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Immunochemiluminometric assays (ICMA) specific for growth hormone releasing hormone 1-44 NH_2 and 1-40 OH

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Summary

We describe specific two-site immunochemiluminometric assays able to directly measure human growth hormone-releasing hormone 1-44 NH_2 and 1-40 OH concentrations in unextracted plasma. A common *N*-terminal antibody was purified from polyclonal rabbit antisera to growth hormone-releasing hormone 1-44 NH_2 on a growth hormone-releasing hormone 1-29 NH_2 linked affinity column and labelled with chemiluminescent acridinium ester. C-terminal specific monoclonal antibodies to growth hormone-releasing hormone 1-44 NH_2 and 1-40 OH were raised in Balb/C mice and used as solid phase antibodies. Assay of fasting specimens from normal individuals gave medians (and ranges) of 23 pg/ml (2–200) and 30 pg/ml (3–134) for growth hormone-releasing hormone 1-44 NH_2 and 1-40 OH, respectively. Samples from a series of acromegalics showed that most have values in the normal range though median values were higher, 56 pg/ml for growth hormone-releasing hormone 1-44 NH_2 ($P < 0.001$) and 52 pg/ml for 1-40 OH ($P < 0.001$). Using these assays it will be possible for the first time to directly study the physiology and pathophysiology of these two peptides.

Introduction

Secretion of growth hormone (GH) from the pituitary is controlled by two neuropeptides, growth hormone releasing hormone (GHRH) which is stimulatory

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and somatostatin which is inhibitory. These peptides are in turn influenced by a variety of neurotransmitter pathways and peripheral feedback signals. It is the interplay of all these factors which determines GH secretion at any one time [1]. Somatostatin was characterised in 1973 [2] and has been extensively studied. All evidence indicates that circulating somatostatin fails to fulfil a classical endocrine role and that its widespread effects are mediated in a paracrine manner. Certainly there is no evidence that peripheral somatostatin concentrations reflect release of the hormone from the hypothalamus [3]. GHRH was eventually isolated from pancreatic tumours causing acromegaly by two groups working independently [4,5]. Total GHRH immunoreactivity is contributed to by the mature peptide GHRH 1-44 NH_2 and the C terminally shortened derivative GHRH 1-40 OH, both of which are present in the human hypothalamus [6].

Because of the low concentrations of GHRH in peripheral plasma, few reliable radioimmunoassays have been described. Most of these recognise GHRH 1-44 NH_2 and GHRH 1-40 OH on an equimolar basis [7–11] though one utilised an antibody specific for GHRH 1-44 NH_2 [12]. Using a chemiluminescent acridinium ester as a tracer and long incubation times we have previously reported a sensitive chemiluminescence immunoassay (CIA) for GHRH in unextracted plasma samples [13]. In common with previously described assays, however, this assay gave no information about the relative contribution of GHRH 1-44 NH_2 and GHRH 1-40 OH to total GHRH immunoreactivity. Since circulating concentrations are too low to permit HPLC characterisation of individual plasma samples little is known about the relative physiology of GHRH 1-44 NH_2 and 1-40 OH.

In order to address this problem, we set out to develop two-site immunochemiluminometric assays (ICMAs) specific for GHRH 1-44 NH_2 and 1-40 OH. A common polyclonal N-terminal antibody was prepared by affinity purification and labelled with chemiluminescent acridinium ester [14]. Monoclonal antibodies specific to the C-termini of GHRH 1-44 NH_2 and 1-40 OH were raised, coupled to diazocellulose and used to separate the immune complex.

Materials and Methods

Laboratory grade chemicals were purchased from the Sigma Chemical Co (Poole, Dorset, UK) and GHRH peptides from Peninsula Laboratories (St Helens, Merseyside, UK) with the exception of GHRH 3-44 NH_2 which was a gift from Sanofi UK (Wythenshawe, Manchester, UK).

