Characterization of Canine Intestinal Cholecystokinin-58 Lacking Its Carboxyl-terminal Nonapeptide

EVIDENCE FOR SIMILAR POST-TRANSLATIONAL PROCESSING IN BRAIN AND GUT*

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An antibody raised against a synthetic cholecystokinin (CCK) analog, (1-27)-(CCK)-33, corresponding to the midregion of CCK-58, detected immunoreactivity in intestinal extracts which eluted between the positions of CCK-33/39 and CCK-58 on high performance liquid chromatography. This peak, lacking carboxyl-terminal cholecystokinin immunoreactivity, was purified by reverse phase and cation-exchange chromatographies. Amino acid, mass spectral, and microsequence analysis established that it was the amino-terminal desnonapeptide fragment of cholecystokinin-58, (1-49)-CCK-58. It was demonstrated further that CCK-58 has less biological activity than CCK-8, suggesting that the amino terminus either sterically hindered the ability of CCK-58 to exert its biological activity or that its amino terminus acted at another site to inhibit release of amylase from rat pancreatic acini. The desnonapeptide of CCK-58 by itself had no biological activity, but did affect CCK-8-stimulated amylase release from isolated rat pancreatic acini, suggesting that the amino terminus shields the carboxyl terminus from expressing its biological activity. Its presence in intestine suggests that it is released into the circulation where it could be detected by midregion antibodies.

The presence of high proportions of (1-49)-CCK-58 indicates that most CCK-8 is directly derived from CCK-58. Its occurrence in brain and intestine indicates similar processing for procholecystokinin in both tissues.

Molecular biological techniques are being used to discover the structure of several important peptide and protein precursors. However, our understanding of how these precursors are processed to form the biologically active peptide is limited. Therefore, predictions of cleavage sites are not always accurate. Cleavage occurring after double basic residues has been recognized as a general processing site, but for several peptide hormones cleavage does not occur after pairs of basic residues. Vasoactive intestinal polypeptide (1) is one of several examples in which double basic residues remain intact in the biologically active peptide. Cholecystokinin-33 (2) is an example in which cleavage is between the basic residues and not after the pair. Recently, it has been proposed that cleavages occur after basic residues if (i) leucine or alanine residues are in the same region as the residue to be cleaved and (ii) there is an arginine (or rarely, a histidine) 3, 5, or 7 residues before the arginine to be cleaved (3). Nevertheless, there are still several known processing sites that would not have been predicted by these rules.

Since the signal peptide and active region of peptide hormones take up only a small portion of their precursor, investigators have tried to find functions for the remaining portions of the precursor. Some structural elements are needed for correct processing. Glycine preceding a double basic residue may signal amidation sites (4). Acidic residues near a tyrosine may signal for sulfation of that tyrosine (5). Serine or threonine residues after an asparagine may signal glycosylation of the asparagine (6). More in-depth knowledge about processing peptide precursors will enhance our ability to predict potential processing products.

The structure of preprocholecystokinin has been determined by cDNA techniques for man (7), rat (8), pig (9), and mouse (10). Since it has been shown that there is only one gene for CCK (7), all processed forms must arise from the same propeptide in all tissues that express cholecystokinin. Preprocholecystokinin can be divided into five regions (Fig. 1) resulting from proteolytic processing. The signal peptide is cleaved off by signal peptidase as preprocholecystokinin crosses the endoplasmic reticulum membrane. An amino-terminal flanking peptide connects the amino terminus of preprocholecystokinin to the largest characterized form of CCK (CCK-58). The position of signal peptidase cleavage has not been determined, so the exact structure of the signal peptide and the amino-terminal flanking peptide is not known. The cleavage to form the amino terminus of CCK-58 occurs after

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1 The abbreviations used are: CCK, cholecystokinin; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography.
of amylase from rat pancreatic acini.

