Radioimmunoassay of cholecystokinin in human plasma

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Summary

A sensitive radioimmunoassay for cholecystokinin (CCK) has been developed. Porcine CCK-33 was labelled by conjugation with 125I-hydroxyphenyl-propionic acid succinimide ester. Antibodies were raised against porcine CCK-33 covalently coupled to egg albumin. Plasma samples were extracted with 96% ethanol prior to assay. Free and bound hormone were separated by dextran-coated charcoal. The antibodies bound CCK-8 and CCK-33 with equimolar potency. The assay detection limit was 1 pmol/l plasma. Within and between assay coefficients of variation were ±12.7 and 13.0% at mean plasma CCK concentrations of 13.2 and 13.6 pmol/l. The concentration of CCK in 47 normal fasting subjects ranged from undetectable to 22 pmol/l. Ingestion of a mixed meal in 9 normal subjects increased the plasma concentration from 8.3 ± 2.5 S.E. to 24.4 ± 6.5 pmol/l.

Introduction

Cholecystokinin (CCK) is one of the three classical gut hormones. Its name was coined in 1928 [1], and it is believed to control pancreatic enzyme secretion, gall bladder contraction [2], intestinal motility [3] and perhaps appetite [4]. However only a few radioimmunoassays for cholecystokinin in human plasma have been described [5–9] and these have been poorly validated and given conflicting results. Rehfeld, using sequence-specific radioimmunoassays [10,11] has demonstrated that CCK in the upper intestinal tract of man is present in several molecular forms including the triacontatriapeptide, CCK-33. A predominant immunoreactive form corresponded to the carboxytermin al octapeptide, CCK-8. It is, therefore, likely that plasma CCK is also heterogeneous. Thus, an assay for CCK should detect both large and small forms of CCK. In the present study a radioimmunoassay for cholecystokinin is described and validated.

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Materials

Pure (99%) porcine CCK-33 was obtained from Prof. V. Mutt, Stockholm through the NIAMDD. Porcine CCK of 10 and 20% purity was purchased from the Karolinska Institute, Stockholm. Synthetic sulphated CCK-8 (SQ19844) and unsulphated CCK-8 (SQ19255) were gifts from Dr. M. Ondetti of the Squibb Institute of Medical Research, Princeton, NJ. Synthetic non-sulphated gastrin-17 was a gift from Prof. M. Grossman, Cure, Los Angeles, CA. Mono $^{125}$I-labelled $p$-hydroxyphenylpropionic acid succinimide ester (Bolton and Hunter reagent) was purchased from New England Nuclear. Human serum albumin was obtained from Behringwerke, A.G., egg albumin from Calbiochem, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (E.D.C.) (from Merck), and gelatin from Parke Davis.

Methods

Radioiodination

Radioiodination of CCK-33 using a modification of the Bolton and Hunter method [12] was performed as previously described [10]. 1 mCi of $^{125}$I-labelled $p$-hydroxyphenylpropionic acid succinimide ester was dried under nitrogen in a tapered glass vial (Perse Chemicals). Porcine CCK-33 (10 µg), was placed in the reactivial and the latter was chilled in an ice-bath. 10 µl of chilled 0.1 mol/l borate buffer, 8.5, were added to the vial which was then mixed vigorously at 4°C for 60 min. The reaction was terminated by the addition of 100 µl of 0.2 mol/l glycine in 0.1 mol/l borate buffer, pH 8.5. After a further 5 min, the mixture was applied to a 60 x 0.9 cm column of Sephadex G50 superfine equilibrated in 0.5 mol/l acetic acid, pH 5, containing 0.2% (w/v) gelatin and eluted with the same buffer at a flow rate of 0.07 ml/min. The fractions were tested for immunoreactivity and the fraction demonstrating the best binding was stored at −20°C in 0.5 mol/l acetic acid pH 5.0 until assay.

Production of antibodies

1 mg of porcine CCK-33 (20%) was dissolved in 200 µl of 0.9 mol/l saline. 2 mg of egg albumin, and 4 mg of ethyl-carbodiimide, each in 200 µl of saline, were then added. The reaction mixture was vortexed and allowed to stand overnight at 4°C. The mixture was then diluted with saline to a concentration equivalent to 200 mg CCK/l and emulsified with an equal volume of Freund's complete adjuvant. 1 ml aliquots of this emulsion were injected subcutaneously into the back of outbred New Zealand white rabbits. Booster injections with the same immunogen were performed six weeks later and the animals bled from the central ear artery 7 days after the second injection.

Sampling and treatment of plasma

Blood samples were collected into chilled, heparinized tubes in an ice-bath. Within 30 min of collection the samples were centrifuged and the plasma stored at −20°C till assayed.

