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Post-translational processing of cholecystokinin in pig brain and gut

(methanol extraction/acid extraction/high-performance liquid chromatography/Sephadex gel filtration)

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ABSTRACT **A sequential extraction method employing methanol extraction of the COOH-terminal fragments of cholecystokinin (CCK) from pig tissues followed by HCl extraction of intact CCK and its NH_2-terminal fragments is described. Radioimmunoassay of extracts and their fractionation by Sephadex chromatography and HPLC demonstrate that the distributions of COOH-terminal and NH_2-terminal immunoreactivities among various regions of brain are similar and independent of the concentrations in individual regions. The distribution in gut differs from that in brain. Greatest concentrations of CCK immunoreactivity are located in cortical tissue in the brain and in duodenal mucosa in gut. Both brain and gut contain CCK octapeptide (CCK8) and an NH_2-terminal fragment that is likely to be desoctapeptide-CCK33. Intact CCK33 is extractable from gut but not from brain. Brain contains another NH_2-terminal immunoreactive molecule lacking COOH-terminal immunoreactivity that may be a peptide with a COOH-terminal extension, as has been described for gastrin, or one that may not be derived from a CCK33-like precursor. This peptide is much less prominent in gut, or may be nonexistent there. The failure to find CCK33 in the brain and the presence in the brain of this as-yet-uncharacterized NH_2-terminal peptide raises the question as to whether the differences between neuronal and mucosal tissues are a consequence of differences in post-translational processing or in the DNA templates.**

It is now generally accepted that there are several peptides common to the brain and to the gastroenteropancreatic axis. Among these, cholecystokinin (CCK) appears to be unique in that comparably high concentrations are found in neuronal and in mucosal tissues (1–18). The various CCK-related peptides, immunochemically distinguishable by a variety of antisera (6, 7, 16), have not been shown to be extracted from tissues with equal efficiency by any single extractant. In this study we describe optimal methods for sequential extraction and radioimmunoassay (RIA) of the CCK peptides by using antisera specific for the NH_2 terminus and COOH terminus, further characterize the properties of these peptides in different physicochemical systems, and determine whether, assuming a common precursor for all CCK forms, there are differences in the post-translational processing in the different tissues.

MATERIALS AND METHODS

Mature pigs weighing 10–20 kg were purchased from Bio-Medical Associates (Friedensburg, PA). They were sacrificed by injecting an overdose of pentobarbital into the heart. The brain and sections of small intestine were quickly removed, dissected, and placed on dry ice. The frozen tissues were stored at −70°C until extraction. In some studies equivalent portions of brain and gut tissues were maintained at ambient temperature for 60 min before freezing.

Tissues were weighed and extracted with 10 vol of absolute methanol in autoclaved Teflon grinders. The extract suspensions were filtered by suction through Whatman no. 50 filter paper. The filter cakes together with 10 vol (based on the original tissue weight) of 0.1 M HCl were placed into a boiling water bath for 3 min and then reextracted with Teflon grinders. Both the methanol and acid extracts were stored at −20°C overnight, brought to 4°C, and centrifuged to remove precipitates before further studies.

RIAs for CCK were performed with three antisera having NH_2- or COOH-terminal specificity. Goat 1 (G1) and rabbit B (RB) have been previously characterized and are specific for the NH_2- and COOH-terminal portions of porcine CCK33, respectively (19). All peptides containing CCK4 are equipotent in the RB assay. A recent bleed (7/16/81) from goat 1 was useful at a final dilution of 1:500,000. Rabbit 71 was immunized with CCK8 coupled to human sera albumin through glutaraldehyde. A recent bleed (8/19/81) was used at a final dilution of 1:500,000. This antiserum has immunochemical specificities similar to those previously described for R72 (19). Both R71 and R72 appear to be directed at the NH_2-terminal portion of CCK8 and have progressively decreasing crossreactivity with COOH-terminal fragments smaller than CCK8. pCCK33 (Kabi, lot no. 69206-03) was used as the standard in assays for acid extracts and CCK8(s) (Sincalide, Squibb) was used as the standard in assays of methanol extracts. These standards have identical molar immunopotency in the RB assay. Some extracts were also assayed with GP438, which is an antiserum specific for gastrin and with which CCK peptides crossreact about 1/1,000th as much on a molar basis as does gastrin.

