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Extraction and immunochemical characterization of cholecystokinin-like peptides from pig and rat brain

(COOH terminus/alkaline extraction/acid extraction)

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ABSTRACT **Two major classes of immunoreactive cholecystokinin peptides (iCCK) have been identified in rat and pig brains: (*i*) large basic peptides (big iCCK) resembling the 33-amino acid porcine cholecystokinin (pCCK33) in size and charge; (*ii*) small acidic peptides (small iCCK) resembling the COOH-terminal fragments of CCK. Boiling 0.1 M HCl maximally extracts big iCCK; boiling 0.1 M NaOH maximally extracts small iCCK. The differences in hormonal forms removed by these extractants are not likely to be due to enzymatic conversion during the extraction procedures. Fractionation on Sephadex G-50 and starch gel electrophoresis combined with radioimmunoassay using three antisera of different specificities—(*i*) directed towards the NH_2 terminus of pCCK33, (*ii*) produced by immunization with COOH-terminal fragment CCK8, (*iii*) produced by immunization with COOH-terminal fragment CCK4—are consistent with the hypothesis that a major fraction of big iCCK may represent intact cholecystokinin with a COOH-terminal extension, as has recently been suggested for gastrin, a molecule having a COOH-terminal pentapeptide identical with that of cholecystokinin.**

Since the discovery of a brain peptide reacting with anti-gastrin antibodies (1), numerous studies have demonstrated the presence of cholecystokinin (CCK)-like peptides in the central nervous system of humans and animals (2–14). Two major classes of immunoreactive cholecystokinin peptides (iCCK) have been identified in brain: (*i*) large basic peptides (big iCCK) resembling the 33-amino acid porcine CCK (pCCK33) in size and charge; and (*ii*) small acidic peptides (small iCCK) resembling COOH-terminal fragments of pCCK33 in size, charge, and immunoreactivity (6, 7). In this communication, we report the optimal conditions for extraction of both types of iCCK and characterize immunochemically and by fractionation in several physicochemical systems the peptides in each class of iCCK found in the cerebral cortex and hypothalamus of pig and rat.

MATERIALS AND METHODS

Preparation of Tissue Extracts. Eight 350- to 450-g male Spraque–Dawley rats were decapitated and the brain, cortex, and hypothalamus were quickly dissected according to the anatomical guidelines of Glowinski and Iverson (15). The brain tissues were immediately frozen on dry ice. Eight whole brains and hypothalami from sexually mature adult pigs were purchased frozen from Pel-Freeze. Slices of frozen parietal cortex and intact hypothalami were placed in extraction vessels on dry ice. While the tissues were still frozen, either 0.1 M HCl or 0.1 M NaOH was added at a concentration of 0.2 g (wet weight) of tissue per ml. Both acid and alkaline extractants were chosen to remove maximal CCK immunoreactivity (6–8). The extraction solutions were immediately placed in a boiling water bath for 5 min and the tissues were then homogenized with a Teflon tissue grinder. Tissue extracts were centrifuged at 10,000 × *g* for 30 min, and the supernatants were removed and frozen at −20°C until assayed for content of peptide hormone within 3 days of preparation. Prior to assay, the extracts were neutralized by dilution (1:4–1:24) in 0.25 M sodium phosphate buffer (pH 7.5) containing 0.25% human serum albumin. The pH of all neutralized extracts was 7.5.

In another series of experiments, four additional frozen slices of porcine parietal cortex were extracted in an identical fashion with 3 M acetic acid. The extracts were neutralized by dilution as described and assayed within 1 day of preparation.

Fractionation of iCCK. iCCK contained in tissue extracts was fractionated in three systems: adsorption to microfine precipitated silica (QUSO), which binds big iCCK but not small iCCK (16); Sephadex G-50 fine column chromatography (6, 7); and starch gel electrophoresis (17).