1 ml samples of plasma were extracted by the addition of 2.0 ml of 96% ethanol. The mixture was vortexed and centrifuged. Duplicated aliquots of either 300 or 600
μl of the supernatant (equivalent to 0.1 or 0.2 ml of plasma respectively) were placed in assay incubation tubes and dried under nitrogen streams in a water bath at 37°C.

**Extraction of porcine duodenal mucosa**

In the frozen state the tissue was cut into pieces weighing a few mg, which subsequently were immersed in boiling redistilled water (10 ml/g tissue, pH 6.6), for 20 min, homogenized and centrifuged (15000 rpm in 10 min). After decantation, 0.5 mol/l acetic acid (10 ml/g) was added. The tissue was again homogenized, centrifuged, and decanted [11].

The supernatant fractions were kept frozen at −20°C until assayed in appropriate dilutions. The results were calculated as the mean of dilutions within the working range of the assay.

**Fractionation of the duodenal extracts**

Extracts (1.0–1.5 ml) were applied to a Sephadex G-50 superfine column (10 mm × 1000 mm) eluted with 0.02 mol/l sodium barbital buffer, pH 8.4, containing 0.1% bovine serum albumin at 4°C at a flow rate of 4 ml/h. Fractions of 1.0 ml were collected. All columns were calibrated with 125I-labelled albumin and 22NaCl (Amersham) for an indication of void volume and total volume. In addition, the columns were calibrated with 99% pure porcine CCK-33, and synthetic COOH-terminal octapeptide.

**Incubation conditions**

The dried ethanol extracts of plasma were reconstituted by adding 200 μl of 0.04 mol/l phosphate buffer pH 7.4 containing 0.5% human serum albumin. The standards were prepared by adding 200 μl of buffer containing 0–20 fmol of CCK-33 to tubes containing dried ethanol extracts of charcoal stripped serum (20 g of Norit A/1 of serum). Both the unknown and standard tubes had equal volumes of extracted plasma, (either 100 or 200 μl). 100 μl of buffer containing CCK antiserum R4 diluted 1:2000 and 25 fmol gastrin-17 were added to each tube with the exception of incubation damage control tubes. After 72 h of pre-incubation approximately 33 dps of 125I-labelled CCK (∼1.4 fmol) in 200 μl of buffer were added to each tube and the incubation continued for a further 48 h at 4°C.

**Separation of free from antibody-bound radioactivity**

At the completion of incubation 2 ml of phosphate buffer, pH 7.4, containing 5 mg of Norit A charcoal, 10 mg bovine albumin, 0.5 mg dextran T 70 and 1.5 mg of methyl cellulose were added to each tube and allowed to react for 10 min at 4°C after which the tubes were centrifuged, the supernatant decanted and the charcoal pellet containing the "free" 125I-labelled CCK was counted in an automatic gamma spectrometer.

**Physiological studies**

Nine normal subjects ingested a mixed meal (cereal, milk, toast and cheese) after an overnight fast. Blood samples were obtained sequentially from an indwelling catheter in an antecubital vein.

In seven normal subjects a 'Crosby' capsule was passed into the second part of the duodenum and the position confirmed by screening. After basal plasma samples
had been obtained, 0.1 mol/l HCl was infused into the duodenum at a rate equivalent to 0.5 meq·kg⁻¹·h⁻¹ over a 20 min period.

Informed consent was obtained from all the subjects and the studies were approved by St. Vincents’ Hospital Ethics Committee.

Analyses of the plasma CCK responses to stimulation were made using Student’s ‘t’ test [13].

Results

Radioiodination

Approximately 40% of the ¹²⁵I-labelled p-hydroxyphenylpropionic acid was conjugated with CCK-33 (Fig. 1). The specific radioactivity was approximately 500 Ci/mmol. Binding to excess antisera was >90%. Incubation “damage” was 5–6%.

Production of antibodies

Seven rabbits produced detectable antibodies at a titre of 1:1000 but only 4 produced antibodies of sufficient affinity for use in a radioimmunoassay. In this series all of the antisera bound CCK-8, CCK-33 and gastrin to varying degrees, but only one of these (R4) bound CCK-8 and CCK-33 with equimolar potency. The titre of R4 which bound 50% of 1.4 fmol of ¹²⁵I-labelled CCK was 1:10000 and the equilibrium constant ($K^0$) was $1.1 \times 10^{11}$ 1/mol. Pre-incubation resulted in an apparent $K^0$ of $5.5 \times 10^{11}$ 1/mol.