Recently, it was reported that a CCK-related peptide similar in size to CCK-58 could be detected in canine intestinal mucosa extracts by midregion CCK immunonassays but not by carboxyl-terminal CCK immunonassays (23). Two modifications of CCK-58 could account for these results. The first is that the carboxyl terminus is not fully processed to form the phenylalanine amide, thus carboxyl-terminal extensions of Gly, Arg-Gly-Arg, or further extensions may exist. Rehfeld and Hansen (24) have concluded from immunological results that glycine extensions exist on CCK peptides extracted from porcine brain. The second possibility is that proteolytic activity has removed the carboxyl terminus. CCK-58 lacking a carboxyl-terminal nonapeptide has been isolated from porcine brain extracts (25). The structure of the cholecystokinin peptide containing midregion but lacking carboxyl-terminal immunoreactivity in the intestine has been postulated based on results of region specific radioimmunonoassays, but it has not been determined chemically (23).

The purposes of the present study were to purity and characterize a peptide related to CCK-58 which was of similar size but lacking carboxyl-terminal immunoreactivity and to study the biological activity of purified CCK-58. We report the isolation and structural analysis of the peptide lacking carboxyl-terminal cholecystokinin immunoreactivity. We have also tested the hypothesis that the amino terminus of canine CCK-58 sterically hinders its carboxyl terminus from expressing its biological activity.

**EXPERIMENTAL PROCEDURES**

**Tissue Extraction**—Mongrel dogs were killed after a 24-h fast by a pentobarbital overdose. The small intestine was obtained within 5 min of death and irrigated quickly with cool water and placed in 3 volumes of boiling water. The intestine was cooled and the mucosa removed from the muscle by scraping. The mucosa was stored frozen at -60 °C until extraction. Frozen intestinal mucosa (1 kg) from the upper third of the small intestine was homogenized in 4% trifluoroacetic acid using two other Rains (27). The mixture was centrifuged at 2,000 x g for 2 h and the supernatant used for peptide purification.

**Radioimmunoassay**—Antibody 5135, a kind gift from Rosenquist and Walsh (28), was used to measure the carboxyl terminus of biologically active cholecystokinin peptides. This antibody recognizes sulfated and unsulfated forms of gastrin and cholecystokinin but does not detect fragments without the carboxyl-terminal phenylalanine amide (27). Antibody 736 to canine (1-27)-CCK-33 was developed in Dr. Eysselein’s laboratory (23). It is specific for the midregion of canine CCK-33 and recognizes CCK peptides with or without amidated carboxyl termini.

**Analytical Chromatography**—Crude extracts were centrifuged at 70,000 x g. A portion of the supernatant (250 ml) was loaded directly onto an analytical Waters reverse phase C-18 column (4.9 x 250 mm) equilibrated in 0.1% trifluoroacetic acid. The column was eluted with an increasing gradient of acetonitrile. The effluent was monitored by the midregion and carboxyl-terminal CCK radioimmunonoassays described above.

**Peptide Purification**—The crude supernatant was loaded directly onto a Rains C-8 reverse phase column (21.4 mm x 25 cm) at a flow rate of 30 ml/min using a Rains HPX’ drive module with preparative (100 ml/min) pump head. After loading, the column was rinsed with 0.1% trifluoroacetic acid until the absorbance at 280 mm neared zero. After rinsing, the column was eluted at 5 ml/min with a linear gradient to 50% acetonitrile in 0.1% trifluoroacetic acid over 100 min using two other Rains HPX drive modules with preparative (50 ml/min) pump heads.

Two peaks were purified further. The peak containing CCK-58 was purified using the carboxyl-terminal antibody 5135 for peak detection. The peptide lacking carboxyl-terminal immunoreactivity was purified using the midregion antibody 736.

**Amino Acid Analysis**—Shortly before and shortly after each biological experiment approximately 100 pmol of the purified peptide was hydrolyzed in 6 M HCl for 22 h at 110 °C. The hydrolysate was...
dried, dissolved in dilution buffer, and applied to a Beckman 6200 amino acid analyzer. The eluted amino acids were derivatized with ninhydrin. The derivatives were detected by absorbance at 440 and 540 nm and quantitated on a Nelson analytical system.

**Microsequence Analysis**—The purified peptides were sequenced on a Beckman 6300A amino acid analyzer. Phenylthiohydantoin derivatives were analyzed using the system described by Hawke et al. (29).