Preparation of N-terminal labelled antibody

Polyclonal antisera to GHRH 1-44 NH_2 were raised in New Zealand White rabbits, using GHRH 1-44 NH_2 conjugated to keyhole limpet haemocyanin (KLH). Each received 20 μg GHRH conjugate in 600 μl sterile phosphate buffered saline (PBS; prepared as follows, 20 ml of a solution containing 315 ml 0.5 M Na_2HPO_4 plus 47 ml 0.5 M NaH_2PO_4 made up to 1 litre with 150 mM NaCl, pH 7.4) emulsified with 1.4 ml complete Freunds Adjuvant (CFA) as 20 intradermal injections. The rabbits were boosted with 10 μg conjugate in incomplete Freunds Adju-

vant (IFA) after 8 weeks. Antibody affinity was assessed by binding of dilutions of antisera to ^{125}I GHRH 1-44 NH_2 prepared by the Chloramine T method [15]. Rabbits were continually boosted until no further rise in affinity was noted.

The most promising antiserum was purified using a GHRH 1-29 NH_2 linked Sepharose 4B immunoaffinity matrix prepared as described previously [16]. An immunoglobulin precipitate of 20 ml polyclonal anti-GHRH serum was prepared by adding sodium sulphate to a final concentration of 1.27 M. After overnight dialysis at room temperature against PBS, the precipitate was passed twice over the column. PBS (50 ml) was passed over the column followed by 10 ml PBS containing 0.25 M NaCl to remove non-specifically bound protein. Bound antibodies were eluted with pH 2 HCl, collecting 1-ml fractions into tubes containing 100 μl 0.5 M Na_2CO_3 to neutralize pH. The column was washed and stored in PBS with 3 mmol l^{-1} Na azide. Fractions 3 and 4 contained 0.22 and 0.54 mg/ml protein, respectively as assessed by OD_{280}. These were pooled, divided into 30-μg portions and stored at -70°C.

Portions of N-terminal specific antibody were labelled with chemiluminescent acridinium ester [16]. The labelled antibody was separated from unreacted label on a 10 cm × 1 cm Sephadex G25 column from which 1-ml fractions were collected during elution with luminescent assay buffer (see below). Non-specific binding was reduced by adsorption with non-immune mouse gamma globulin coupled to diazocellulose for 30 min at room temperature. After centrifugation at 2,000 × *g* for 20 min at room temperature the supernatant was aliquoted and stored at -70°C.

Production of specific C-terminal antibodies

Monoclonal antibodies to GHRH 1-44 NH_2 and 1-40 OH were produced using similar methodology with the exception that the original immunogens and the screening tests were specific for each peptide. Balb/C female mice were immunised by subcutaneous injection of a 100-μl volume containing 10 μg peptide conjugated to KLH in 25 μl PBS emulsified with 75 μl CFA. After 21 days, the mice were boosted with 10 μg peptide conjugate emulsified in IFA. Positive mice were identified by specific ELISA. Each well of a 96-well microtitre plate (Life Technologies Ltd, Paisley, Scotland) was coated overnight with 100 μl of a 1 μg/ml solution of peptide dissolved in coating buffer (15 mmol l^{-1} Na_2CO_3, 35 mmol l^{-1} $NaHCO_3$, pH 9.6). Non-specific binding was blocked using 100 μl 5 g l^{-1} bovine serum albumin (BSA) in PBS containing Tween 20 (0.5 ml l^{-1}) for 1 h. After washing with PBS/Tween, blood obtained by retro-orbital bleeding was added to the well for a 2-h incubation. After washing again with PBS/Tween, 100 μl 1:1,000 goat anti-mouse IgG conjugated to alkaline phosphatase (Bio Rad Laboratories, Hemel Hempstead, Herts, UK) in PBS was incubated for a further hour. After a final wash, the reaction was developed by the addition of 38 mmol l^{-1} *p*-nitrophenyl phosphate in 10 mmol l^{-1} diethanolamine containing 5 mmol l^{-1} $MgCl_2$ (pH 9.5). Plates were read on a Titertek Multiscan (ICN Flow, High Wycombe, Bucks, UK). Positive mice selected for fusion received a final intraperitoneal boost of 20 μg peptide in 0.5-ml sterile PBS. After boosting, splenocytes were hybridised with SP2-o-Ag14 myeloma cells [17] using polyethylene glycol as a fusogen [18]. A ratio of 5:1 spleen and myeloma