Acid extracts were chromatographed on Sephadex G-50 superfine (Pharmacia) columns (1 × 50 cm) equilibrated with 0.1 M HCl. Methanol extracts were prepared for chromatography by addition of an equal volume of 0.02 M sodium barbital buffer, pH 8.6. The methanol was evaporated in a vacuum centrifuge before application of the extract to Sephadex G-50 superfine columns (1 × 50 cm) equilibrated with 0.02 M barbital.

HPLC analysis was performed with equipment from Waters Associates. Acid extracts were filtered through a glass fiber prefilter and 0.45-μm pore nylon membrane filter prior to analysis by HPLC. One milliliter of filtered extract was injected onto a 3.9 mm × 30 cm μBondapak C_{18} column (Waters Associates). Immunoreactive CCK was eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Two-milliliter fractions were collected and portions were assayed for CCK by the three RIAs.

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Abbreviations: RIA, radioimmunoassay; CCK, cholecystokinin (CCK33 indicates the 33 amino acid residue form, etc.).

Table 1. CCK-like immunoreactivity (pmol/g) in tissues of adult pigs

No. of animals		Methanol (CCK8 standard)			0.1 M HCl (CCK33 standard)		
		G1	R71	RB	G1	R71	RB
	Brain						
3	Cortex	<3	315 ± 16	320 ± 27	356 ± 18	80 ± 9	68 ± 6
3	Olfactory bulb	<3	99 ± 29	113 ± 20	131 ± 47	45 ± 3	34 ± 10
3	Hypothalamus	<3	25 ± 5	46 ± 10	38 ± 9	12 ± 4	4 ± 1
3	Thalamus	<3	14 ± 1	42 ± 8	26 ± 2	6 ± 1	2 ± 1
3	Midbrain	<3	25 ± 4	50 ± 10	37 ± 5	10 ± 2	3 ± 1
2	Pons	<3	6 ± 1	17 ± 3	17 ± 5	5 ± 2	<2
3	Medulla	<3	6 ± 1	15 ± 3	16 ± 3	5 ± 2	<2
	Gut						
3	Duodenal mucosa	<3	318 ± 141	—	983 ± 271	295 ± 13	—
1	Duodenum	<3	118 ± 21	—	442 ± 31	240 ± 13	—
1	Jejunum	<3	63 ± 2	—	347 ± 31	161 ± 1	—
1	Ileum	<3	5 ± 1	—	45 ± 4	23 ± 5	—

Results are presented as mean ± SEM; — indicates not determined.

RESULTS

The concentrations of immunoreactive CCK in methanol and in acid extracts of various regions of the adult pig brain and gut as determined with NH_2-terminal (G1) and COOH-terminal (R71 and RB) specific antisera are shown in Table 1. Only COOH-terminal immunoreactivity is measurable in the methanol extracts. The concentrations as determined with R71 and RB are quite comparable. The observed concentrations of COOH-terminal immunoreactivity in the acid extracts are considerably less than the NH_2-terminal activity, averaging about one-half in all regions of the gut and only about one-fourth in all regions of the brain. NH_2-terminal immunoreactivity in acid extracts of duodenum and jejunum are comparable to the levels found in brain cortex. However, the concentrations in duodenal mucosa are considerably higher. Compared to the cortex, concentrations are only about one-third in olfactory bulb, one-tenth in hypothalamus and midbrain, and less in the other regions of the brain. The minimal gastrin immunoreactivity detected by GP438 in methanol or acid extracts is due entirely to the cross-reactivity of the CCK peptides present in the extracts.

The time between removal and freezing of tissues on CCK content did not appear to be critical. There was no significant loss of either NH_2- or COOH-terminal immunoreactivity when tissues remained at room temperature for 60 min prior to freezing (Table 2).

