCK8ns	<2.0%	>95%		<0.1%	<1.0%
CCK33	<2.0%	>95%		<0.1%	<1.0%
G13-gly[d]	<2.0%	<0.1%		100%	100%
G6-gly	<2.0%	<0.1%		>90%	>95%
CCK8-gly	<2.0%	<0.1%		<0.1%	>95%

[a] Gastrin peptides.

[b] Sulfated gastrin peptides.

[c] Cholecystokinin peptides, ns = non-sulfated.

[d] Glycine-extended peptides.

Antibody 1611 (kind gift of J. Walsh, Los Angeles, CA) that was specific for the carboxyl-terminal pentapeptide of amidated gastrin, exhibited greater than 95% cross-reactivity with amidated, nonsulfated human gastrins 14, 17 and 34 and sulfated human gastrin 17, as well as rat gastrin 17 and less than 2% cross-reactivity with CCK33, sulfated and nonsulfated CCK8, G13-gly, G7-gly, or CCK-gly [10]. Antibody 5135 (kind gift of G. Rosenquist, Los Angeles, CA), was characterized as specific for the carboxyl octapeptide of amidated gastrin and CCK and found to cross-react 100% with all forms of CCK greater than 6 amino acids [9,12,13] except for CCK58. Rat CCK58 is 5–10-times less immunoreactive than CCK8 with antibody 5135 [15]. In our laboratory, antibody 5135 was found to cross-react equally well with all of the amidated human and rat gastrins, sulfated and nonsulfated CCK8 and CCK33. Antibody 5135 cross-reacted less than 0.1% with any of the glycine-extended peptides (G13-gly, G7-gly and CCK-gly). Antibody 2605 (kind gift of J. Rehfeld, Copenhagen, Denmark) recognized all forms of nonsulfated amidated gastrin (G14, G17 and G34), but cross-reacted less than 10% with sulfated G17 [14]. This antibody was used to confirm the elution volume of sulfated gastrin peptides analyzed by ion-exchange chromatography and RP-HPLC [16]. All three radioimmunoassays for amidated peptides were performed utilizing ^{125}I-Leu^{15}-human gastrin 17 as the radiolabeled peptide and Met^{15} human G17 as standard. Thus, we were able to distinguish amidated CCK-like immunoreactivity from amidated gastrin immunoreactivity by subtracting immunoreactivity obtained with 1611 (gastrin) antibody from that obtained from antibody 5135 (CCK + gastrin) as previously described [17,18].

Glycine-extended peptides were quantified utilizing two antisera that have been previously characterized [4,9]. These antisera were recharacterized for our use with the peptides described above. Antibody 7207 (kind gift of J. Rehfeld, Copenhagen, Denmark) recognizes both sulfated and nonsulfated glycine-extended gastrins, but cross-reacts less than 0.1% with CCK8-gly or any of the amidated forms of gastrin and CCK [9]. Antibody 8237 recognizes CCK-gly, G13-gly and G6-gly equally well, but cross-reacts less than 1% with any amidated gastrin or amidated CCK [4]. As was the case for amidated CCK, we were able to distinguish CCK-gly immunoreactivity from G-gly immunoreactivity by subtracting immunoreactivity obtained from antibody 7207 (G-gly) from that obtained from antibody 8237 (CCK-gly + G-gly) [17].

Characterization of molecular forms

Aliquots of the water extracted supernatants were applied to a 1 × 120 cm Sephadex G-50 superfine (Pharmacia, Piscataway, NJ) column equilibrated with 50 mM sodium barbital buffer (pH 8.6) at 4°C. Aliquots of the water/acetic acid extracts were applied to a similar column equilibrated in 0.5 M ammonium acetate (pH 5.5). Fractions (1 ml) were collected and assayed for peptide content with the various antisera. Both columns were calibrated with human G17, G34 and CCK8 and with extracts from rat gastric antrum.

