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Preprocholecystokinin processing in the normal human anterior pituitary

(cell differentiation/peptide hormone/radioimmunoassay)

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ABSTRACT **The processing of preprocholecystokinin in human pituitary extracts was investigated using gel and ion-exchange chromatography monitored by sequence-specific radioimmunoassays before and after incubation with trypsin, carboxypeptidase B, and arylsulfatase. Whereas the neural lobe contained only the bioactive α-carboxyamidated cholecystokinin (CCK) peptides (32 pmol/g), of which CCK-8 predominated, the anterior lobe contained substantial amounts of three large nonamidated procholecystokinin fragments (95 pmol/g; M_rs, 9000, 7000, and 5000) and small amounts of α-amidated CCK (8.3 pmol/g). The latter occurred only in the following large molecular forms: component I, CCK-58, and traces of CCK-33. Corticotrophic tumors processed the large forms to small CCK-8-like forms as are found in the brain and in the gut. The results show that a hormone gene, although translated, is expressed only to a limited extent as mature, active peptide outside the principal production region(s). Thus the processing of CCK to small α-amidated peptides in the less-differentiated tumor tissue supports the hypothesis that differentiation of endocrine cells may be sustained also at the posttranslational level.**

Cholecystokinin (CCK) is a potent hormone and neurotransmitter synthesized in several bioactive forms in the gut and the brain (1, 2). Although the relative abundance of each form varies among regions of the gut and between gut and brain (1–5), both tissues synthesize the same forms of which the sulfotyrosine octapeptide amide (peptide CCK-8) usually is the most abundant (1–6).

The multiple forms reflect multiple steps in the processing of preprocholecystokinin (preproCCK) (7–10). PreproCCK contains an amidation site in which glycine is followed by a pair of arginines [residues 104–106 of human preproCCK (10)]. Amidation at this site is required for biological activity. To follow the generation of bioactive CCK, we have developed a library of radioimmunoassays that monitor the maturation of preproCCK at and in the N-terminal direction from the amidation site (refs. 6 and 11; see also Fig. 1).

In previous studies on pituitary CCK neurons (12), CCK was also found in the human adenohypophysis. Characterization of adenohypophyseal CCK with the radioimmunoassay library (6, 11) has revealed a processing pattern that suggests a role for posttranslational modifications in the differentiation of endocrine cells.

MATERIALS AND METHODS

Tissue Sampling and Extraction. Ten pituitaries from human subjects without known endocrine or neurological disease were obtained 6–16 hr postmortem. They were kept on ice until dissection into anterior and neural lobes as described (12). Two pituitary corticotrophic tumors from patients with Nelson syndromes were obtained by transsphenoidal resection. The tumor specimens and the normal pituitary lobes were frozen in liquid nitrogen and stored at −80°C until extraction. The frozen tumors or lobes were minced (each lobe separately), boiled for 20 min in redistilled water (pH 6.6, 10 ml/g of tissue), homogenized, and centrifuged. The pellet was extracted in an equal volume of 0.5 M CH_3COOH at 10°C and centrifuged; the supernatants were stored at −20°C until analysis. The neutral and acid extracts diluted from 1:3 to 1:10,000 were assayed with sequence-specific CCK radioimmunoassays before and after treatment with trypsin and carboxypeptidase B (6, 11). For controls, surgical biopsies of histologically verified normal human cerebral cortex and jejunal mucosa were also extracted in boiling water and acetic acid as described above.

Chromatography. Neutral and acid extracts (1 ml) were applied separately or together to Sephadex G-50 superfine columns (10 × 1000 mm) before and after trypsin and carboxypeptidase B treatment. The columns were eluted at 4°C with 0.02 M barbital buffer (pH 8.4) at a flow rate of 4 ml/hr and 1.0-ml fractions were collected. The columns were calibrated with extracts of human jejunal mucosa or of cerebral cortex, with sulfated porcine tritriacontapeptide CCK (CCK-33; a kind gift from V. Mutt, Stockholm, Sweden), and with sulfated and nonsulfated CCK octapeptides and nonsulfated tetrapeptides (CCK-8 and CCK-4; kind gifts from J. S. Morley, Cheshire, U.K.). Void and total volumes of the columns were determined with ^{125}I-labeled albumin and ^{22}NaCl. Trypsin-treated extracts were also subjected to anion-exchange chromatography on Whatman DE32 columns (10 × 150 mm) eluted at 20°C with a 0.05–0.50 M NH_4HCO_3 gradient at a flow rate of 30 ml/hr, and 2.5-ml fractions were collected.

