POSTNATAL DEVELOPMENT OF THE CHOLECYSTOKININ-GASTRIN FAMILY OF
PEPTIDES IN THE BRAIN AND GUT OF RAT

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Summary

The average levels of CCK-8- and CCK-like peptides present in aqueous and acidic extracts of the brain of the newborn rat were, respectively, 5.3 and 2.9 pmol/g wet weight. These low levels increased 20- and 10-fold, respectively, so as to attain, at 25-30 days post partum, values comparable to those found in adult brain. There was also a rapid deposition of CCK-gastrin-like peptides in the whole gut between day 20 post coitum and day 10 post partum, when maximal concentrations were attained. Later, less CCK-gastrin peptides than other gut components were accumulated during the progressive weaning of the young rats, so that the average concentrations of CCK-gastrin-like peptides were, at day 30, similar to those observed in adult gut.

Cholecystokinin-pancreozymin (CCK), originally isolated from porcine jejunum as a triacontatriapeptide (CCK-33) and a triacontanonapeptide (CCK-39) (1), shares with gastrin the same biologically active C-terminal pentapeptide amide sequence GLY-TRP-MET-ASP-PHE-NH₂. Like a number of other gut peptides, CCK is also present in brain. Vanderhaeghen et al. described in 1975 the presence of a major brain peptide reacting with gastrin antiserum and having a molecular size smaller than gastrin 17 (2). This peptide was later identified as being CCK-8 (3-7). It now appears that CCK in different forms is present in both brain and gut, with CCK-8 and CCK-4-like forms predominating over CCK-33 and CCK-39 (7,8). In particular, large amounts of CCK peptides are widely distributed in most brain regions (7) where they are synthesized (9) and stored in synaptic vesicles (10) from which they are released following depolarization with K⁺ in the presence of Ca²⁺ (11). The present study documents the postnatal development pattern of CCK-gastrin-like peptides in brain and gut in the rat.

Materials and methods

1. Animals.

Wistar rats were obtained from our breeding colony. Foetuses were obtained by cesarean section from the mother after anaesthesia with ether. Their ages were determined from mating time. Birth dates of neonates were recorded 22 days after mating by twice-daily inspections of the animal house. Rats began to nibble at the standard chow offered to the mothers when 15 to 18 days-old. No selection was made with regard to the sex up to 32 days of age. Young adult rats used for comparison were 120-150 g males.

Abbreviations : CCK-39, CCK-33, CCK-8, CCK-4 : C-terminal triacontanona-, triacontatria-, octa-, and tetrapeptide of cholecystokinin-pancreozymin.

2. Extraction procedure.

Animals were weighed and killed by decapitation. The entire gut was excised from pylorus to rectum, slit open, washed, weighed, and immediately frozen in liquid nitrogen. The whole brain was resected without brain stem and cerebellum, and rapidly frozen in liquid nitrogen. Rats ranged in age from 20 days gestation to 90 days after birth. For fetuses and for neonates less than 4 days-old, guts and brains from 3 animals were pooled. Between 4 and 6 days, the tissues from 2 animals were pooled, and after 8 days of age, the organs were treated individually. CCK peptides were extracted by the sequential method of Rehfeld (7) as modified by Larsson and Rehfeld (12). The frozen tissues were subsequently dropped into boiling distilled water for 10 min (10 ml/1-3 brains and 10 ml/1-3 guts, depending on the age). The tissue samples were then homogenized in a Potter homogenizer equipped with a Teflon pestle, and centrifuged for 30 min at 100,000 x g. The supernatants were collected and frozen at -20° C. The pellets were further extracted with 3 ml 0.5 M acetic acid. After 15 min at 20° C, the pellets were homogenized and the homogenate was centrifuged for 30 min at 100,000 x g. The acidic supernatants were neutralized with ammonia and kept frozen at -20° C. It can be inferred from data in refs (7,12) that this sequential extraction allows the recovery of CCK-8 and CCK-4 in the aqueous extracts, and the desorption of larger molecular forms such as CCK-39, and CCK-33 from the acidified pellets.