**Mass Spectral Analysis**—The purified peptide (200–400 pmol) was analyzed with a JEOL HR 100HF mass spectrometer operating at a 3-kV accelerating potential and a nominal resolution of 1,000. Sample ionization was accomplished using a 6-kV Xenon atom beam. A solution of diethyliothreitol/dithioerythritol (5:1) spiked with camphor sulfonic acid (6 mm) was used as a sample matrix. Positive ion spectra were collected over the mass range m/z = 1650–7500 using a JEOL DA5000 data system. The mass value assigned to the sample is for the average mass of the protonated molecular ion and is accurate to ± 1.0 mass units.

**Preparation of Pancreatic Acini and Bioassay of CCK**—The bioactivities of CCK-8 and (1–49)-CCK-58 were determined by their ability to stimulate amylase release from isolated rat pancreatic acini as described previously (30). Briefly, pancreatic acini were prepared from Sprague-Dawley rats weighing 250–280 g according to the method of Williams and co-workers (30, 31). Krebs-Henseleit bicarbonate buffer containing 0.1 mg/ml soybean trypsin inhibitor and 0.1 mg/ml chromatographically purified collagenase was injected into the pancreatic parenchyma, and the tissue was incubated at 37 °C for 50 min. The acini were then dissociated by passage through pipettes with restrictive orifices and purified by centrifugation through step gradients of buffer containing 4% bovine serum albumin. The peak containing the middle region of midregion immunoreactivity and major amounts of absorbance were not detected, which indicated that the peptide ends in His-Arg-Ile-Ser-Asp-COOH. Tyrosine, arginine, and methionine were low in both CCK-58 and its desnonapeptide.

**Microsequence Analysis**—Microsequence analysis of the peptide lacking carboxyl-terminal immunoreactivity was continuous for 29 residues (Table III). All of the amino acids identified were the same as those determined for CCK-58 (12). The low absorbance at 280 nm is consistent with a peptide lacking tryptophan, which is located four amino acids from the carboxyl terminus of CCK-58.

**Mass Spectral Analysis**—The final assignment of the structure as CCK-58 desnonapeptide was based on the molecular weight obtained by mass spectral analysis. A prominent protonated molecular ion was observed at m/z 5407.6, which is in good agreement for the calculated value of 5408.3 for (1–49)-CCK-58.

**Biological Activity of CCK-8, CCK-58, and CCK-58 Desnonapeptide on Pancreatic Acini**—HPLC-purified canine CCK-58, CCK-58 desnonapeptide, and synthetic CCK-8 were diluted with 0.1% acetic acid containing 0.1% bovine serum albumin until the final dilution. The final dilution into assay buffer (Tris-Ringer buffer containing 6.5% bovine serum albumin) was done, and the peptides were incubated with pancreatic acini. CCK-8 stimulated amylase release in a dose-dependent manner (Fig. 4). At CCK-8 concentrations greater than 100 pm a reduced response on amylase release was observed. This biphasic response has been well demonstrated for CCK (31, 33). In contrast, CCK-58 desnonapeptide did not stimulate amylase release from rat pancreatic acini (Fig. 4).

The ability of CCK-58 to release amylase from rat pancreatic acini was compared with CCK-8. CCK-58 also caused release of amylase in a dose-dependent manner, with higher concentrations showing the biphasic response seen for CCK-8 (Fig. 4). However CCK-58 was 3–4 times less potent than CCK-8.

To determine if CCK-58 desnonapeptide had an effect on stimulated amylase secretion from pancreatic acini, concentrations of 30–1000 pm CCK-58 desnonapeptide were incubated with acini in the presence of 30 pm CCK-8. CCK-58 desnonapeptide had neither a stimulatory nor inhibitory effect on CCK-8 stimulated amylase release (Table IV).

**DISCUSSION**

It has been reported previously that a peptide containing midregion but no carboxyl-terminal cholecystokinin immunoreactivity is detected in extracts of canine intestines (23). Eng and co-workers (25) have published that brain contains...
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**Fig. 2.** Analytical HPLC of canine intestinal extracts. The same elution fractions were measured with canine midregion-specific radioimmunoassay, using antibody 736 (23) (A) and carboxyl-terminal-specific radioimmunoassay, using antibody 5135 (26) (B).