Enzyme Analysis. To measure CCK precursors extended beyond the glycine residue in the amidation site [Gly-Arg-Arg-, residues 104–106 of human preproCCK (10)], tissue extracts and chromatographic fractions were incubated sequentially with trypsin and carboxypeptidase B (6, 11). *O*-sulfation of tyrosine, a normal derivatization of CCK (13), was examined by incubation with limpet arylsulfatase as detailed elsewhere (6) and by measurement with antisera requiring tyrosine *O*-sulfation of CCK peptides (14). To ensure that large α-carboxyamidated CCK peptides contained the active COOH-terminal octapeptide amide, the large forms were subjected to exhaustive tryptic cleavage (15) and then characterized by cellulose DE32 ion-exchange chromatography.

Sequence-Specific Radioimmunoassays. Development and characteristics of radioimmunoassays for preproCCK have been described elsewhere (6, 11, 14). The sequence specificity of the four antisera used in this study is shown in Fig. 1. Briefly, antiserum 1561 binds residues 86–90, antiserum

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Abbreviations: CCK, cholecystokinin; preproCCK, preprocholecystokinin.

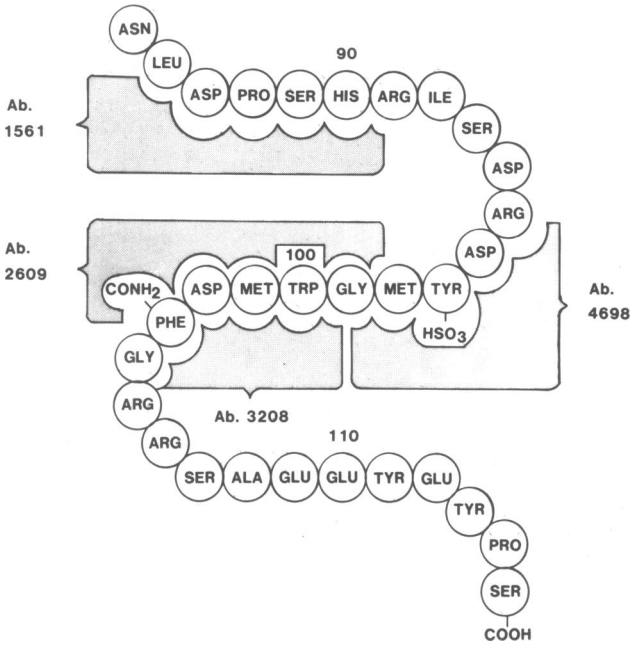

FIG. 1. Sequence specificity of radioimmunoassays toward the COOH-terminal sequence, residues 85–115, of human preproCCK. The minimal active site of CCK is residues 100–103. Activation of this site requires carboxyamidation of the COOH-terminal phenylalanine. Accordingly, the subsequent Gly-Arg-Arg- sequence is also a typical amidation site with glycine as amide donor. Antiserum (Ab.) 2609 is specific for the amidated bioactive tetrapeptide sequence and binds consequently only bioactive products of preproCCK. Antiserum 3208 requires COOH-terminal glycine and thus does not measure bioactive peptides. Antiserum 4698 is specific for the *O*-sulfated tyrosine sequence residues 95–99. It binds the octapeptide amide (residues 96–103) and further NH_2-terminal extended peptides with equimolar potency, whereas glycine and further COOH-terminal-extended products of preproCCK are bound with poor affinity. Antiserum 1561 is specific for residues 86–90 and independent of known posttranslational modifications of preproCCK.

4698 binds residues 95–99, antiserum 2609 binds residues 99–103, and antiserum 3208 binds residues 99–104 of human preproCCK. Antiserum 4698 is dependent on *O*-sulfation of tyrosine-97; antiserum 2609 requires α-carboxyamidation of phenylalanine-103; and antiserum 3208 requires a free carboxyl group on glycine-104 of preproCCK. Hence, antiserum 2609 binds only bioactive products of preproCCK.