To confirm the precise nature of the peptides extracted from rat tissues that nearly coelute on gel filtration, water and water/acetic acid extracts were further analyzed by ion-exchange utilizing Fast Protein Liquid Chromatography (FPLC, Pharmacia, Piscataway, NJ). Extracts were diluted 10-fold in 50 mM Tris-HCl (pH 8.2) containing 10% acetonitrile (buffer A) and applied to a HR 5/5 MonoQ anion-exchange column. A linear gradient of buffer B (buffer A + 1 M NaCl) was run for 10 min to 20% B, then for 30 min to 40% B and an additional 10 min to 50% B. Flow rate was 1 ml/min and 1 ml fractions were collected for assay of peptide content. The column was calibrated utilizing synthetic standard human gastrins 17 and 34, sulfated human G17, rat G17, sulfated CCK8, nonsulfated CCK8-gly and extracts from rat gastric antrum. Recovery of 5 pmol of CCK8 and CCK33 standards from the column was 80% and 60%, respectively. The recoveries of

G34 and G17 were 85%. Elution volumes of sulfated amidated gastrins were confirmed by radioimmunoassay with antibody 2605 [16].

Rat tissues were also extracted in 2% TFA and analyzed by RP-HPLC [9]. After homogenization extracts were immediately filtered through a 0.25 μm filter and aliquots applied directly to an FPLC HR 5/5 pepRPC (C18 reverse-phase column, Pharmacia) previously equilibrated in 0.1% TFA. After the absorbance at 280 nm had returned to baseline following sample application, the column was eluted with a linear gradient to 50% acetonitrile over 40 min at a flow rate of 1 ml/min and 1 ml fractions collected.

Results

Rat duodenum

Water and water/acetic acid extracts of various segments of the rat duodenum contained significant amounts of both 5135 (gastrin + CCK) immunoreactivity and 1611 (gastrin) immunoreactivity. The amount of 1611 immunoreactivity was less than the 5135 immunoreactivity suggesting the presence of amidated molecular forms of both gastrin and CCK in the distal segments of the rat duodenum (Table II).

TABLE II

Immunoreactive gastrin (G) and cholecystokinin (CCK) peptides in segments of rat small intestine

Peptide content (pmol/g, mean, $n = 2$)	Segment No.				
	1	2	3	4	5
Water extract					
G + CCK	53	6.5	5.0	5.2	4.0
Gastrin	53	4.4	2.5	1.9	1.9
Water/acetic acid extract					
G + CCK	24	6.6	2.6	2.5	2.7
Gastrin	24	6.3	1.5	1.0	1.8

Surprisingly, in the most proximal segment of the rat duodenum the amount of immunoreactivity obtained with antibody 5135 (gastrin + CCK) was equal to that obtained with antibody 1611 (gastrin), suggesting a virtual absence of amidated CCK in this region (Table II). In order to ensure that there was not a small amount of amidated CCK present in the extracts they were analyzed by gel filtration and ion-exchange chromatography. Characterization of the 5135 (gastrin + CCK) immunoreactivity in water/acetic acid extracts in the rat proximal duodenum demonstrated the presence of immunoreactive peaks corresponding to G17 and G34 by gel filtration (Fig. 1A) and sulfated an nonsulfated forms of G17 and G34 by ion-exchange (Fig. 1B) chromatography. No 5135 or 1611 immunoreactivities were detected in the void volume of the ion-exchange column. Tissue extracted in 2% TFA was also analyzed by RP-HPLC (Fig. 1C) and demonstrated the presence of gastrin but not CCK molecular forms. All peaks detected with antibody 5135 (gastrin + CCK) were detected in similar amounts with antibody 1611 (gastrin). Furthermore, these gastrin molecular forms coeluted with synthetic gastrin standards and with water extracts of the rat antrum when analyzed by gel filtration (Fig. 1D), ion-exchange chromatography (Fig. 1E), or RP-HPLC (Fig. 1F). No amidated molecular forms of CCK (5135 immunoreactivity without 1611 immunoreactivity) were seen by either gel filtration, ion-exchange chromatography, or RP-HPLC in either the proximal duodenum or the antrum.