Five milligrams of QUSO G32 (Philadelphia Quartz) were added to a portion of the tissue extracts (0.3 ml) suspended in 0.9 ml of 0.25 M phosphate buffer (pH 7.5) containing 0.25% human serum albumin. The mixture was swirled on a Vortex mixer for 30 sec and centrifuged at 2000 × *g* for 15 min at 4°C, and the supernatants were decanted and assayed for iCCK. The absorbed CCK was eluted from the QUSO by addition of 0.5 ml of 0.1 M HCl to the residual pellet. The mixture was swirled on a Vortex mixer and centrifuged, the supernatant was decanted, and 1- to 50-μl portions of eluates were assayed for iCCK.

On two occasions, a portion (2 ml) of the HCl or NaOH extract (0.2 g/ml) of the porcine or rat cerebral cortex was fractionated on a 1 × 50 cm Sephadex G-50 fine column. The column used to fractionate the HCl extracts had been equilibrated with and was eluted with 0.1 M HCl. The column used to fractionate the NaOH extracts had been equilibrated with and was eluted with 0.02 M sodium barbital buffer (pH 8.6) containing 0.25% human serum albumin. Blue dextran 2000 (Pharmacia, Sweden) and $Na^{125}I$ were applied as molecular markers. Fractions (1 ml) were collected and portions were immediately assayed for CCK. The Sephadex G-50 fine chromatography system permits distinction among pCCK33, CCK12, CCK8, and the COOH-terminal tetrapeptide (CCK4) (18). COOH-terminal fragments CCK7 through CCK5 elute approximately with CCK8 (18).

Portions of tissue extracts were also subjected to starch gel electrophoresis as described (17). This electrophoretic system

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Abbreviations: CCK, cholecystokinin; iCCK, cholecystokinin-like immunoreactive peptides; pCCK33, 33-amino acid porcine cholecystokinin; CCK3 through CCK12, COOH-terminal peptide fragments of CCK33 containing the indicated numbers of residues; pG1, porcine heptadecapeptide gastrin (unsulfated); QUSO, microfine precipitated silica; RIA, radioimmunoassay.

clearly distinguishes between sulfated CCK8 and other COOH-terminal fragments, including unsulfated CCK8 (18). The nature of the iCCK migrating in the postalbumin region of the starch gels was established by refractionation of the eluates on a 1 × 50 cm Sephadex G-50 fine column.

Radioimmunoassay (RIA). Tissue extracts, supernatants of tissue extracts after QUSO adsorption, HCl eluates of the QUSO pellets, and Sephadex column and starch gel eluates were assayed for iCCK by using three different CCK antisera: (*i*) a species-specific NH_2-terminal goat anti-pCCK33 serum (G1), (*ii*) a rabbit anti-CCK8 serum (R72), and (*iii*) a rabbit anti-CCK4 serum (RB).

G1 is used in a RIA employing highly purified pCCK33 (generously supplied by V. Mutt) as standard and ^{125}I-labeled pCCK33 as tracer (6, 7, 19). CCK8 (Squibb) is used as standard and ^{125}I-labeled pG1 (heptadecapeptide gastrin, pG1, a gift from R. A. Gregory through the courtesy of M. I. Grossman, Wadsworth Veterans Administration Medical Center) is used as tracer in the RIA employing RB (6, 7). The RIA using R72 is performed similarly to the RB assay in that it uses the same standard, tracer, incubation buffer, and separation conditions.

Serial dilutions of tissue extracts and of all eluates from the QUSO pellets are superposable on the standard curves for each of the three CCK RIAs.

RESULTS

Extraction and Characterization of iCCK from Porcine Brain. The iCCK contained in HCl and NaOH porcine cerebral cortical and hypothalamic extracts as measured in each of the three CCK RIAs are shown in Table 1. The greatest amount of iCCK was that measured in the G1 assay in the cerebral cortical HCl extracts. About 75% of this immunoreactivity was adsorbed to QUSO and about half of the adsorbed immunoreactivity was eluted by 0.1 M HCl. The amount of G1-assayable iCCK extracted in 3 M acetic acid was comparable to that extracted in 0.1 M HCl. Although only about 1/5th of the total immunoreactivity determined in the same acid extracts in the G1 assay was measured in the R72 assay, the patterns of absorption to the elution from QUSO were similar. Much less iCCK was measured in the RB assay, and this material was not adsorbed by QUSO.