was used. Cells were maintained in RPMI 1640 medium + 20% foetal calf solution (Life Technologies Ltd) and hybridoma cells were selected by addition of Hypoxanthine 1×10^{-4} M, Aminopterin 4×10^{-5} M and Thymidine 1.6×10^{-5} M to the medium. Medium from the wells was tested in the ELISA and cells from positive wells cloned by the method of limiting dilutions.

C-Terminal specificity of the antibodies produced was assessed by incubation of medium with plates coated with GHRH 1-44 NH_2, 1-40 OH and 1-29 NH_2. Two cell lines gave positive results only in the GHRH 1-44 NH_2 plate and five were positive only in the GHRH 1-40 OH plate. 2×10^6 cells from each of these lines were injected intraperitoneally into mice which had received 0.5 ml of the tumour promoting agent tetramethylpentadecane (Sigma) intraperitoneally 10 days previously. Dilutions of the resulting ascitic fluid re-checked in the ELISA, gave results identical to those obtained in the screening tests. The most robust and productive lines were chosen, 1C5 A4 D1 for GHRH 1-44 NH_2 and 6B7 A2 C11 A12 for GHRH 1-40 OH. A large quantity of each of these antibodies was produced by intraperitoneal propagation. Immunoglobulin fractions were coupled to diazocellulose [19] and stored at 4°C.

Assay protocol

GHRH 1-44 NH_2 and 1-40 OH standards were diluted in foetal calf serum from 10 ng to 10 pg/ml. Standard or unextracted sample (100 µl) was incubated overnight with 100 µl labelled anti GHRH 1-29 NH_2 diluted in assay buffer at 4°C. (10.5 mmol l^{-1} Na_2HPO_4; 30 mmol l^{-1} $NaH_2PO_4 \cdot 2H_2O$; 74 mmol l^{-1} NaCl; human serum albumin 0.5 g l^{-1}; BSA 4.5 g l^{-1}; rabbit gamma globulin 0.05 g l^{-1}; 1.5 mmol l^{-1} Na Azide). 50 µl of the C-terminal solid phase antibodies were added at 1:10 dilution for 60 min at room temperature, after which two wash steps were performed with 1 ml wash buffer (11.5 mmol l^{-1} $NaH_2PO_4 \cdot 2H_2O$; 150 mmol l^{-1} NaCl; BSA 0.5 g l^{-1}; 1.5 mmol l^{-1} Na Azide; Triton X100 10 ml l^{-1}), centrifuging at 3,000 × *g* for 20 min at 4°C in between. After decanting, tubes were blotted dry and luminescence counted in a Magic Lite Analyser (Ciba Corning, Halstead, Essex, UK). The number of counts bound is directly proportional to the amount of analyte present and the zero analyte dose corresponds to the NSB of the assay.

Assay validation

Assay performance was assessed by calculation of precision profiles [20]. Cross-reactivity experiments were performed using the two assays on several separate occasions. In addition to a range of GHRH derivatives, a variety of hypothalamic and gastro-entero-pancreatic peptides were assessed for their ability to produce binding in the assays. Recovery experiments were performed in which known quantities of GHRH were added to portions of plasma samples which were then both assayed.

Extraction and HPLC of hypothalamic GHRH

Postmortem hypothalamic specimens from two females (80 and 73 years) were obtained. The median eminence and pituitary stalk was dissected away, placed in 5 ml