### Table 1
Purification of CCK-58 desnonapeptide

| Step column | Peptide loaded | Peptide recovered | Amount in pooled peak | % Recovery |
|-------------|----------------|-------------------|-----------------------|------------|
| C-8 Prep    | 14.0           | 8.4               | 4.2                   | 60         |
| C-18 Semi   | 3.3            | 4.2               | 2.9                   | 127        |
| C-4 Semi    | 4.7            | 4.4               | 3.2                   | 95         |
| C-18 Anal   | 1.2            | 1.1               | 1.1                   | 92         |
| C-4 Anal    | 0.9            | 0.9               | 0.9                   | 100        |
| C-18 Anal   | 0.8            | 0.6               | 0.6                   | 75         |
| C-4 Anal    | 0.3            | 0.2               | 0.2                   | 67         |
| FPLC Mono S | 1.3            | 1.3               | 1.3                   | 80         |

several cholecystokinin peptide fragments without the biologically active carboxyl terminus. A similar peptide in the intestine could account for the midregion immunoreactivity not associated with carboxyl-terminal immunoreactivity. However, an extended carboxyl terminus could also account for this immunoreactivity. The intestinal midregion cholecystokinin immunoreactivity was purified by several reverse phase HPLC steps and a cation-exchange FPLC step. The purified peptide was characterized by microsequence, amino acid, and mass spectral analysis. Its structure was that of (1–49)-CCK-58. Its presence in intestinal extracts indicates that similar processing occurs in intestine and brain, where it had been characterized previously (25).

The amino-terminal structure of this peptide was AVQKVDGEPRAH \ldots , which is the same structure as reported for canine intestinal (12) and brain CCK-58 (14). This sequence is different from the partial structure first reported for canine CCK-58, AQKVNSGEPRAH (34). We have sequenced the amino terminus of CCK-58 from single intestines of three dogs subsequent to the reported structures, and Eng has sequenced the amino terminus of CCK-58 from single intestines of five dogs.2 The amino terminus in these eight structural determinations were all AVQKVDGEPRAH \ldots , indicating that an error was probably made in the original partial sequence determination of canine CCK-58 (34) but in agreement with the full sequence, which was reported later (12).

CCK-58 is less potent than CCK-8 for release of amylase from rat pancreatic acini. It was therefore conceivable that the desnonapeptide amino-terminal fragment could diminish

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2 J. Eng, personal communication.
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**Fig. 3.** Final step of CCK-58 desnonapeptide purification by reverse phase HPLC. The solid line shows the absorption profiles at 220 and 280 nm; the dashed line, the gradient profile; and the dotted line, the midregion CCK immunoreactivity.

**TABLE II**

| Amino acid | Ratio residue/peptide | Theoretical pmol/peptide |
|------------|-----------------------|--------------------------|
| Asp        | 4.5                   | 5                        |
| Thr        | 0.1                   | 0                        |
| Ser        | 3.5                   | 4                        |
| Glu        | 5.2                   | 5                        |
| Pro        | 3.4                   | 3                        |
| Gly        | 3.7                   | 3                        |
| Ala        | 6.2                   | 6                        |
| Val        | 1.0                   | 3                        |
| Met        | 0.2                   | 1                        |
| Ile        | 2.7                   | 3                        |
| Leu        | 5.3                   | 5                        |
| Tyr        | 0.1                   | 1                        |
| Phe        | 0.2                   | 0                        |
| His        | 1.9                   | 2                        |
| Lys        | ND                    | 3                        |
| Arg        | 3.1                   | 5                        |
| Trp        | ND                    | 0                        |

ND means that the residues were not determined. The lysine was in a region in which an unknown peak emerged, making the determination of lysine impossible. Tryptophan is destroyed by acid hydrolysis.

the bioactivity of carboxyl-terminal CCK-8. However, CCK-58 desnonapeptide had no effect on CCK-8-stimulated release of amylase from rat pancreatic acini nor did it affect basal release. Specific binding of CCK-58 desnonapeptide is still a possibility, but if such binding does occur it does not appear to affect amylase release. It is possible that the amino terminus of CCK-58 shields the carboxyl terminus from reacting fully with pancreatic receptors because of tertiary structures not seen in smaller cholecystokinin peptides. Shielding could also account for the fact that CCK-58 is less immunoreactive than CCK-8 when carboxyl-terminal-directed assays are used to compare immunoreactivity (11, 22). This tertiary structure could also shield the biologically active carboxyl terminus.