RESULTS

There are substantial amounts of CCK (95.2 pmol/g) in the human anterior pituitary as measured with antiserum 1561 (Table 1). However, only 8.6% of this immunoreactivity is α-carboxyamidated and thus biologically active (Table 1). In addition, the anterior pituitary contains traces of glycine-extended (1.9 pmol/g) and further COOH-terminal-extended peptides (0.5 pmol/g) as measured with antiserum 3208 before and after cleavage with trypsin and carboxypeptidase B (Table 1).

The preproCCK products chromatographed on Sephadex G-50 elute in three peaks corresponding to molecular weights of 9000, 7000, and 5000 (Fig. 2). The relative distribution of immunoreactivity in the three peaks varies considerably between antiserum 1561, which mainly measures peptides eluting in positions corresponding to CCK-58 and CCK-33, and the remaining antisera that measure mainly component I- and CCK-58-like material (Table 1 and Fig. 2). The components measured by antiserum 1561 consist, however, of so much peptide that they include the carboxyamidated and glycine-extended peaks measured with the remaining antisera (Fig. 2). Tryptic cleavage of the large α-carboxyamidated CCK peptides released tyrosine *O*-sulfated CCK-8 from each of the three components (Fig. 3) demonstrating that they contain the usual active site of CCK.

The neural lobe of the human pituitary contains 32 ± 13 pmol/g of carboxyamidated and tyrosine-sulfated CCK peptides as measured with antisera 4698 and 2609. The concentrations of glycine-extended CCK and further COOH-terminal-extended peptides as measured with antiserum 3208 before and after cleavage with trypsin plus carboxypeptidase B were below the detection limit of the assay. The immunoreactivity measured with antiserum 1561 was 27 ± 11 pmol/g. It eluted in the same fraction with the "desoctapeptides" CCK-58 and CCK-33 (refs. 5 and 6; data not shown). The molecular pattern of the carboxyamidated CCK in the human neural lobe resembles that of other central nervous system regions like the cerebral cortex in which CCK-8 is the predominant molecular form (Fig. 4). The human jejunal mucosa also contains substantial amounts of CCK-8, but in addition there is more component I, CCK-58, and CCK-33 (Fig. 4). Thus, adenohypophyseal CCK differs from that of other CCK synthesizing tissues being devoid of CCK-8 (Fig. 4).

Pituitary corticotrophic tumors associated with the Nelson syndrome contain carboxyamidated CCK in concentrations 10^3 times higher than normal adenohypophyseal tissue (16). In contrast with the normal anterior pituitary that is devoid of CCK-8, these tumors process preproCCK to intermediate-size CCK, CCK-8, and smaller forms, and they contain none of the large molecular forms (Fig. 5). Thus, the CCK pattern from the corticotrophic tumors resembles the patterns from the small intestine and the brain (Figs. 4 and 5).

DISCUSSION

The present study has shown that the human anterior pituitary synthesize large preproCCK fragments devoid of an α-amidated COOH terminus. The fragments occur in con-

Table 1. Concentration of CCK peptides in human anterior pituitaries

Antiserum and standard peptide	Total concentration, pmol equivalents of CCK standard/g	PreproCCK products, fraction of total concentration		
		Component I	CCK-58-like	CCK-33-like
1561 (CCK-33, sulfated)	95.2 ± 36.7	0.06 ± 0.03	0.37 ± 0.16	0.56 ± 0.22
2609 (CCK-8, sulfated)	8.3 ± 3.4	0.54 ± 0.21	0.32 ± 0.14	0.15 ± 0.07
3208 (CCK-8+Gly, nonsulfated)	1.9 ± 0.8	0.58 ± 0.26	0.28 ± 0.10	0.14 ± 0.07
3208* (CCK-8+Gly, nonsulfated)	0.5 ± 0.3	0.41 ± 0.18	0.46 ± 0.20	0.11 ± 0.05

Data are expressed as mean ± SEM (n = 10). An equivalent of CCK standard peptide is the amount of endogenous peptide necessary to show the same immunoreactivity as the standard peptide used.
*Expressed in equivalents of glycine-extended CCK-8 after cleavage with trypsin and carboxypeptidase B.