Fractionation by Sephadex gel chromatography and HPLC was employed to identify the nature of the various immunoreactive hormonal forms. Sephadex G-50 superfine gel chromatography of methanol extracts of both brain (Fig. 1 *Upper Left*) and gut (Fig. 1 *Upper Right*) reveals a major peak in the region of $^{125}I^-$, the elution volume of authentic CCK8. There is an additional peak in gut extract that elutes later than CCK33 but earlier than CCK12. It is not a gastrin-like peptide because it has little immunoreactivity in the gastrin-specific assay. Acid

Table 2. CCK-like immunoreactivity (pmol/g) in tissues frozen immediately or maintained at room temperature for 60 min before freezing

Tissue	Time, min	Methanol G1	Methanol R71	Methanol RB	0.1 M HCl G1	0.1 M HCl R71	0.1 M HCl RB
Cortex	0	<3	349	275	307	102	61
	60	<3	307	304	392	80	65
Duodenum	0	<3	138	—	411	227	—
	60	<3	97	—	472	252	—

—, Not determined.

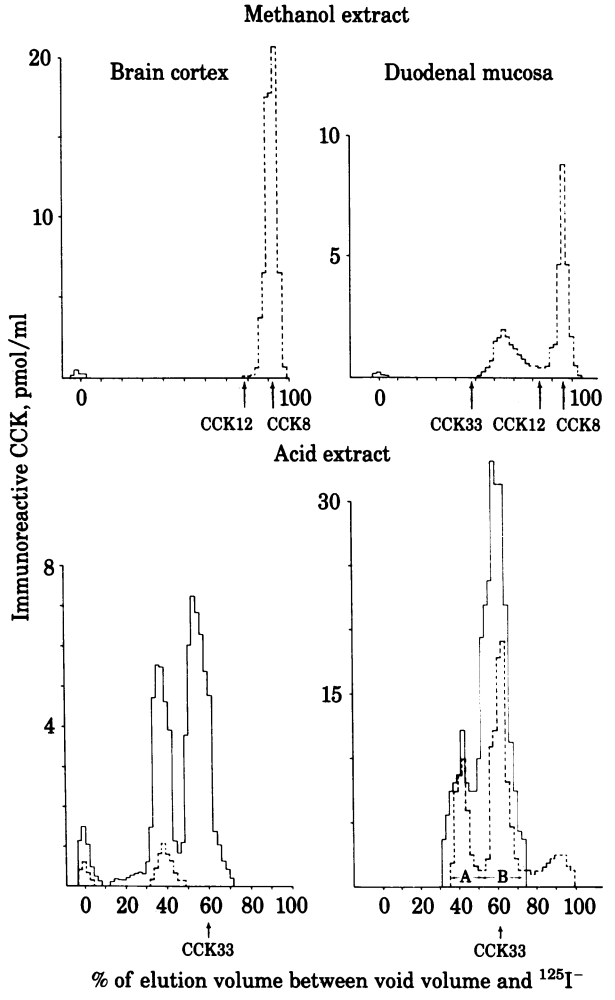

FIG. 1. Sephadex G-50 superfine gel filtration of methanol (*Upper*) and acid (*Lower*) extracts of pig brain cortex (*Left*) and duodenal mucosa (*Right*). CCK8 was used as the standard in assays of methanol extracts and CCK33 was used as the standard for acid extracts. The column eluant for the methanol extracts was 0.02 M barbital; that for the acid extracts was 0.1 M HCl. Immunoreactive CCK shown in this and the following figures as solid lines was that determined with G1, an NH_2-terminal antiserum; that shown in the histograms with broken lines was determined with R71, a COOH-terminal antiserum. Assay with RB antiserum gives a histogram similar to R71. Shown also are the elution volumes for authentic CCK8, CCK12, and CCK33. Pooled peaks A and B from the acid extract of duodenal mucosa were subsequently refractionated (see Fig. 4).