In contrast to the data for amidated gastrin and CCK in the proximal duodenum, 8237 immunoreactivity (G-gly + CCK-gly) was twice that of 7207 immunoreactivity (G-gly) demonstrating the presence of both G-gly and CCK-gly in proximal duodenal extracts (Table III). As expected, glycine-extended forms of G17 and G34 (7207 immunoreactivity) were seen in water/acetic acid extracts of the proximal duodenum (Fig. 2A and B) that coeluted with the forms seen in the antrum (Fig. 2C and D) with both gel filtration and ion-exchange chromatography.

There were peaks of 8237 (G-gly + CCK-gly) immunoreactivity that coeluted with G17-gly and G34-gly

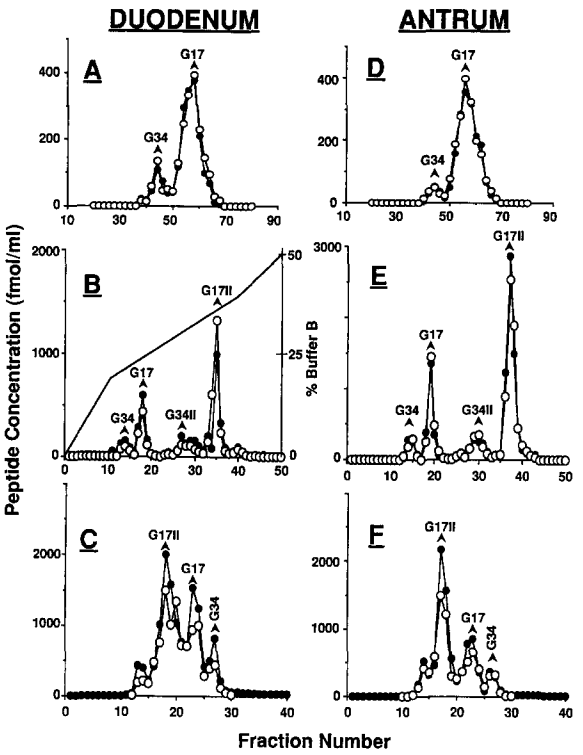


Fig. 1. Characterization of amidated CCK and gastrin molecular forms in the rat duodenum and gastric antrum. Water/acetic acid extracts by gel filtration chromatography (A and D) and ion-exchange chromatography (B and E) as outlined in Materials and Methods. Fractions were collected and assayed with antibody 5135 (gastrin + CCK, closed circles) and antibody 1611 (gastrin, open circles). The gel filtration column was calibrated by denoting the elution position of G17, G34 and CCK8 as shown in panel A. The MonoQ-Q ion-exchange column was calibrated with synthetic rat gastrin 17 as well as human G17, G17II G34 and CCK8 and a rat antral extract. Tissues extracted in 2% TFA were also analyzed by RP-HPLC (C and F) as outlined in Materials and Methods. This C18 column was calibrated with synthetic rat G17, human G17II, G34 and CCK8. Note that all peaks detected with antibody 5135 were also detected with antibody 1611 and did not co-elute with the amidated molecular forms of CCK seen in the distal small bowel (Fig. 3). The identification of the sulfated forms of gastrin was confirmed with antibody 2605. All peaks of 5135 immunoreactivity were detected with antibody 1611 in water extracts as well (data not shown). The chromatograms shown are representative of at least 2 others showing similar results.

that were also detected by antibody 7207 (G-gly). There were, however, additional peaks of 8237 (G-gly + CCK-gly) immunoreactivity in the proximal duodenal extracts that were not detected by antibody 7207 (G-gly) after both gel filtration and ion-exchange chromatography. These additional peaks were not seen in the antrum and presumably represent the various molecular forms of CCK-gly in the proximal duodenum.