Detection limit

The smallest amount of CCK which could be differentiated from zero hormone concentration with 95% confidence was 0.2 fmol/tube equivalent to 1 pmol/l of plasma.

Accuracy

(a) Recovery of CCK-33 added to serum prior to extraction over a range of 1.25–40 pmol/l and recovery of CCK-8 over the range 2.5–40 pmol/l was $105.9 \pm 1.4\%$ S.E. and $87.7 \pm 1.3\%$ respectively (Figs. 2 and 3).

(b) Parallelism. Plasma samples containing high levels of endogenous CCK (following ingestion of a protein meal) demonstrated parallelism when diluted with serum, extracted and equated to the standard curve (Fig. 4).

![Graph](attachment:image.png)

Fig. 1. Elution profile of ¹²⁵I-labelled p-hydroxyphenylpropionic acid-CCK on Sephadex G50 superfine. The unreacted CCK elutes earlier than the conjugated CCK.
Fig. 2. Recovery of CCK-8 from ethanol extracts of human plasma.

Fig. 3. Recovery of CCK-33 from ethanol extracts of human plasma.

**Precision**

Within and between assay coefficients of variation were 12.7 and 13.0% at mean CCK levels of 13.2 and 13.6 pmol/ml, respectively (N = 6).

**Specificity**

CCK-8 and CCK-33 produced equal displacement of the \(^{125}\text{I}\)-labelled CCK (Fig. 5). Following addition of 25 fmol of gastrin-17 (non-sulphated) per assay tube, cross-reaction by non-sulphated gastrin-17 and non-sulphated CCK-8 were negligible (1: \(1.7 \times 10^3\) and 1: \(6.4 \times 10^2\), respectively). However sulphated gastrin-17 cross-reacted more with the CCK antiserum R-4 ([ID\(_{50}\)CCK-33]/[ID\(_{50}\)gastrin-17(8)] = 0.034) and was unaffected by prior addition of non-sulphated gastrin-17. The gel chromatography of the duodenal extracts shows that the CCK assay using antiserum R4 measures at least four major CCK components (Fig. 6): component I is larger than CCK-33 and -39. Component II corresponds to CCK-33; it may also contain

Fig. 4. Parallelism between the standard curve and serially diluted plasma obtained after a mixed meal.

Fig. 5. Cross-reaction of CCK-8 and CCK-33 using antiserum R4. Bo was 32% of total radioactivity.
Fig. 6. Gel chromatography of cholecystokinin extracted from the first part of porcine duodenal mucosa. 1 ml extract was applied to Sephadex G-50 superfine columns (1000 x 10 mm) eluted at 4°C with 0.02 mol/L veronal buffer, pH 8.4, containing 0.1% bovine serum albumin. Fractions of 1 ml were collected at a rate of 5 ml/h. The columns were calibrated with 125I-labelled albumin (for indication of void volume (V₀)), 99% pure porcine CCK-33 and -8, and 22NaCl (for indication of total volume (V_t)). The elution was monitored by a CCK-assay using antisera R4. The upper panel illustrates the acetic acid extract, and the lower panel the boiling water extracts. Roman numbers (I-IV) indicate the different CCK-components.

CCK-39 which, by gel chromatography, is eluted in a position identical with that of CCK-33 [11]. Component III has a size intermediate between CCK-33 and -8. It may also contain CCK-12. Component IV corresponds to CCK-8. The identity of

Fig. 7. Distribution of plasma CCK concentration in normal subjects.

Fig. 8. Plasma CCK response to a mixed meal. (Mean ± 1 S.E.)
the four components was corroborated by the previously described sequence-specific radioimmunoanalysis for CCK [10,11].

Stability

Incubation of plasma at 37°C was accompanied by a progressive fall in the immunoreactivity with a T1/2 of 3.8 and 7.0 h for CCK-8 and CCK-33, respectively. The loss of immunoreactivity was not affected by Apronitin (Trasylool) at a concentration of 1000 μg/ml.

Basal concentration of CCK in plasma

The CCK concentration in plasma from 47 normal fasting subjects ranged from undetectable (< 1 pmol/l) to 22 pmol/l. The distribution was skewed with a mean of 6.3, a mode of 4 and a median of 3 pmol/l (Fig. 7).

Physiological studies

(a) Ingestion of a mixed meal by 9 normal subjects led to a significant (p < 0.05) rise in plasma CCK concentration from 8.3 ± 2.5 S.E. to 16.1 ± 4.6 pmol/l within 10 min reaching a peak of 24.4 ± 6.5 after 90 min (Fig. 8).