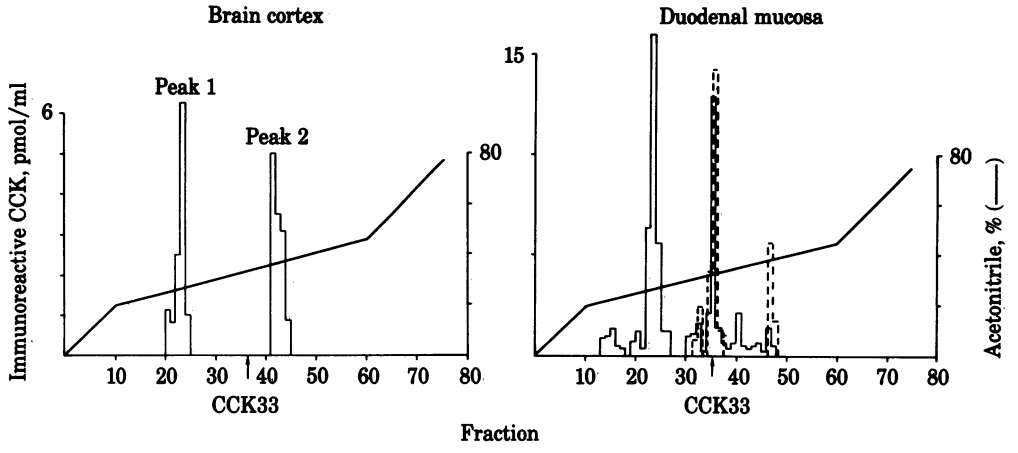

FIG. 2. HPLC of acid extracts of pig brain cortex (*Left*) and duodenal mucosa (*Right*). Immunoreactive CCK was eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Shown is the immunoreactivity in 2-ml fractions as determined with G1 (——) and R71 (----). The concentration of acetonitrile in the fractions is shown by (——). Peaks 1 and 2 were pooled and refractionated (see Fig. 3).

extracts are chromatographed with an acid eluant in order to ensure that recovery from the columns is better than 80%. There are two major peaks in acid brain extract, with elution volumes of 37% and 54%, respectively, as determined with G1, the NH_2-terminal antiserum (Fig. 1 *Lower Left*). A minor component of COOH-terminal immunoreactivity coelutes with the first NH_2-terminal peak. The elution pattern of acid gut extract is more complex (Fig. 1 *Lower Right*). As in brain there are two peaks of NH_2-terminal activity (A and B) but unlike in brain the second peak is always predominant in size and contains a substantial fraction of COOH-terminal activity. In addition, unlike in brain, the COOH-terminal immunoreactivity in peak A is comparable to the NH_2-terminal immunoreactivity. A small

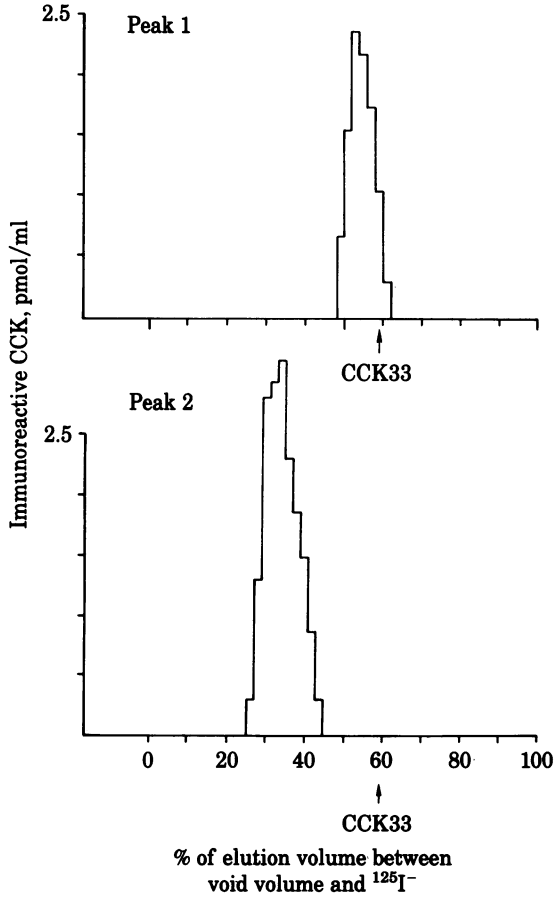

FIG. 3. Sephadex G-50 superfine chromatography of peaks 1 and 2 from HPLC of acid extracts of brain cortex. The eluant was 0.1 M HCl. Immunoreactive CCK was measurable only with G1. Shown also is the elution volume of CCK33. The earlier-eluting peak on HPLC is that which elutes later on Sephadex and vice versa.