Table 1. iCCK in pig brain

	pmol pCCK33 or CCK8 equivalents per g (wet weight)		
	Total	After QUSO adsorption	QUSO eluate
Cerebral cortex (n = 4)			
HCl extracts			
G1	204 ± 38	52 ± 17	80 ± 11
R72	44 ± 6	18 ± 8	28 ± 16
RB	24 ± 6	26 ± 15	<5
NaOH extracts			
G1	50 ± 5	<2	—
R72	92 ± 9	89 ± 7	—
RB	87 ± 5	98 ± 6	—
Hypothalamus (n = 4)			
HCl extracts			
G1	22 ± 6	<5	5 ± 3
R72	<5	<5	<3
RB	<10	<10	<5
NaOH extracts			
G1	3 ± 2	<2	—
R72	9 ± 2	13 ± 4	—
RB	10 ± 1	10 ± 3	—

Results are given as mean ± SEM.

G1 immunoreactivity in NaOH extracts averaged only about 1/4th of that found in HCl extracts and it was adsorbed by QUSO. In contrast, R72 and RB immunoreactivities in the NaOH extracts were more than double the immunoreactivity in HCl extracts and were not adsorbed by QUSO.

The iCCK in the porcine hypothalamic extracts was only about 10% of that in the cerebral cortical extracts, but the patterns of extraction and of QUSO adsorption were similar.

Sephadex G50 chromatography of the acid and alkaline brain extracts revealed peaks of iCCK primarily in two regions: big iCCK coeluting with authentic pCCK33 was maximally extracted by HCl and was measured in highest concentration in the G1 assay (Fig. 1*A*); small iCCK peaked close to the $^{125}I^-$ region, was maximally extracted by NaOH, and appeared to be about equally immunoreactive in the RB and R72 assays (Fig. 1*B*). Virtually no immunoreactivity was observed in the CCK4 region in the RB assay. There was no G1 immunoreactivity in either extract in the $^{125}I^-$ region. Relatively small amounts of RB immunoreactivities were found in the big iCCK region. R72 immunoreactivity in the big iCCK region averaged only about 1/5th of that measured in the G1 assay (Fig. 1*A*), consistent with the lesser immunoreactivity of the extract itself as measured in the R72 system (Table 1).

The recovery of big iCCK from Sephadex columns was diminished from 64% to 22% when the same HCl porcine cortical extracts were eluted with an alkaline eluent rather than with 0.1 M HCl. In addition, there was some big iCCK in the void volume as well as in the usual position of pCCK33. Because no void volume iCCK was found with 0.1 M HCl as eluent, acid-extracted iCCK found in the void volume with the barbital eluent may represent nonspecific adherence of the large, basic forms of CCK to tissue proteins. Thus void volume iCCK found in NaOH brain extracts may simply be a chromatographic artifact rather than a larger form of iCCK.

Small iCCK contained in both the acid and alkaline tissue extracts was quantitatively fully recovered from the Sephadex G-50 columns with either 0.1 M HCl or 0.02 M barbital buffer as eluent.

Starch gel electrophoresis of the alkaline extracts revealed only a single peak with mobility similar to that of sulfated CCK8 (Fig. 2*A*). This peak contains equal amounts of CCK as determined in both the RB and R72 assays. There is no immunoreactivity in the CCK4 region. A lesser amount of iCCK is found on starch electrophoresis of the acid extracts. In addition to a peak in the sulfated CCK8 region there was a minor postalbumin immunoreactive peak (Fig. 2*B*). Refractionation of the latter peak on a Sephadex G-50 fine column revealed a peak with an elution volume resembling that of unsulfated CCK8. Thus this minor immunoreactive peak found in acid cortical extracts is not CCK12, which also migrates in this region on starch gel, but is probably unsulfated CCK8 artifactually produced from sulfated CCK8 by boiling in an acid extractant (18).