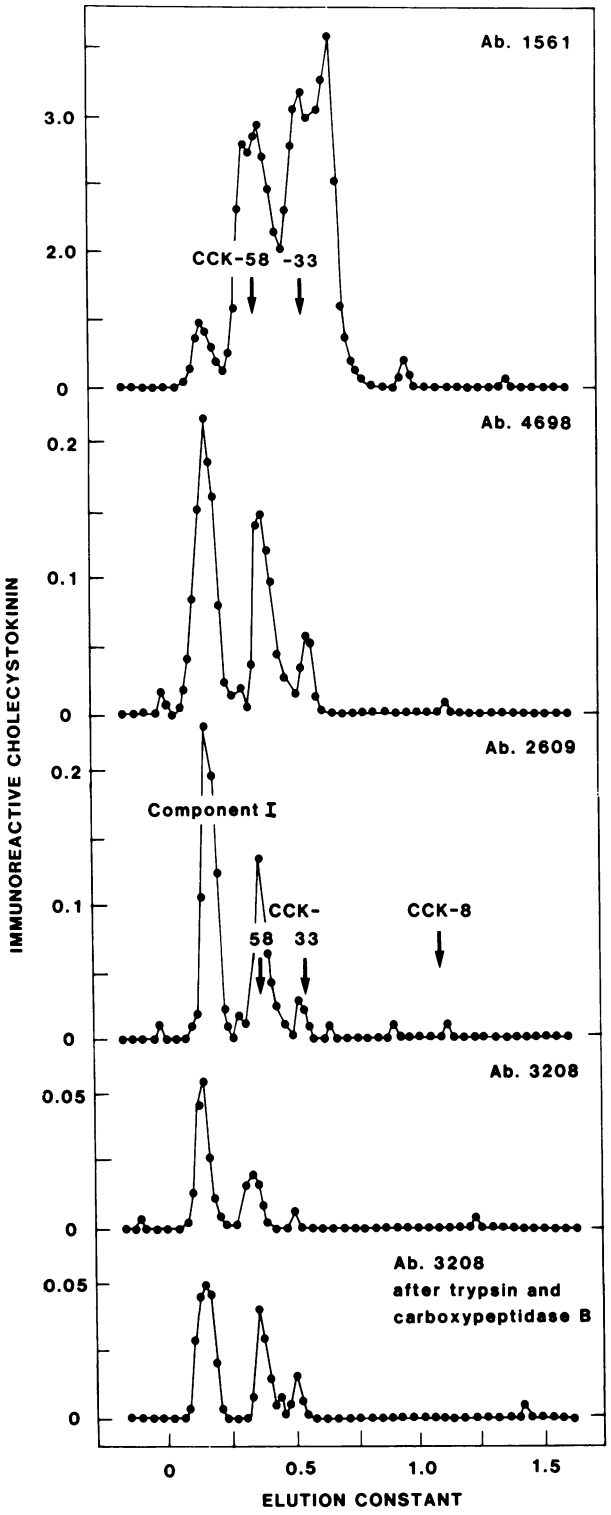

FIG. 2. Gel chromatography of combined neutral and acid extracts of normal human anterior pituitary. Extract (1 ml) was applied to a Sephadex G-50 superfine column (100 × 1 cm), and CCK was eluted with 0.02 M sodium barbital, pH 8.4, containing 0.1% bovine serum albumin. The fractions were monitored with radioimmunoassays using antisera with a specificity for preproCCK sequences as indicated in Fig. 1. The abscissa is given as the elution constant $K_{av} = (V_e - V_o)/(V_t - V_o)$. The concentrations indicated on the ordinate are given in equivalents of standard peptides (nmol of immunoreactive CCK per liter). Hence, highly purified sulfated CCK-33 was used with antiserum 1561 (*Top*), synthetic sulfated CCK-8 with antisera 4698 and 2609 (second and third curves from the *Top*), and synthetic, nonsulfated glycine-extended CCK-8 with antiserum 3208 (two curves at *Bottom*). For comparison, note that antiserum 4698 binds sulfated CCK-33 and -8 with equimolar potency.