Rat distal small bowel

Because we were surprised to find virtually no amidated CCK in the rat proximal duodenum despite multiple molecular forms of CCK-gly, we extended our studies to characterize both CCK and CCK-gly in the rat distal small bowel. Both water and water/acetic acid extracts of the rat distal small bowel contained more 5135 (gastrin + CCK) than 1611 (gastrin) immunoreactivity and more 8237 (G-gly + CCK-gly) than 7207 (G-gly) immunoreactivity indicating the presence of amidated and glycine-extended CCK molecular forms and a paucity of the various gastrin forms (Table III). Further analysis of the extracts by gel filtration confirmed that amidated gastrin was scarcely present in the distal small bowel in that there were no identifiable molecular forms of amidated gastrin (1611 peaks), although major peaks of CCK (5135 peaks) were noted (Fig. 3A). There was a peak that coeluted with standard CCK8 and other peaks eluting earlier than CCK8, presumably larger molecular forms of amidated CCK. These results were confirmed by ion-exchange chromatography which again revealed the presence of amidated forms of CCK, but no amidated forms of gastrin (Fig. 3B). Rat distal bowel 2% TFA extracts analyzed by RP-HPLC revealed multiple peaks of 5135 immunoreactivity one of which coeluted with CCK8 (Fig. 3C). As in the case of the amidated forms of CCK and gastrin, no glycine-extended gastrin forms were seen (7207 peaks) in the rat distal small bowel, although multiple forms of CCK-gly (8237 peaks) were detected by both gel filtration (Fig. 4A) and ion-exchange chromatography (Fig. 4B).

TABLE III

Immunoreactive gastrin (G) and cholecystokinin (CCK) peptides in intestinal extracts from rats, hogs and dogs

Tissue extract	Peptide content (pmol/g, mean ± S.E.)			
	G + CCK	G	(G + CCK)-gly	G-gly
Rat ($n = 5$)				
duodenum				
water	54 ± 8	53 ± 5	32 ± 4	18 ± 3
water/acetic acid	24 ± 4	25 ± 5	23 ± 5	10 ± 2
small bowel				
water	3 ± 1	1 ± 1	35 ± 7	8 ± 1
water/acetic acid	8 ± 1	3 ± 1	36 ± 2	7 ± 1
Hog ($n = 3$)				
proximal duodenum				
water	164 ± 35	92 ± 45	4 ± 1	1 ± 1
water/acetic acid	47 ± 4	12 ± 3	22 ± 5	1 ± 1
distal duodenum				
water	57 ± 5	13 ± 2	2 ± 1	1 ± 1
water/acetic acid	25 ± 1	3 ± 1	21 ± 4	1 ± 1
Dog ($n = 3$)				
proximal duodenum	50 ± 1	6 ± 1	11 ± 7	2 ± 1
water	50 ± 1	6 ± 1	11 ± 7	2 ± 1
water/acetic acid	26 ± 1	3 ± 1	14 ± 7	7 ± 3
distal duodenum				
water	50 ± 6	5 ± 1	1 ± 1	1 ± 1
water/acetic acid	40 ± 3	3 ± 1	16 ± 5	1 ± 1

Hog and dog duodenum

In view of the unexpected finding that the rat proximal but not distal duodenum contained CCK-gly, but no amidated CCK, we extended our studies to two other species. As was the case with the rat distal duodenum, the hog and dog distal duodenum contained significantly greater amounts of 5135 (gastrin + CCK) immunoreactivity than 1611 (gastrin) immunoreactivity, suggesting the presence of amidated forms of both gastrin and CCK (Table III). Likewise, the proximal duodenum of both the hog and dog was similar to the distal segments in both species in that there was substantially more 5135 immunoreactivity than 1611 immunoreactivity. This suggests the presence of amidated CCK in the hog and dog proximal duodenum and is in direct contrast to our findings in the rat proximal duodenum. As expected, both the hog and dog proximal and distal duodenal extracts contained more 8237 (gastrin-gly + CCK-gly) than 7207 (gastrin-gly) immunoreactivity, demonstrating the presence of both CCK-gly and G-gly. Gel filtration of the hog proximal (Fig. 5A) and distal (Fig. 5B) duodenal water extracts showed multiple peaks of 5135 (gastrin + CCK) immunoreactivity, only some of which were detectable with antibody 1611 (gastrin) confirming the presence of both amidated CCK and gastrin. As was the case with the hog, gel filtration chromatograms of dog