Starch gel electrophoresis of either the alkaline or acid porcine extracts revealed a very small amount of immunoreactivity measured in the G1 assay that remained at the origin or moved slightly cathodally (Fig. 2). This resembles pCCK33 and, like it, is poorly recovered from starch gel (18). In contrast, small iCCK contained in both the acid and alkaline porcine tissue extracts was quantitatively recovered from the starch gel.

Extraction and Characterization of iCCK from Rat Brain. iCCKs in HCl and NaOH rat cerebral cortical and hypothalamic extracts were determined in both the RB and R72 assays (Table 2). As previously reported (19), rat brain extracts do not react with the species-specific G1 antiserum. More iCCK in the acid

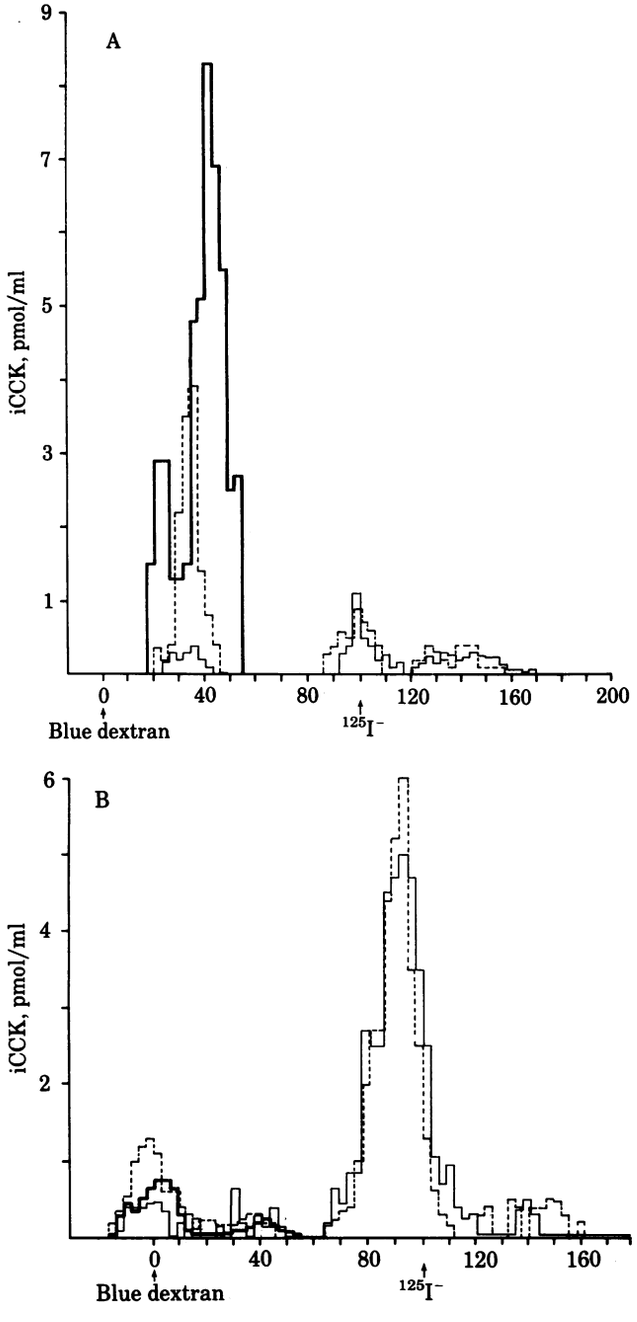

FIG. 1. Sephadex G-50 gel filtration of extracts of porcine cerebral cortex. The eluates were assayed with three different antisera: G1 (——), RB (——), and R72 (---). The reactivity of G1 is directed toward the NH_2 terminus of pCCK33, because it does not react with peptides containing the COOH-terminal sequences including CCK4, CCK8, CCK12, and pG1. Furthermore, it is species specific, because it does not crossreact with gut or brain extracts of any animal species but pig (7, 19). The minimal detectable concentration of pCCK33 immunoreactivity is 1 pM (19). RB was prepared in a rabbit by immunization with CCK4 coupled to bovine serum albumin. The bleeding of the RB antiserum used crossreacted almost identically on a molar basis with pG1, pCCK33, synthetic sulfated CCK12 and CCK8, synthetic unsulfated CCK8 and CCK7, as well as synthetic CCK6, CCK5, and CCK4 (18). The minimal detectable concentration of CCK8 immunoreactivity in this system is about 5 pM (18). R72 was prepared in a rabbit by immunization with unsulfated CCK8 coupled to bovine serum albumin. Antiserum R72 crossreacts almost identically on a molar basis