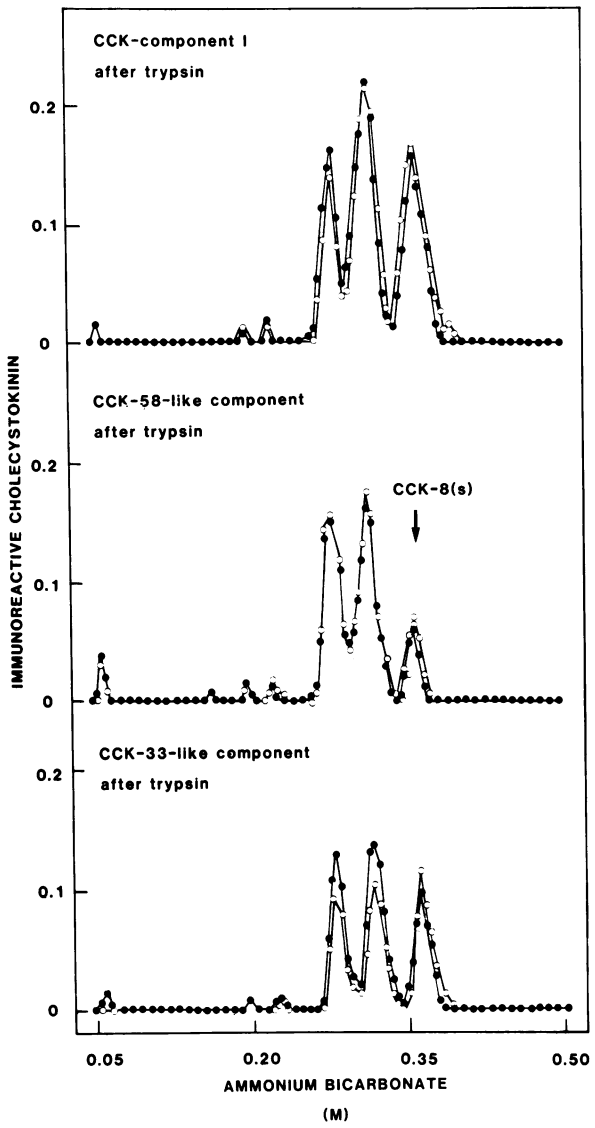

FIG. 3. Release of tyrosine-sulfated and carboxyamidated CCK-8 from the large amidated forms of pituitary CCK shown in Fig. 2. Aspartic acid-96, the NH_2-terminal residue of CCK-8, follows an arginine (Fig. 1). CCK-8 is, therefore, released from the large forms by tryptic cleavage. The large molecular forms isolated by gel chromatography (second and third curves from the *Top* in Fig. 2) were separately incubated by trypsin and then chromatographed on DE32. The fractionations were monitored with radioimmunoassay using antiserum 4698 (○), specific for the tyrosine-sulfated CCK, and antiserum 2609 (●), specific for the amidated COOH terminus of CCK (see also Fig. 1), and data is expressed in nmol equivalents of CCK-8 per liter. The abscissa is given in gradient concentrations. Data showing shift in elution positions to those of nonsulfated CCK-8 after incubation with arylsulfatase are not shown.

centrations >10-fold above those of the amidated CCK peptides (Table 1 and Fig. 2). Thus, beyond the established pituitary hormones, some pituitary cells synthesize precursors of other hormones that apparently do not—or only to a minor extent—reach the final steps of maturation.

Only one CCK mRNA has been isolated from man (10). Therefore, the anterior pituitary CCK peptides are derived from the single human preproCCK (10). PreproCCK—also from pig (8) and rat (7, 9)—contains several monobasic and a three dibasic cleavage sites. Accordingly, brain and gut tissues in which the CCK gene (17) is efficiently expressed synthesize several α-amidated, i.e., bioactive, forms of which CCK-8 usually is most abundant (1–6). The brain and the gut synthesize, however, also smaller forms (CCK-5 and