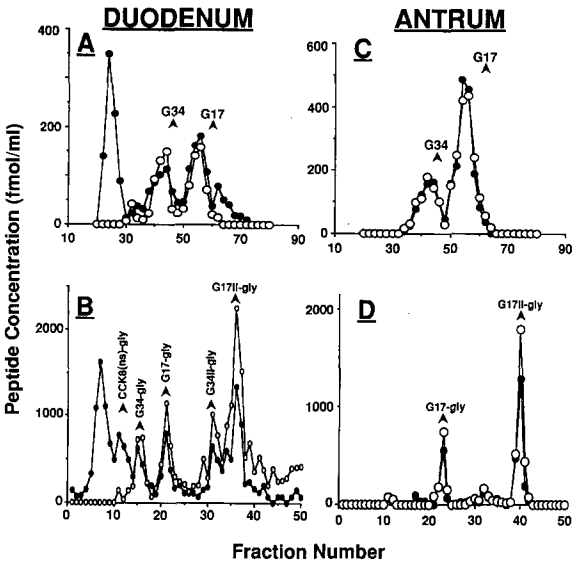


Fig. 2. Characterization of glycine-extended gastrin and CCK molecular forms in the rat duodenum and gastric antrum water/acetic acid extracts by gel filtration chromatography (A and C) and ion-exchange chromatography (B and D) as outlined in Materials and Methods. Fractions were collected and assayed with antibody 8237 (G-gly + CCK-gly, closed circles) and antibody 7207 (G-gly, open circles). The columns were calibrated as described in Materials and Methods and the elution positions for G17, G17II G34 and CCK8 synthetic standards are denoted. The peaks corresponding to G17-gly, G17II-gly, G34-gly and G34II-gly shown in panel B were determined by calibration with a rat antral water extract (D). All 4 glycine-extended gastrin molecular forms eluted just after the corresponding amidated molecular form with ion-exchange chromatography. The chromatograms shown are representative of at least 2 others showing similar results.

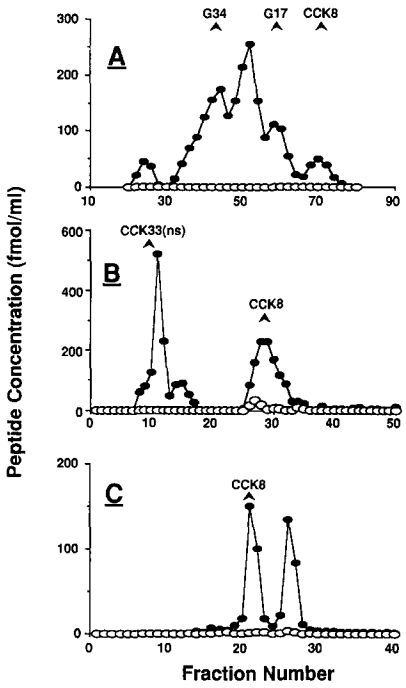


Fig. 3. Characterization of amidated CCK molecular forms in the rat distal small bowel water/acetic acid extracts by gel filtration chromatography (A) and ion-exchange chromatography (B) as outlined in Materials and Methods. Fractions were collected and assayed with antibody 5135 (gastrin + CCK, closed circles) and antibody 1611 (gastrin, open circles). The columns were calibrated as described in Materials and Methods and the elution positions for G17, G17II G34 and CCK8 synthetic standards are denoted. Tissues extracted in 2% TFA were also analyzed by RP-HPLC (C) as outlined in Materials and Methods. The chromatograms shown are representative of at least 2 others showing similar results.

proximal duodenal water (Fig. 6A) and water/acetic acid (Fig. 6B) extracts showed multiple peaks of amidated gastrin and CCK.