(b) Infusion of 0.1 mol/l HCl intraduodinally at 0.5 mol·kg⁻¹·h⁻¹ in seven normal subjects led to a significant (p < 0.05) increase in plasma CCK from 4.9 ± 1.3 S.E. to 9.3 ± 1.6 pmol/l (Fig. 9).

Discussion

The main problem hindering the development of radioimmunoassays for CCK has been the difficulty in producing radioiodinated CCK which has retained immunoreactivity. Previously all CCK tracers have been produced by oxidative iodination (Chloramine “T” method) [14] which leads to sulfoxidation of methionine residues of which CCK has 3. Even mild oxidation of CCK produces great or almost total loss of immunoreactivity [10]. However, since occasional antisera have recognized, although poorly, the oxidized CCK tracers [5–9], it was not initially realised that the “poor immunogenicity” of CCK [15] was really an artefact obtained by using poorly immunoreactive tracers. One of us subsequently [10] showed that high quality CCK tracers could be prepared by conjugation labelling with the Bolton and
Hunter Reagent (125I-labelled p-hydroxyphenylpropionic acid succinimide ester), which is preiodinated and does not involve any oxidation of the protein. The suitability of this method has since been confirmed by others [16,17] and again in this paper. Rehfeld also noted previously that, whereas antibodies had been undetectable using oxidised CCK tracers, all immunized animals were found to have detectable antibodies against CCK when tested with tracers prepared by conjugation labelling [10]. This paper also confirms the view that CCK is, in fact, an excellent immunogen since in this series, 7 out of 16 rabbits produced detectable antibodies at a titre of 1:1000.

Like other gastrointestinal hormones CCK has been shown to be present in mucosa in multiple molecular forms [11] and therefore perhaps, is also present in multiple forms in plasma. Thus antibodies used for the radioimmunoassay of CCK should be characterized with respect to their sequence specificity, which again will inform about their component specificity. The small fragment, CCK-8 which is a major component in mucosal extracts [11], resembles the C-terminal heptadecapeptide of gastrin and it could be expected that gastrin may cross-react with antisera capable of detecting CCK-8. This phenomenon was observed with all antisera in the present series, but in R4 the cross-reacting antibodies against non-sulphated gastrin appeared to be a subpopulation, which could be almost completely saturated with gastrin without affecting the reactivity with CCK. In contrast however, cross-reaction by sulphated gastrin persisted after saturation with gastrin indicating that this CCK antisera has specificity towards the N-terminal tyrosyl sulphate residue of the octapeptide of CCK-8.

This cross-reaction by sulphated gastrin may be of concern when using R4 to determine plasma concentration of CCK. The ratio of sulphated to non-sulphated gastrin in human antral mucosa is 2:1 [18] and the same ratio is present in plasma [19]. Thus the cross-reaction by the "total" plasma gastrin in the CCK assay is only 1-2%, i.e. a peak post-prandial plasma gastrin of 50-100 pmol/l would only artificially elevate the plasma CCK concentration by 1 pmol/l, provided that sulphated gastrin is fully extractable in ethanol. However, gastrin is poorly extracted in 96% ethanol making interference from this hormone negligible in the present CCK assay.

The plasma concentrations of CCK detected by the present assay are lower (up to 50 times) than previously reported [5-9], but in keeping with the concentration of other gut hormones such as gastrin and secretin. The lower levels of CCK observed with the present assay are a reflection of a greater assay sensitivity and abolition of plasma artefacts by prior extraction of samples.

For a radioimmunoassay to be acceptable it must be shown that appropriate stimulation produces the expected plasma hormone response. In this paper it has been shown that plasma CCK rises appropriately after ingestion of a mixed meal. In addition there is biological evidence that acid may release a small amount of CCK [20]. Intraduodenal infusion of acid at rates equivalent to maximal gastric secretion did produce a small but significant rise in immunoreactive CCK.

Our assay is now being used to study further the physiology of CCK.

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References

hæmorrhæmurus. Only four of twenty patients with severe initial symptoms were not totally relieved by day fourteen. Three of six patients whose ulcers remained unhealed had never had severe symptoms. Of these four, two healed by ten weeks; a fifth by sixteen and a single patient was submitted to surgery.

We conclude that Dernd and cimetidine are of equal efficacy in the treatment of chronic duodenal ulcer and that continued abuse of alcohol, tobacco and analgesics does not appear to prevent healing during treatment with either agent.