3. Radioimmunoassay.

The antiserum was originally raised in a rabbit against gastrin I-(2-17) covalently coupled to bovine serum albumin by carbodiimide. The first bleeding, called 3737 b, was originally used for gastrin radioimmunoassays (13). Its I₅₀-human gastrin I-(1-17)/I₅₀-CCK-8 ratio was as low as 0.0028 (Fig. 1). After boost injections of gastrin I-(2-17) in the same rabbit, the capacity to recognize CCK-8 increased 60-fold. The characteristics of the less specific new antiserum 3737 f (Fig. 1) indicate a binding capacity for all CCK and gastrin-like peptides containing the COOH-terminal pentapeptide amide common to CCK and gastrins. The I₅₀-human gastrin I-(1-17)/I₅₀-CCK-8 ratio was now as high as 0.08. This antibody bound CCK-8 and gastrin I-(11-17) with equimolar potency. It reacted more poorly with CCK-33 and caerulein. It had almost no affinity for CCK-4 and for the mid region of human gastrin I-(1-17), as shown with gastrin I-(1-13). In the present study, the peptides in tissue extracts were measured with antiserum 3737 f (except when stated otherwise), synthetic CCK-8 and pure natural porcine CCK-33 as standards, and ¹²⁵I-gastrin as tracer. Synthetic human gastrin I-(1-17) was radiiodinated using the chloramine-T method (14). The moniodinated form was separated on DEAE-Sephadex, according to Brown et al. (15). Bound and free tracer were separated with charcoal coated with human serum. In practice, 0.25 ml of sample, 0.05 ml of tracer containing 5,000 cpm of ¹²⁵I-gastrin I-(1-17), and 0.3 ml of antiserum solution in 0.1 M phosphate buffer (pH 7.4) enriched with 1 % bovine serum albumin were mixed and incubated for 2-3 days at 4° C. Antibody-bound and unbound tracer were separated by centrifugation after addition of 20 mg of charcoal. The extracts were assayed in triplicate and the results expressed in pmoles of CCK-8 equivalents/g wet weight of original tissue for the aqueous extracts, and in pmoles of CCK-33 equivalents for the acidic extracts.

4. Peptides.

Synthetic CCK-8 (SQ 19844) was a gift from Dr. S.J. Lucania (Squibb Institute for Medical Research, Princeton, N.J., U.S.A.). Natural cholecystokinin-pancreozymin (99 % pure CCK-33) was generously supplied by Dr. V. Mutt (Gastrointestinal Hormone Research Unit of the Karolinska Institutet, Stockholm, Sweden). Caerulein was a gift from Dr. de Castiglione (Farmitalia, Montedison, Milano, Italy). Human gastrin I-(1-17) desulfated, human gastrin I-(2-17) desulfated, human gastrin I-(1-13) tridecapeptide desulfated, and heptagastrin I-(11-17)-heptapeptide desulfated were synthesized by Dr. Morley (Imperial Chemical Industries, Macclesfield, England). CCK-4 was purchased from Senn

Chemicals (Dielsdorf, Switzerland). Na¹²⁵I (16.45 mCi/ μ g iodine) was supplied by the Radiochemical Centre (Amersham, England). All other reagents were of the highest grade available.

FIG. 1

Standard calibration curves for immunoreactive peptides of the CCK-gastrin family. The following standards, with respect to displacement of moniodinated ¹²⁵I human gastrin I-(1-17) from antiserum 3737 b (---) and antiserum 3737 f (—) were used: human gastrin I-(1-17) desulfated: ● and ○; CCK-8: ▲ and △; human heptagastrin I-(11-17)-heptapeptide desulfated: □; CCK-33: +; caerulein: ■; human gastrin I-(1-13) tridecapeptide desulfated: ◆; CCK-4: ◇. The final antiserum dilution was 1:40 000; B₀ around 50%; 5 000 cpm of moniodinated human gastrin I was added per test tube.

Results

Levels of CCK-like peptides in brain during development.

CCK-like peptides were already present at birth (Fig. 2) in aqueous extracts: 5.3 ± 0.7 pmoles of CCK-8 like peptides/g wet weight as well as in acidic extracts: 2.9 ± 0.3 pmoles of CCK-33 equivalents/g wet weight (mean $\pm$ S.D., $n = 4$). These levels increased continuously and markedly from birth to the 30th day (Fig. 2) when they reached the adult values of 67.7 ± 3.4 pmoles of CCK-8 equivalents/g wet weight for aqueous extracts, and 24.2 ± 1.5 pmoles of CCK-33 equivalents/g wet weight for acidic extracts (mean $\pm$ S.D., $n = 5$). In both aqueous and acidic extracts, half-maximal levels of CCK peptides were attained around the 15th day. There was a striking parallelism in the accumulation pattern of CCK peptides in aqueous and acidic extracts, except in brains of animals younger than 4 days, where the large molecular forms in acidic extracts appeared to be somewhat more abundant: at one day of age, for instance, the concentration in acidic extracts represented 12 % of adult levels while the concentration in aqueous extracts represented 8 % of adult levels.