Discussion

The crucial importance of the amidation reaction that converts glycine-extended peptides into amidated peptides is illustrated by the observation that deamidated forms of gastrin and CCK have markedly reduced potency in biological assays when compared to their corresponding amidated forms [19–21]. The molecular forms of fully processed carboxyl-terminally amidated gastrin and its glycine-extended post-translational processing intermediates have been well characterized in the gastric antrum and amidated G34 and G17 have been localized in the duodenum of a variety of species [22,23]. Moreover, multiple molecular forms of CCK of 58, 39, 33, 22, 8 and 5 amino acids in length have been identified in

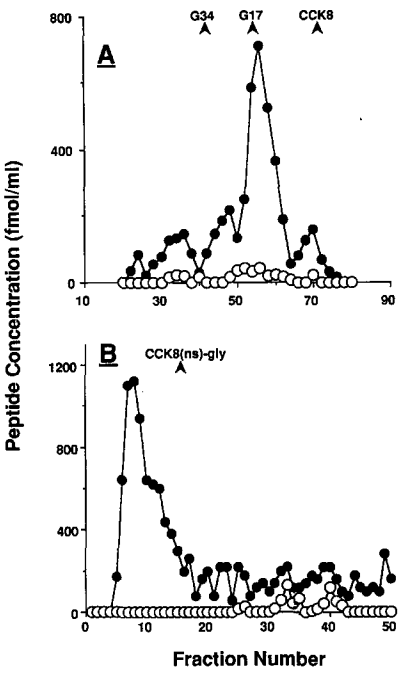


Fig. 4. Characterization of glycine-extended gastrin and CCK molecular forms in the rat distal small bowel water/acetic acid extracts by gel filtration chromatography (A) and ion-exchange chromatography (B) as outlined in Materials and Methods. Fractions were collected and assayed with antibody 8237 (G-gly + CCK-gly, closed circles) and antibody 1611 (G-gly, open circles). The columns were calibrated as described in Materials and Methods and the elution positions for G17, G17II G34 and CCK8 synthetic standards are denoted. The chromatograms shown are representative of at least 2 others showing similar results.

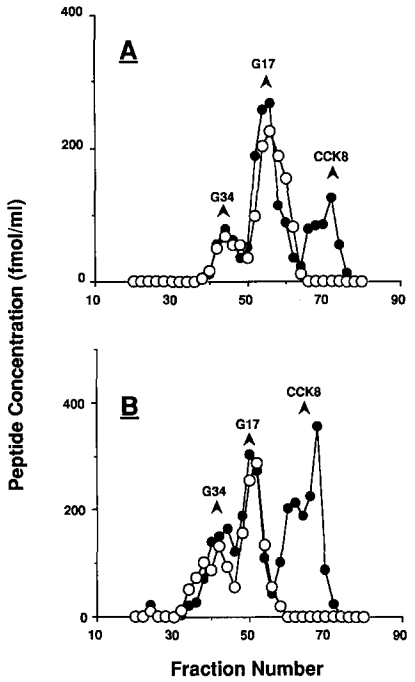


Fig. 5. Gel filtration profiles of hog proximal (A) and distal (B) duodenal water extracts (B). Fractions were collected and assayed with antibody 5135 (gastrin + CCK, closed circles) and antibody 1611 (gastrin, open circles). The gel filtration column was calibrated by denoting the elution positions of G17, G34 and CCK8. The chromatograms shown are representative of at least 2 others showing similar results.

portions of mammalian intestine [6,9,24–33] and although CCK-gly is felt to be released after a meal [34] the molecular forms of intestinal CCK-gly and G-gly have yet to be determined. We therefore sought to characterize these glycine-extended processing intermediates in the small intestine. As in other tissues, multiple amidated molecular forms of gastrin and CCK and their corresponding glycine-extended forms were detected throughout the small bowel. Surprisingly, we detected substantial amounts of CCK-gly in the proximal rat duodenum without the corresponding amidated molecular forms. In contrast, the proximal duodenum of dog and hog contained both glycine-extended and amidated CCK. These findings suggest that there may be peptide-, tissue- and species-specific differences in expression and activity of the peptide α-amidating enzyme, the enzyme responsible for converting glycine-extended peptide precursors to their biologically active amidated forms.