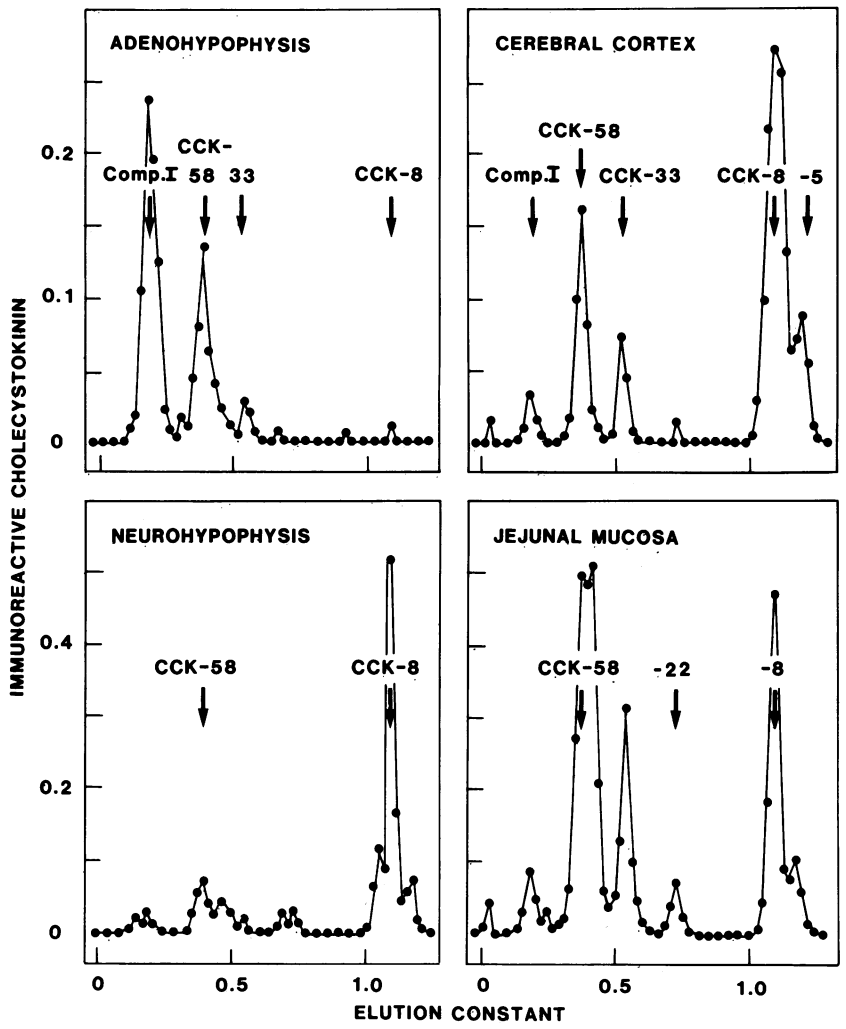

FIG. 4. Comparison of the amidated, bioactive CCK peptides in the two lobes of the human pituitary with those of the human cerebral cortex and human jejunal mucosa. Combined neutral and acid extracts from the four tissues were applied to Sephadex G-50 superfine columns (100 × 1 cm) eluted as described in the legend of Fig. 2. The fractionations were monitored with a radioimmunoassay using antiserum 2609 specific for the amidated COOH terminus of bioactive CCK peptides (see also Fig. 1). The abscissa and ordinate are given in units as described in the legend of Fig. 2.

-4) in significant concentrations (1, 2, 6). Thus, with respect to α-amidated CCK peptides, the anterior pituitary pattern differs completely from that of other tissues (Fig. 4).

The occurrence of large preproCCK fragments devoid of the α-amidated COOH terminus is also distinctive. These peptides did not react with antibodies specific for glycine-extended CCK neither before nor after treatment with trypsin and carboxypeptidase B. Presumably, these peptides, therefore, contain neither the COOH-terminal nonapeptide sequence [residues 107–115 of preproCCK (Fig. 1)] nor just an additional glycine residue. Immunochemically and chromatographically they resemble the "desoctapeptide" fragments of component I, CCK-58, and CCK-33 from brain tissue (5, 6). But with such identity it is hard to explain the lack of complementary COOH-terminal fragments such as CCK-8 that is easily detected in other CCK-synthesizing tissues (1, 6, 12). Therefore, the COOH-terminal part of most preproCCK in the anterior pituitary is processed differently from that of other tissues to avoid synthesis of bioactive CCK (18). Probably some yet unknown amino acid modification close to the amidation site prohibits normal activation of preproCCK.