Levels of CCK-gastrin peptides in gut during development.

Radioimmunoassay measurements in gut reflected the levels of both CCK- and gastrin-like peptides (see Discussion). Twenty days post coitum (Fig. 3,

FIG. 2

Developmental changes of CCK peptides in aqueous and acidic extracts of rat brain. The results were calculated in CCK-8 equivalent/g wet weight for aqueous extracts (●—●) and in CCK-33 equivalents/g wet weight for acidic extracts (○—○) and were expressed in percent of adult values. The number of brain extracts investigated is mentioned under brackets. For animals younger than 6 days, two or more brains were pooled for a single determination. B = birth. A = 120- to 150-g male adults.

Panel A), the levels of CCK-gastrin peptides in aqueous extracts were 4-times higher and those of CCK-like peptides in acidic extracts were similar to those observed in adult gut, when expressed per g wet weight of tissue. These concentrations increased in both aqueous and acidic extracts from birth to day 4 post partum. Maximal values remained relatively constant until the 14th day and then decreased rapidly so that adult levels were attained around the 30th day in aqueous extracts, and the 20th day in acidic extracts. The average concentrations of CCK-gastrin peptides finally attained in the entire gut at day 30 were, on a molar basis, similar to those present in the adult gut. The adult concentrations were 73.4 ± 15.1 pmoles/g wet weight for aqueous extracts and 20.4 ± 3.7 pmoles/g wet weight for acidic extracts (mean $\pm$ S.D., $n = 5$). The accumulation pattern of CCK-gastrin peptides in gut is made more obvious in Fig. 3, Panel B, where data are expressed per whole gut.

Discussion

1. Validity of the radioimmunoassays.

Considering the antisera utilized, it is clear that peptides of the CCK family were only measured accurately in the absence of gastrin(s). In addition, the present data took only partially into account the heterogeneity of molecular forms (7) and species differences. The estimation of the absolute concentrations of the different CCK peptides in rat tissues must await their purification, characterization, and subsequent use as appropriate rat standards. In preliminary experiments, we observed that rat brain CCK-8 was indistinguishable from porcine CCK-8 on the basis of its charge, size, immunological, and biological properties. Another fraction separated by DEAE-cellulose chromatography from rat brain was more acidic than porcine CCK-33 but cochromatographed with porcine CCK-33 on Biogel P-2, yielded CCK-8 upon trypsin digestion, and

might well be rat CCK. A similar species specificity has already been reported in the NH_2 -terminal portion of porcine, monkey, and dog CCK (16) and of porcine, human, and rodent gastrins (17,18).

FIG. 3

Developmental changes of CCK-gastrin peptides in aqueous and acidic extracts of rat gut. The results were calculated in CCK-8 equivalents/g wet weight for aqueous extracts (●—●) and in CCK-33 equivalents/g wet weight for acidic extracts (○—○), and were expressed in percent of adult values. The number of gut extracts investigated is mentioned in Panel A. For animals younger than 6 days, two or more guts were pooled for a single determination. B = birth. A = 120- to 150-g male adults.

In brain, CCK in different molecular forms is known to be widely distributed in the neurons of most brain regions, except the cerebellum, epithalamus, and pituitary (7,9,12,19,20) and, so far, only vasoactive intestinal peptide has also been localized to neocortical regions (21). In the present study, the average concentration of 68 pmoles of CCK-8 equivalents/g wet weight found in aqueous extracts of whole adult rat brains agreed with the figure of 50-150 pmoles/g rat brain reported by Dockray (3). These levels of brain CCK exceed those of any other known brain peptide (7). A significant contribution of CCK-4 to this value was most unlikely, considering the very low affinity for CCK-4 of the antiserum used (Fig. 1). The value of 24 pmoles/g wet weight CCK-33 equivalents found here in acidic extracts is indicative only since acidic extracts are heterogeneous (12). Furthermore, we did not dispose of rat standards in adequate amounts. True gastrin did not interfere with the present radioimmunoassays in brain, since it is located, in small amount only, in the hypothalamus, infundibulum, and the lower part of the medulla oblongata (9) while being a major form in the hypophysis (22) and in the vagal nerves (23). In adult gut, the concentrations of CCK-gastrin peptides were comparable to those found in brain. Endocrine cells rich, respectively, in CCK (I cells), CCK-4 (TG cells), and gastrin-34 (GI or Ga cells) are reported to coexist in the small intestine while CCK- and gastrin-nerves are numerous in the large intestine and distal ileon (12,24,25). In the present study, where the entire gut was taken, it is clear that CCK-gastrin peptides originating from both endocrine cells and nerves were extracted. CCK-4 could not be detected (Fig. 1) but members of the gastrin family, that are easily solubilized with boiling

water at neutral pH (26), were able to interfere with the measurement of CCK-8-like peptides in aqueous extracts (see below).