Since we sought to characterize the glycine-extended and amidated molecular forms of gastrin and CCK we utilized an extraction procedure (boiling water/acetic acid) that has previously been shown to extract multiple molecular forms of both gastrin and CCK efficiently [6–8]. For our studies we chose antisera specific for the carboxyl- rather than amino-terminus so that we could simply but clearly distinguish amidated gastrin and CCK molecular forms

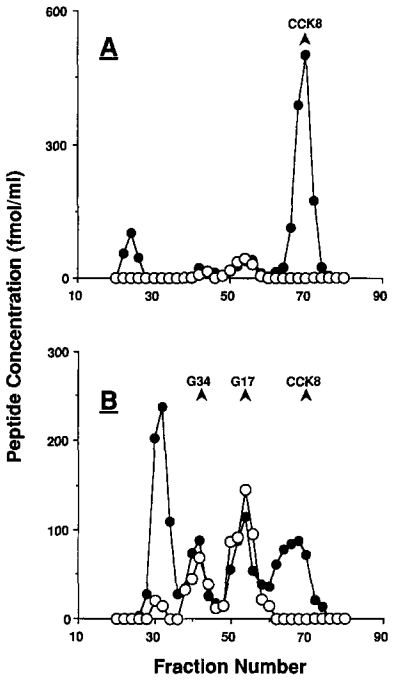


Fig. 6. Gel filtration profiles of dog proximal duodenal water (A) and water/acetic acid extracts (B). Fractions were collected and assayed with antibody 5135 (gastrin + CCK, closed circles) and antibody 1611 (gastrin, open circles). The gel filtration column was calibrated by denoting the elution positions of G17, G34 and CCK8. The chromatograms shown are representative of at least 2 others showing similar results.

from their glycine-extended processing intermediates. To distinguish amidated CCK from amidated gastrin in the duodenum and small bowel we used subtraction assays with antibodies 1611 and 5135. These antibodies have been well characterized by others [4,9–14] and their characterization was confirmed in our laboratory using synthetic standards. This subtraction assay provides a reliable method for measuring CCK immunoreactivity because antibody 5135 recognizes gastrin and virtually all sulfated and nonsulfated forms of CCK larger than CCK 4–6 equally well [9,13] and because antibody 1611 cross-reacts less than 0.1% with any form of CCK. Although the validity of the subtraction assay has been questioned in plasma assays where the concentrations of CCK are very small, antibodies 5135 and 1611 have detection limits of less than 2 fmol/ml enabling the detection of the relatively large quantities of CCK in tissue extracts [17]. Thus, subtraction of 1611 (gastrin) immunoreactivity from 5135 (gastrin + CCK) immunoreactivity allows us to detect amidated CCK-immunoreactivity in our extracts. Furthermore, this subtraction technique has been used by others to examine the processing of CCK in the developing rat gut [17]. In an analogous manner, antibody 7207 recognizes sulfated and nonsulfated forms of glycine-extended gastrin and does not cross-react with CCK-gly [9], whereas antibody 8237 recognizes both G-gly and CCK-gly equally well [4,17]. Subtraction assays using these two antibodies permitted the measurement of CCK-gly immunoreactivity. Confirmation that the radioimmunoassays and extraction procedures described above were reasonably accurate can be found in the fact that the total tissue content of amidated CCK that we measured in the small bowel was in agreement with several previous authors using similar antisera in the hog [9,35] and dog [9,33]. Because other authors have identified greater amounts of CCK in the rat small bowel [9,36], we utilized a TFA extraction technique felt to aid in the extraction of larger molecular forms of CCK [9]. As was the case with the water/acetic acid extracts, we were unable to identify substantial amounts of amidated CCK in the rat proximal duodenum. A possible explanation for the lower levels of amidated CCK seen in the rat bowel in our studies when compared to others include the location of the bowel chosen since CCK content decreases in distal portions of the small bowel [9,17,35].

We identified amidated gastrin molecular forms (G17, G17II, G34 and G34II) in the rat proximal duodenum as reported previously by others [22,23]. To our surprise our data indicated the virtual absence of amidated CCK in the rat proximal duodenum. To confirm that there was not a small amount of amidated CCK present in either the water, water/acetic acid, or TFA extracts, they were analyzed further by gel filtration, ion-exchange, or RP-HPLC