In spite of a careful search with sensitive radioimmunoassays (6, 11, 14) for the related human preprogastrin (19), we have found only insignificant traces (<0.5 pmol/g) of preprogastrin products in the human anterior pituitary (data not shown). Thus, in contrast with the pig (18, 20, 21), the gastrin gene is poorly expressed in the human adenohypophysis. However, preproCCK processing in the human anterior pituitary shows striking analogies to preprogastrin processing in the pig pituitary (18, 21). First, the adenohypophysis from both species contains prohormone (100–200 pmol/g). Second, only 8% and 1% of the precursors are α-amidated. Third, the amidated peptides are relatively long (≤33 amino acid residues) in comparison with the products CCK-8 and gastrin-17 that predominate in the principal production sites, the gut and the brain. Presumably, the processing of pituitary preproCCK and preprogastrin serve the same purpose, i.e., to maintain a high degree of differentiation for endocrine cells. In other words, the synthesis of certain bioactive peptides may be inhibited in cells that by their localization are inexpedient for release of these peptides.

The use of autopsy material might pose a problem. However, several observations indicate that the CCK pattern presented here is not modified by autolysis. First, pig pituitaries sampled during anesthesia or immediately postmortem contain also large nonamidated preproCCK fragments (ref. 18 and unpublished studies). Second, there are no structural modifications of CCK peptides in human brains sampled 6–16 hr postmortem in comparison with the CCK of freshly frozen biopsies from neurosurgery (22). Finally, autolytic proteolysis should produce small fragments rather than the exclusive occurrence of large molecular forms (Figs. 2–4).

This study has not identified which cells synthesize CCK in the anterior pituitary. Available evidence, however, suggests that it is corticotrophs. Thus, examination of 87 human pituitary tumors showed increased amounts of CCK only in corticotropin-producing tumors (16). Moreover, antibodies specific for the common COOH terminus of CCK and gastrin stain only corticotrophs in the anterior pituitary (23, 24).

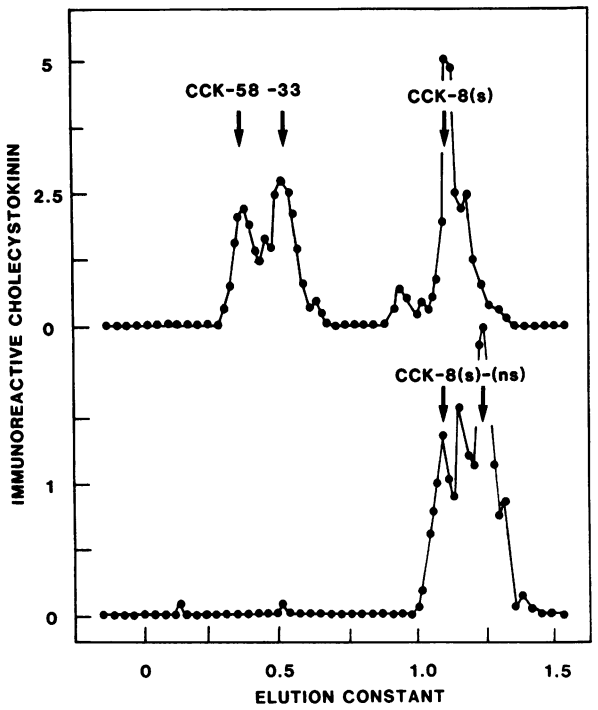

FIG. 5. Gel chromatography of combined neutral and acid extracts of anterior pituitary tumors from two patients with Nelson syndrome. Tumor extract (1 ml) was applied to Sephadex G-50 superfine columns (100 × 1 cm) eluted as described in the legend of Fig. 2. The fractionations were monitored with a radioimmunoassay using antiserum 2609 specific for the amidated COOH terminus of bioactive CCK peptides (see also Fig. 1). The abscissa and ordinate are given in units as described in the legend of Fig. 2. Each curve shows the elution pattern from one tumor.

In conclusion, it should be emphasized that different processing of the same primary translation product in different cells (cell-specific processing) is a well-known phenomenon. The variable processing of the corticotropin precursor (proopiomelanocortin) in corticotrophs, melanotrophs, and extrapituitary cells is an example (for review, see ref. 25). Therefore, the major finding of this study is not that the corticotrophic processing of preproCCK differs from that of other CCK-synthesizing cells (Fig. 4). It is rather that a highly differentiated cell tailored to secrete corticotropin and other hormonal products of the proopiomelanocortin gene also contains products of unrelated hormone genes, but processes these prohormones poorly. Thus, the findings suggest that endocrine cells maintain their differentiation by incomplete processing of precursors for unrelated hormones. In other words, control of cell differentiation may be exerted post-translationally and not only at the transcriptional level.

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