Developmental pattern of CCK-like peptides in rat brain and gut.

In brain, peptide accumulation was made especially obvious by representing the data as % of adult values (Fig. 2). The small (in aqueous extracts) and large (in acidic extracts) molecular forms of CCK present at birth increased 20- and 10-fold, respectively, to attain adult levels at 4 weeks after birth. This developmental pattern covered the neonatal as well as the late suckling period. The maximal rate of increment was at 0-6 days of age, a period marked by neuronal multiplication. Adult concentrations were reached at 20 days, i.e. when the period of proliferation of neuronal processes, myelination of axons, and multiplication of oligodendroglia was mostly over (27). This considerable accumulation of CCK peptides in the neurons of the developing rat brain probably reflected increased rates of peptide synthesis and/or low peptidase activity. In vivo biosynthesis of CCK peptides indeed occurs in rat cerebral cortex (9), and the large forms may represent biosynthetic precursors of CCK-8 and CCK-4, since enzymes converting CCK-33 into CCK-12 and CCK-8 are present in brain (16,28). It is of interest to note that the ratio of large to small CCK peptides was constant throughout post-natal development, except for the period around birth when the large molecular forms were more abundant. It is tempting to speculate that the proteolytic cleavage of precursors was less active during this period.

The present pattern of accumulation of CCK peptides differed from that of other brain-gut peptides. We have shown (29) that the low level of VIP present at birth in rat brain (17 pmoles/g wet weight) remains constant during the first two weeks and then doubles progressively to reach at 20 days a value comparable to that found in adult animals. For substance P also, a slow rise during the first two weeks of life is followed by larger increments between day 14 and 28 post-partum (30).

In gut, the pattern of accumulation of CCK-gastrin peptides differed markedly from that observed in brain. Maximal concentrations were observed in neonates, between the 4th and the 15th day post-partum, and then decreased to reach adult value around day 20-30 (Fig. 3). Larsson, using histochemical methods (25,31), has described peak frequencies of gastrin cells and CCK(I) cells, respectively between day 1-4 and 10-15, in the rat duodenum, which are followed by a progressive decrease in frequency between day 15 and day 18, i.e. when the small intestine of the rat shows a striking increase in the rate of cellular proliferation (32). Since the timing of development of CCK-gastrin peptides preceded that of rapid growth of the intestines (Fig. 3), it is conceivable that the release of more gastrointestinal hormones, at the onset of the weaning period, and the development of CCK-gastrin nerves, contributed to this cell proliferation in the intestinal mucosa (33). It is noteworthy that the average concentrations of CCK-gastrin peptides in adult gut were comparable to those observed in brain.

In order to examine the contribution of gastrin to the present developmental curves, we used antiserum 3737 b with which CCK-8 reacted very poorly (Fig. 1). Based on the assumption that rat gastrin(s) behave as human gastrin I-(1-17), the gastrin contents in aqueous extracts of guts from 4 days-old, 11 days-old and adult rats were always low: 2.2, 4.8, and 12.6 pmoles gastrin I-(1-17) equivalents, respectively, per total gut. With the less specific antiserum 3737 f, these gastrin concentrations should correspond to 18, 63, and 240 pmoles of CCK-8 equivalents, as derived from standard calibration curves (Fig. 1). Since the corresponding total CCK-8-like concentrations were in fact 126, 339, and 839 pmoles, it appears that true gastrin(s) contributed only 14 % of the total at 4 days, 19 % at 11 days, and 29 % in the adult state. It can thus be concluded that the accumulation pattern between days 4 and 11 in aqueous extracts (Fig. 3) was mainly due to CCK-8-like peptides. This accumulation pattern of CCK-gastrin peptides in gut differed from that of VIP (34) and substance P (30) whose contents in the small intestine increase

mainly during the third week of life,

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