ENTERAL FEEDING IN GASTROENTEROLOGICAL PRACTICE

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Malnourishment is common in gastroenterological practice and increases morbidity and mortality. Nutritional support requires identification of patients at risk, assessment of needs and effective administration. General nutritional status is rarely assessed although most measurements are simple (height, weight, urinary urea and creatinine excretion, plasma albumin and lymphocyte count). Vitamin and mineral assays identify specific deficiencies. Patients requiring support fall into three categories: malabsorption or inflammatory disease; mechanical problems; anorexia. Both dietitian and physician should determine needs and the method of administration. Parenteral nutrition is expensive, has associated risks and many patients can be fed enterally. Oral intake is ideal but often inadequate in sick patients especially with current hospital catering services and special dietary formulations. Fine bore tube feeding can supplement or supply nutritional needs in hospital or at home. Tubes are available commercially or can be made from radio-opaque PVC or radiolucent silicone tubing. Feed administration is usually nasogastric (intermittent or continuous) or nasoenteric (continuous). A pump reduces supervision and allows overnight infusion at home. Inadvertent IV administration is avoided by distinctive containers, feed colours and special giving sets. Feeds are in two categories: routine—local (premixed, sterile), Ensure (powder, flexible formulation); special—"elemental", Triglyde etc. In practice, a sterile prepacked feed is adequate for routine ward and home use and costs the same as special diets. A team of physician, dietitian, pharmacist and nursing staff makes the fine bore enteral feeding simple, safe, cheap and effective.

RADIOIMMUNOASSAY OF CHOLECYSTOKININ

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The main difficulty hindering development of radioimmunoassays for cholecystokinin (CCK) has been the loss of immunoreactivity during oxidative denaturation. Recently conjugation labelling has been reported to produce a satisfactory tracer without loss of immunoreactivity.1 This paper reports our preliminary studies on the development of a radioimmunoassay for CCK in human plasma.

Pure natural CCK (Mutt) was iodinated using the conjugation method of Bolton and Hunter2 followed by purification on Sephadex G50 and CM 25 columns. Sixteen rabbits were immunised at 6 weekly intervals with 50 μg of CCK (either 10% or 20% purity) conjugated covalently to egg albumin.

The 125I-CCK had a specific activity of 500 μCi/mmol and was stable for at least 2 months at −20°C. Seven of the rabbits produced antibodies against CCK. One antisera (R4) bound 35% of 125I-CCK at a final dilution of 1:10,000 and showed equal affinity for CCK P and CCK 33. The sensitivity of the assay was 0.25 pmol/tube (95% confidence).

Cholecystokinin was found to be unstable in plasma and required extraction with ethanol prior to assay. Recovery of CCK 33 and CCK 8 added to plasma was 100% and 86% respectively.

Preliminary studies reveal normal plasma levels of CCK to lie between 1 and 22 pmol/l. A biphasic rise in CCK occurs after ingestion of a mixed meal.

References

WHAT CONTROLS FOOD STIMULATED PANCREATIC POLYPEPTIDE RELEASE? STUDIES AFTER GASTRIC SURGERY

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Pancreatic polypeptide (PP) a 36 amino acid peptide is released from the pancreas by a protein meal, vagal stimulation, gastric distension and gastrointestinal hormones. We and others have previously shown that there is both a primary and secondary phase of release following a protein meal. To attempt to elucidate the mechanisms involved, PP release in response to food was studied in 20 patients with unoperated duodenal ulcer (DU). 14 patients following truncal vagotomy and pyloroplasty for duodenal ulcer (TV&P); 8 patients following Billroth II gastrectomy for duodenal ulcer (BII) and 4 patients following a total gastrectomy (TG). After an overnight fast an indwelling catheter was inserted into an antecubital vein and kept patent with heparinised saline and serum obtained at 15 minute intervals for 30 minutes before and 2 hours after a standard protein rich meal. PP was measured by radioimmunoassay in duplicate. Basal and peak (15 or 30 minute) PP responses following food were DU 47±3±8·1 pmol/l and 151±21±4 pmol/l; TV&P 25±7±4±5 pmol/l and 42±5±6±7 pmol/l; BII 15±8±2±2 pmol/l and 41±6±8±9 pmol/l; TG 24±4±1±5 pmol/l and 115±26±1±5 pmol/l. Each of the groups had a significant rise in PP level from basal to peak (P<0.01) but this rise is significantly inhibited following either vagotomy or gastrectomy without vagotomy. Thus vagal section or partial gastric resection without vagotomy leads to significant diminution in the primary phase of food stimulated PP release. This suggests vagal denervation, interruption of a neural arc or removal of a source of gastric hormones may be responsible factors. The marked primary release of food stimulated PP following a total gastrectomy suggests a powerful duodeno-PP axis. Such an axis is probably responsible for the significant secondary peak of food stimulated PP release seen more than one hour after food in patients with intact vagal and normal gastric integrity.