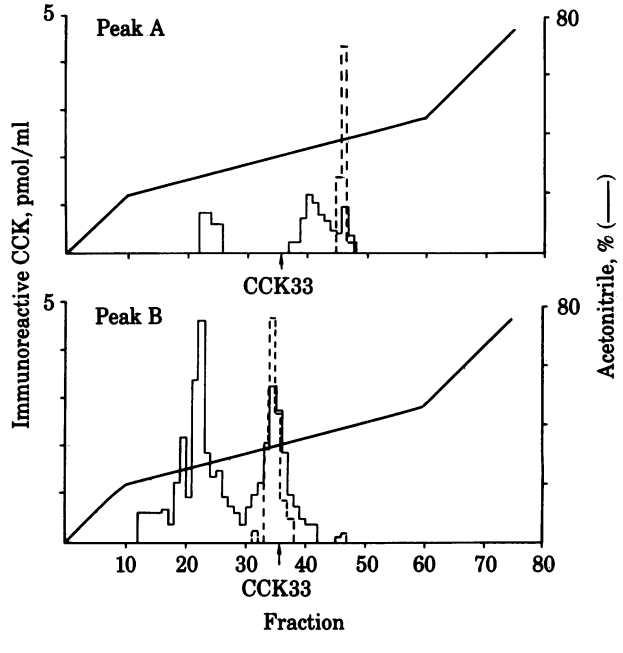

FIG. 4. Refractionation on HPLC of Sephadex column pooled eluates A and B. Refractionated peak B (*Lower*) contains a component that coelutes with CCK33 and that contains about equivalent amounts of COOH- and NH_2-terminal immunoreactivity. There is another peak containing only NH_2-terminal immunoreactivity with an elution volume resembling that of peak 1 in brain cortex and a corresponding peak in the duodenal mucosa. This is probably the NH_2-terminal portion of CCK after cleavage of CCK8. Peak A (Fig. 1) contained about equal amounts of COOH- and NH_2-terminal immunoreactivity. However, refractionation reveals (*Upper*) that the COOH- and NH_2-terminal immunoreactivities appear to reside on different molecules. The peak around fraction 22 resembles peak 1 of brain cortex and probably is spillover from peak B. The second peak containing NH_2-terminal immunoreactivity elutes in the same region as peak 2 of the brain cortex. There is a third peak with virtually only COOH-terminal immunoreactivity, seen also in Fig. 2 *Right*, that has not yet been further identified.

peak of COOH-terminal immunoreactivity quite close to the salt peak is not found in brain extracts.

Fractionation of acid brain extract on HPLC reveals two peaks (1 and 2) with NH_2-terminal immunoreactivity but no COOH-terminal immunoreactivity (Fig. 2 *Left*). We have been unable to account for the failure to recover from HPLC the COOH-terminal immunoreactivity measurable after Sephadex fractionation. Refractionation of these two peaks on Sephadex reveals that the later peak on HPLC has an earlier elution volume on Sephadex and vice versa (Fig. 3). The HPLC pattern of the gut is more complicated (Fig. 2 *Right*). There is a central peak that has the same retention time as CCK33 and contains both COOH- and NH_2-terminal immunoreactivities. There is also a peak of NH_2-terminal activity eluting before CCK33 that corresponds to peak 1 in the brain extracts and a peak of COOH-terminal immunoreactivity eluting later.

The correspondence between the Sephadex and HPLC elution patterns of acid gut extract was examined by pooling the fractions from the Sephadex peaks and refractionating them on HPLC. The elution pattern of peak A from Sephadex on HPLC reveals that the NH_2- and COOH-terminal immunoreactivities of that peak do not reside on the same molecule (Fig. 4). Contained within peak A are three different fragments, one with COOH-terminal immunoreactivity and two having NH_2-terminal immunoreactivity. The first of the NH_2-terminal activity peaks (fractions 22–26) is probably spillover from peak B because refractionation of peak B on HPLC reveals a major NH_2-terminal activity peak in this region. The second NH_2-terminal activity peak may correspond to the peak 2 found in brain extracts. HPLC fractionation of peak B yields a major peak with both COOH- and NH_2-terminal activities that corresponds to authentic CCK33.