with pG1, pCCK33, and synthetic sulfated CCK12 and CCK8, as well as synthetic unsulfated CCK8. Unsulfated CCK7, CCK6, and CCK5 react progressively weaker in this system, and CCK4 and CCK3 appear to be nonreactive. The minimal detectable concentration of CCK8 immunoreactivity in the R72 assay is about 5 pM. (*A*) Extracts were prepared in 0.1 M HCl and the column was equilibrated with and eluted with 0.1 M HCl. Note that the predominant peak of iCCK is in the big iCCK region and is largest when measured with G1. (*B*) Extracts were prepared in 0.1 M NaOH and the column was equilibrated with and eluted with 0.02 M barbital buffer (pH 8.6) containing 0.25% human serum albumin. The predominant peak is in the $^{125}I^-$ region and is about equal as measured with RB and R72.

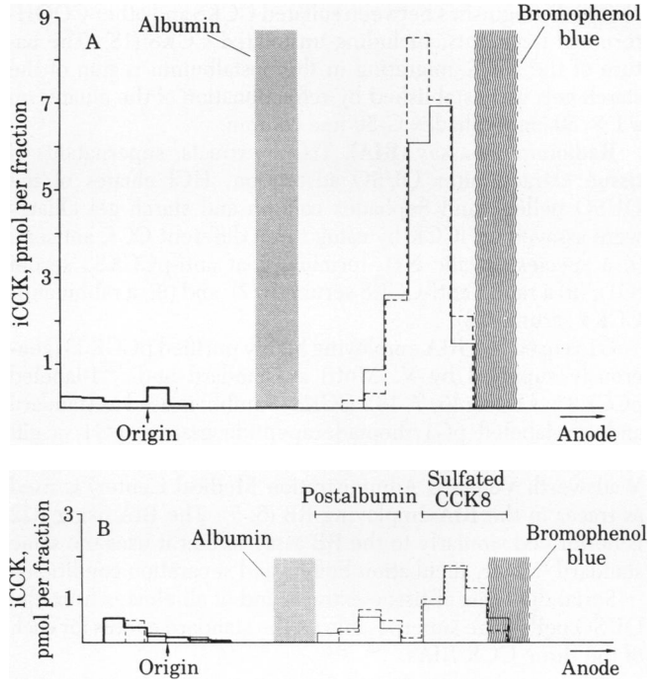

FIG. 2. Starch gel electrophoresis of extracts of porcine cerebral cortex, assayed with the three antisera described in the legend of Fig. 1. ——, G1; ——, RB; ----, R72. (*A*) Extract was prepared in 0.1 M NaOH. Note that with R72 and RB there appears to be virtually identical immunoreactivity in a single peak in the region of sulfated CCK8 and that with G1 there is only a minor peak at, or cathodal to, the origin. (*B*) Extract was prepared in 0.1 M HCl. In addition to the minor peak seen by G1 at or cathodal to the origin, there are two peaks between albumin and bromophenol blue seen equally by R72 and RB. The postalbumin peak is in the region of sulfated CCK12 or unsulfated CCK8.

extracts was measured in the R72 assay than in the RB assay. On the average, about one-third to one-half of the R72 immunoreactivity in these extracts could be adsorbed to and eluted from QUSO. The iCCK in rat NaOH cortical extracts was considerably greater than in acid extracts, and equal amounts were measured in the RB and R72 assays. Essentially none of this immunoreactivity was adsorbed by QUSO.