(Fig. 1). We observed that G34 is relatively more abundant in the rat duodenum than antrum but that G17 remains the predominant form. We were, however, unable to detect any peaks of 5135 (gastrin + CCK) immunoreactivity without 1611 (gastrin) immunoreactivity in the proximal 2 cm of the rat duodenum using any of three different chromatographic techniques. While it is possible that our assays may not have detected small quantities of CCK of less than 8 amino acids in length (inasmuch as the cross-reactivity of 5135 with these forms is 25–30%), these peptides could only be present in exceedingly small amounts since our assay is able to detect concentrations of amidated CCK to as low as 2 fmol/ml. Although previous reports by others have identified CCK in rat duodenum, the portion of intestine employed for extraction either has generally included more distal segments of the duodenum and/or the entire segment of small bowel [9,28,36]. Furthermore, amidated forms of CCK were detected in extracts of more distal segments of the rat duodenum, indicating that the extraction technique used for duodenal tissues was valid. For additional confirmation of the validity of our findings we applied identical techniques to the hog and dog proximal duodenum and detected substantial quantities of both amidated CCK and CCK-gly. Further supporting evidence is provided by the fact that we did not detect amidated CCK in 2% TFA rat duodenal extracts when analyzed by RP-HPLC. Thus, the virtual absence of CCK in the rat proximal duodenum appears to be species specific.

We also characterized the molecular forms of CCK in the rat distal small bowel by gel filtration chromatography (Fig. 3). We found a similar distribution of CCK molecular forms as reported by others utilizing the same antisera (5135) and TFA/acetonitrile extraction technique [9]. We were able to define the elution volumes of these amidated CCK forms on ion-exchange FPLC and RP-HPLC as distinct from amidated rat antral gastrins. In distal small bowel extracts we detected substantial amounts of CCK-gly immunoreactivity and demonstrated multiple molecular forms by both gel filtration and ion-exchange chromatography. As reported previously for G-gly [3], our findings suggest that a molecular form of CCK-gly can be found for each molecular form of amidated CCK in the intestine. The availability of additional CCK-gly standards will enable us to optimize our extraction techniques and to quantitate these forms in future studies.

In previous studies, we and others have characterized the post-translational processing of carboxyl-terminally amidated peptide hormones such as gastrin and CCK and observed that glycine-extended intermediates of prohormone processing are found, stored and co-secreted with their amidated counterparts [31,34,37]. Thus, the absence of CCK producing I cells is not the sole explanation for our observation that CCK-gly was abundantly present in the same extracts that were noted to contain no amidated CCK. Rather, the presence of CCK-gly in the proximal duodenum despite the absence of CCK indicates that the crucial step in post-translational processing, specifically the α-amidation, of rat CCK is diminished. The amidation enzyme is known to require Cu^{2+} and ascorbate as co-factors and in their absence amidation is markedly attenuated [38,39]. However, the presence of amidated gastrin in the proximal duodenum suggests that the conditions in this region of intestine are appropriate for amidation to occur. The possibility that the amidation enzyme is absent specifically in CCK-cells in general is excluded by the clear documentation of CCK-gly conversion to CCK in the distal small bowel. One explanation for our findings may be that the amidation enzyme in the proximal rat duodenum has different substrate specificity from the enzyme found in the distal small bowel. In previous studies we have demonstrated that the amidating enzyme shows remarkable specificity for glycine-extended substrates. For example, in vitro, various molecular forms of the enzyme from both brain and gut tissues exhibit nearly a 10-fold greater affinity and V_{max} for converting glycine-extended pancreatic polypeptide (PP) to amidated PP than for converting G-gly to gastrin [40].

In similar fashion, it is possible that the amidating enzyme in the proximal rat duodenum exhibits a greater affinity for G-gly over CCK-gly if both prohormones were expressed in the same cell in this portion of the duodenum. Alternatively, it is possible that there is a relative reduction of amidating enzyme activity in CCK-cells of the proximal duodenum. The physiologic implications of the observation are as yet unclear but it is possible that glycine-extended peptide precursors may possess biological functions distinct from those of the mature, amidated peptide as in the case of thyrotropin releasing hormone. The precursor peptide (cryptic peptide) of thyrotropin releasing hormone has a physiologic function distinct from that of the mature peptide [41]. The mechanisms for the lack of CCK amidation in the rat proximal duodenum require additional studies for elucidation.

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