Sephadex G-50 superfine analyses of methanol and acid extracts from other regions of brain and gut show patterns of distribution of COOH- and NH_2-terminal CCK immunoreactivity identical to those of the cortex and duodenum, respectively. Thus, subsections of brain and gut differ only in the concentrations of CCK rather than in the distribution of hormonal forms.

DISCUSSION

Since the report by Vanderhaeghen *et al.* (1) of a brain peptide that crossreacts with anti-gastrin antibodies there have been numerous reports concerning the concentrations, hormonal forms, and physiologic role(s) of CCK-like peptides in the brains of various animal species as well as comparisons of these peptides with those found in the gut, the traditional site for CCK (2–21). The lack of agreement among laboratories is due in part to the unavailability of CCK33 or CCK39 from species other than the pig and the marked species specificity of antisera that are directed against the NH_2-terminal portion of this molecule. Thus most laboratories base their CCK determinations on assays with COOH-terminal antisera.

The problem has been further complicated by the diverse methods used for extraction. Gastrin and the CCK COOH-terminal peptides, primarily CCK8, are readily extractable from tissues in boiling water. More recently NaOH (16) and methanol (14, 18) have also been used as extractants for CCK8 and related peptides such as caerulein (22). Generally, acid extraction has been considered optimal for extraction of intact CCK33 (23). In previous studies the tissues to be assayed generally have been divided in half, one part extracted for the acidic COOH-terminal fragments and the other extracted with an acid extractant for the more basic intact CCK or its NH_2-terminal fragments (12, 16, 21, 24). The method described in the present report has two principal advantages over those previously described: the amount of tissue available for extraction is effectively doubled because both acidic and basic peptides are extracted from the same tissue; and methanol is equally as effective as boiling water or alkali in extracting the COOH-terminal fragments while minimizing the extraction of the larger CCK forms that occurs with the other extractants (16, 24).

The rather extensive studies concerned with the distribution of hormonal forms of CCK in the rat and human brain and gut have been complicated by the unavailability of an NH_2-terminal antiserum. There is general agreement that CCK8 (21, 24) is found both in the brain and in gastrointestinal mucosa. It has been reported that CCK4 is also prominent in the brain (20) and in the gut (25, 26). However, our studies (16) and those of most others (5, 14, 27) have failed to confirm this finding. Initially Lamers *et al.* (12) reported the presence of CCK33 in rat brain in amounts of about 10% that of CCK8. In a subsequent study from the same laboratory no CCK33 was detected in rat or human brains (17).

We found a major peak eluting in the region of CCK33 on Sephadex gel filtration. However, immunoreactivity was measurable only with an NH_2-terminal antiserum and not with either of the COOH-terminal antisera. Furthermore, this immunoreactive peak is clearly distinguishable from CCK33 on HPLC and probably is desoctapeptide-CCK33—i.e., the NH_2-terminal fragment remaining after CCK8 has been cleaved off. Both this fragment and CCK33 are prominent in gut mucosal tissue. The nature of the other peak with only NH_2-terminal activity that is found on fractionation of acid extracts of brain has not been determined. It is not a major component in gut as it is in brain. Because it does not have COOH-terminal activity, it could be a large CCK peptide with a COOH-terminal extension as has been described for gastrin (28). It is not likely to be the NH_2-terminal fragment remaining after cleavage of CCK4 or CCK12 because these and other COOH-terminal fragments would have been readily detected with RB and were not found. A possibility that must be considered is that it represents a peptide that contains a major portion of the NH_2-terminus of CCK but is derived from other than a CCK33-like precursor. The ultimate nature of this peptide is not likely to be resolved definitively until it has been purified and its sequence has been determined.

Although brain and gut mucosa appear to have in common CCK8 and a peptide that may be desoctapeptide-CCK33, the failure to find CCK33 in the brain and the presence in the brain of an NH_2-terminal CCK peptide that is not prominent in the gut raises the question as to whether the differences between brain and gut are simply a consequence of post-translational processing or are attributable to the existence of different DNA templates in neuronal and mucosal tissues.

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