Rat hypothalamic extracts contained about one-third the iCCK found in the cortical extracts. This is in contrast to the pig brain, in which the hypothalamic extracts contained only about 10% as much iCCK as the cortical extracts.

Sephadex G-50 chromatography of rat cortical extracts also revealed two fractions of iCCK (Fig. 3). Big iCCK, eluting in the position of pCCK33, was maximally extracted by 0.1 M HCl and displayed more immunoreactivity when measured in the R72 assay than in the RB assay (Fig. 3*A*). Small iCCK, eluting in the region of the salt peak, was maximally extracted by 0.1 M NaOH (Fig. 3*B*) and had about the same immunoreactivity in the R72 and RB assays. There was virtually no immunoreactivity in the CCK4 region as determined by the RB assay.

Starch gel electrophoresis of NaOH rat cortical extracts showed that small iCCK migrates in two distinct peaks, the most

Table 2. iCCK in rat brain

	pmol CCK8 equivalents per g (wet weight)		
	Total	After QUSO adsorption	QUSO eluate
Cerebral cortex (n = 4)			
HCl extracts			
R72	89 ± 2	51 ± 19	24 ± 8
RB	53 ± 3	49 ± 9	5 ± 2
NaOH extracts			
R72	236 ± 27	203 ± 23	—
RB	226 ± 16	219 ± 24	—
Hypothalamus (n = 3)			
HCl extracts			
R72	38 ± 8	18 ± 12	11 ± 2
RB	16 ± 6	14 ± 3	<5
NaOH extracts			
R72	70 ± 6	61 ± 6	—
RB	72 ± 19	67 ± 11	—

Results are given as mean ± SEM.

prominent corresponding to sulfated CCK8 (Fig. 4). The post-albumin peak, with an electrophoretic mobility resembling that of sulfated CCK12 or unsulfated CCK8, contains about one-third the total immunoreactivity. This peak, when subjected to refractionation on a Sephadex G-50 column, has an elution volume similar to that of CCK8 or CCK7. Thus, this minor peak of small iCCK is likely to be unsulfated CCK8. However, one cannot rule out the possibility that it might be sulfated CCK7. Because boiling sulfated CCK8 in alkaline tissue extracts did not cause noticeable desulfation or peptide cleavage (18), this minor form of rat small iCCK may have been present initially in the tissue and not have been produced artifactually during the extraction process.

Rat small iCCK contained in both acid and alkaline tissue extracts was quantitatively recovered from Sephadex G-50 columns and starch gels.

DISCUSSION

There have been numerous reports of the concentrations and hormonal forms of CCK-like peptides in the brains of various animal species (1–7, 9–12, 14, 20). Because a variety of extractants and different antisera with different specificities have been employed, there has generally been little agreement among the various laboratories. Part of the problem can be attributed to the fact that only porcine CCK33 or CCK39 has been available for the development of RIAs that measure the NH_2-terminal portion of the molecule. In this report we confirm and extend our earlier investigations on porcine brain CCK (6, 7), in which we found that there were two components, one resembling pCCK33 in size and charge and NH_2-terminal immunologic specificity (big iCCK) and the other resembling CCK8 (small iCCK). The former was shown to be preferentially extracted in boiling 0.1 M HCl and the latter in boiling water (6, 7). What we failed to note in the earlier reports was that big iCCK was virtually nonreactive with RB, which reacts equally well with all CCK-like peptides from CCK4 through CCK39. Thus porcine big iCCK cannot be pCCK33 or pCCK39. It might be an NH_2-terminal fragment that remains after a COOH-terminal fragment, perhaps CCK4, is cleaved off. However, CCK4 was not detected in the RB assay, in which this fragment is fully immunoreactive. Alternatively, it might be pCCK33 or pCCK39 with a COOH-terminal extension causing a marked diminution in RB immunoreactivity.