**Fig. 4.** Biological activities of CCK-8, CCK-58, and CCK-58 desnonapeptide on isolated rat pancreatic acini. Increasing concentrations of CCK-8, CCK-58, or CCK-58 desnonapeptide were incubated for 30 min with isolated acini. The amylase released into the medium was measured and results expressed as the percent of total amylase content of each incubation vial. Values are the mean ± S.D. of triplicate determinations.

**TABLE IV**

| CCK-58 desnonapeptide | Amylase release (pm) |
|-----------------------|----------------------|
| 0                     | 17.2 ± 1.7           |
| 30                    | 16.3 ± 1.9           |
| 50                    | 16.6 ± 2.0           |
| 100                   | 17.0 ± 1.2           |
| 1,000                 | 16.6 ± 1.2           |
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from degradation in the circulation, in interstitial fluid, or in the vicinity of the receptor.

Even though CCK-58 is less active on the in vitro pancreas, preliminary studies suggest that in vivo circulating CCK-58 is responsible for most of the endocrine activity of cholecystokinin. In preliminary studies, when the two peptides were given as an intravenous bolus, CCK-58 was more potent than CCK-8 on exocrine pancreatic secretion and gastric motility.\(^5\) The increased in vivo biological activity of CCK-58 can be explained by a longer circulating half-life or resistance to degradation after leaving the circulation. The abundance of CCK-58 in the circulation after endogenous stimulation in man (35) and dog (36) further supports the hypothesis that CCK-58 expresses most of the in vivo bioactivity of cholecystokinin. The desnonapeptide of CCK-58, which is presumably released with other CCK forms into the circulation, does not alter in vitro basal and cholecystokinin-stimulated pancreatic secretion (Fig. 4 and Table IV). Thus, it is unlikely that the desnonapeptide affects bioactivity of circulating cholecystokinin.

It is important to consider the implications of these studies for the immunological detection of circulating cholecystokinin. It is probable that CCK-58 desnonapeptide is released into blood concomitantly with biologically active peptides. This occurs, midregion radiomunooassays would not detect cholecystokinin biological activity levels accurately. Cantor and Rehfeld (37) have reported recently that relatively large amounts of amino-terminal fragments of cholecystokinin are released into pig plasma and into duodenal perfusates. These fragments did not contain the biologically active carboxyl terminus. These authors also stressed the potential problems associated with attempting to measure cholecystokinin immunoreactivity with mid- or amino-terminal region antibodies. The other difficulty in the measurements of immunoreactiv-

\[^{5}\] H. Raybould and J. R. Reeve, Jr., unpublished results.

\[^{4}\] J. R. Reeve, Jr., V. E. Eysselein, G. A. Eberlein, P. Chew, and F.-J. Ho, unpublished results.
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The activity that forms CCK-8 from CCK-58. The amino terminus of CCK-58 causes a decrease in its biological activity versus the much higher ratio of CCK-58 to CCK-8 in the intestine and brain. This indicates similar post-translational processing in the course of these experiments and writing of the manuscript.

Dynamic studies such as pulse-chase labeling experiments are required to determine the processing pathways of preprocholecystokinin. To date, these are difficult experiments because tissue experiments are hindered by the low proportion of CCK cells in intestine and brain. Fig. 5 reflects possible processing pathways based on chemically characterized peptides of brain and gut. The major assumption in constructing this figure is that high amounts of a peptide indicate that it is a major intermediate in the processing of procholecystokinin. CCK-58 comprises nearly 30% of the total immunoreactivity in canine and porcine brain (14, 16), and more than 70% of the total immunoreactivity in canine (12), human (11), and feline (47) intestinal extracts. These immunoreactive data are major intermediates in the processing of procholecystokinin and feline (47) intestinal extracts.

These immunoreactive standards for CCK-58 and (1-49)-CCK-58 are available. CCK-58 comprises nearly 30% of the total immunoreactivity and CCK-33/39 and CCK-8 for 29 and 14%, respectively (Fig. 2B). The midregion immunoreactivity had only peaks when compared with other cholecystokinin forms. However, this shielding of the carboxyl terminus may actually by protecting the biologically active carboxyl terminus from degradation.

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