CCK belongs to the group of peptides terminating with an amino acid amide rather than a free α-carboxyl group (21). Suchanek and Kreil (22) have proposed that posttranslational processing involving cleavage of a COOH-terminally extended precursor molecule and a transamidase-like reaction may take place

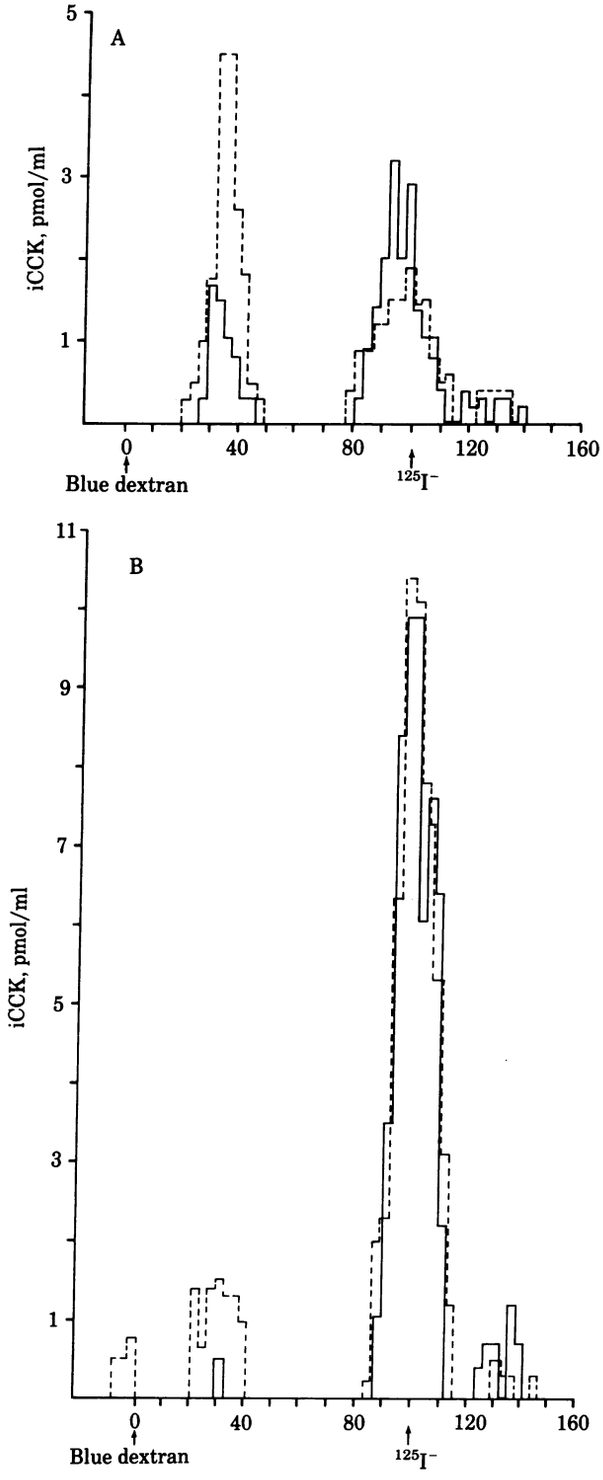

FIG. 3. Sephadex G-50 gel filtration of extracts of rat cerebral cortex. Because rat brain extracts do not react with G1, the eluates were assayed only with RB (——) and R72 (----). (*A*) Extracts were prepared and eluted as in Fig. 1*A*. Note that, in the region of big iCCK, R72 immunoreactivity predominates and that, as in Fig. 1, the peaks are equipotent with both antisera in the $^{125}I^-$ region. (*B*) Extracts were prepared and eluted as in Fig. 1*B*. Note that the predominant peak is in the $^{125}I^-$ region and seen equally with both antisera.

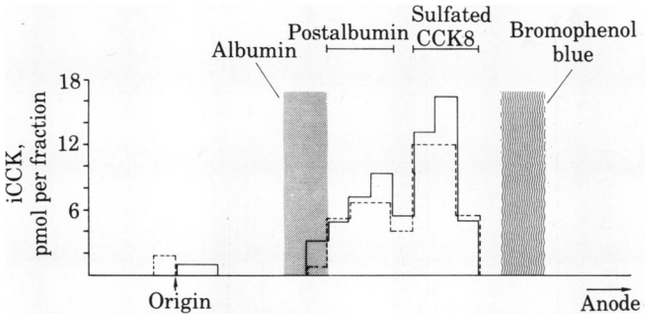

FIG. 4. Starch gel electrophoresis of NaOH extracts of rat cerebral cortex. Immunoreactivity was assayed with RB (——) and R72 (----). Note the major peak in the region of sulfated CCK8 as in Fig. 2A. However, the postalbumin peak was not seen on starch gel electrophoresis of NaOH extracts of porcine cerebral cortex.

with all peptides ending with a COOH-terminal amino acid amide. Recently, using a small oligonucleotide probe to isolate gastrin mRNA, Noyes *et al.* (23) have presented data indicating that gastrin may be initially synthesized as a large peptide requiring both NH_2- and COOH-terminal posttranslational processing to produce active hormone. These authors suggested that the precursor molecule reacts poorly with several gastrin antisera despite containing the gastrin peptide sequence (23). Our inability to measure in acid extracts the same amount of iCCK with an NH_2-terminal antiserum as with a COOH-terminal antiserum could occur because of the presence of either an NH_2-terminal fragment or a COOH-terminal extension of CCK33. Bioassay would be of no value in making the distinction, because both an NH_2-terminal fragment and a CCK molecule without the COOH-terminal amide would be biologically inactive. However, our observation that R72 measures more immunoreactivity in the acid extracts in the big iCCK region than does RB suggests an explanation. It is likely that R72 detects the amino acid sequence corresponding to the NH_2 terminus of CCK8, because it does not crossreact with CCK4. Thus it could crossreact with a CCK33-like peptide with the COOH-terminus extended but is unlikely to crossreact with an NH_2-terminal fragment that does not contain a portion of CCK8. However, because RB, with which CCK4 is equally as reactive as the other CCK peptides, does not measure significant immunoreactivity in the CCK4 chromatographic or electrophoretic region, it is unlikely that CCK4 has been cleaved from the intact hormone. Thus the reactivity of R72 with big iCCK in pig and rat brain is most consistent with the hypothesis that a major portion of this immunoreactive peak corresponds to CCK33 with a COOH-terminal extension.

Acid extraction is more effective in removing big iCCK and alkaline extraction is more effective in removing small iCCK. Because both extractions are performed in boiling solutions, enzymatic conversion between the two forms is unlikely to account for the different hormonal forms of iCCK extracted. It is thus likely that total porcine cortical iCCK is the sum of that measured by G1 and RB and is therefore at least as great as 300 pmol/g (wet weight) of tissue. There have been no reports concerning the availability of an antiserum specific for the NH_2-terminal portion of rat CCK33. R72, prepared by immunization with unsulfated CCK8 coupled to bovine serum albumin, appears to be more efficient in detecting big iCCK in both pig and rat brains than is RB, which was prepared by immunization of CCK4 also coupled to albumin. However, R72 measures only about 1/5th of the total big iCCK in the pig, and it may well be that it underestimates rat big iCCK, perhaps by the same amount. If so, then total rat big iCCK might be as much as 400 pmol/g. This is not unreasonable, because rat small iCCK is more than twice pig small iCCK.

The quantitative aspects of this study suggest that rat cerebral cortical iCCK is about twice that found in the adult pig, and that rat hypothalamic iCCK is about one-third that of rat cerebral cortical iCCK, whereas pig hypothalamic iCCK is only 10% that of pig cerebral cortex. Because brain iCCK has been suggested to be related to feeding patterns and because the rat consumes daily an amount of food much greater in relation to its weight than does the pig, these differences may be related to differences in feeding patterns. However, until additional animal species are studied in a systematic fashion, one cannot rule out the possibility that these differences are simply species related.

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