1 M acetic acid and homogenised for 5 min then heated at 100°C in a water bath. Protein content was measured [21]. One-millilitre portions were centrifuged at 13,600 × *g* for 5 min at room temperature, the supernatant extracted using a Sep Pak C18 cartridge eluting with 80% acetonitrile in 0.013 M trifluoric acid (TFA) and evaporated to dryness. One portion was reconstituted in assay buffer and measured in the two ICMAs and in the GHRH CIA [13]. A further portion was reconstituted in 0.013 M TFA and subjected to HPLC. This was performed on a reverse-phase C4 column (Dynamax 300A 4.6 × 250 mm). An isocratic concentration of 29% acetonitrile gave optimum separation of GHRH peptides at a flow rate of 1 ml/min. Forty 1-ml fractions were collected, dried, reconstituted in assay buffer and measured in the assays. Since previous studies had suggested partial oxidation of GHRH at the 27 (Met) position by extraction prior to HPLC [16], the retention time of Met(O)27 GHRH peptides, produced by incubation with $NaIO_4$, 10^{-3} M in 8.7 M acetic acid for 30 min at room temperature, were also measured.

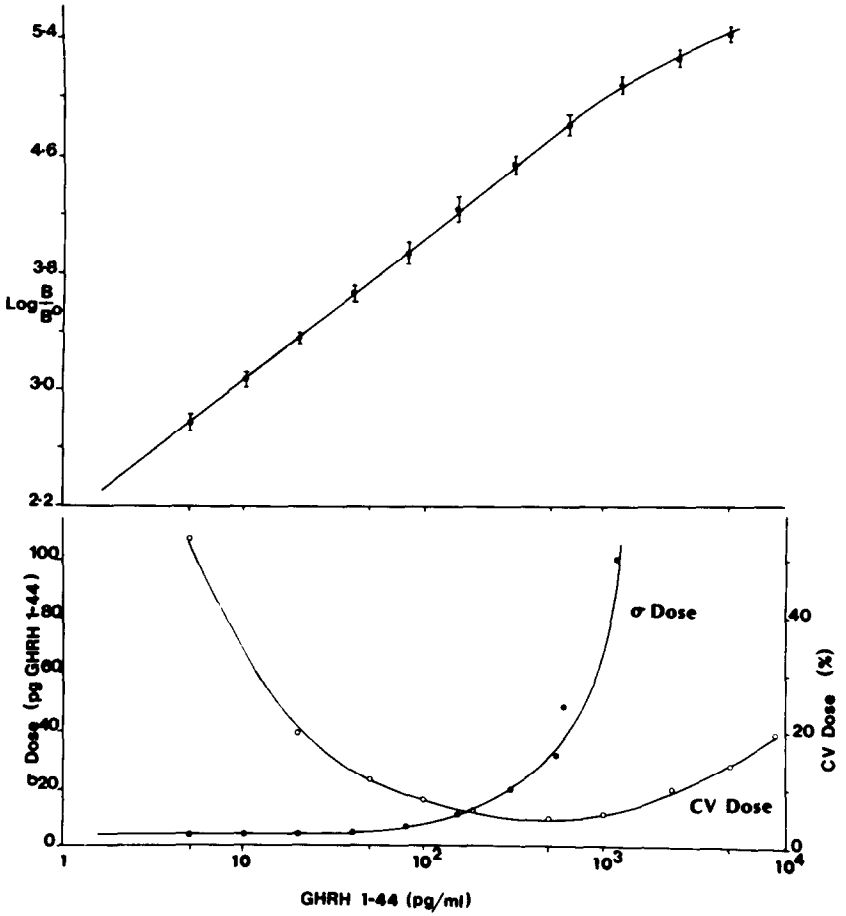

Fig. 1. Mean standard curve and precision profiles for the GHRH 1-44 ICMA.

Samples from normal individuals and acromegalics

With informed consent, samples from 85 normal individuals (69 male, 16 female, aged 19–71 years) and 25 acromegalics (10 male, 15 female, aged 26–72 years) were obtained in the fasted state between 0900 h and 1000 h. The samples were assayed in the specific GHRH 1-44 NH_2 and 1-40 OH assays, with some of the samples also being measured in the total GHRH CIA. Statistical evaluation was by the Mann-Whitney test.

Results

Assay performance

Mean standard curves for the two assays are shown in Figs. 1 and 2 along with their precision profiles, each calculated from 6 consecutive standard curves. The ab-

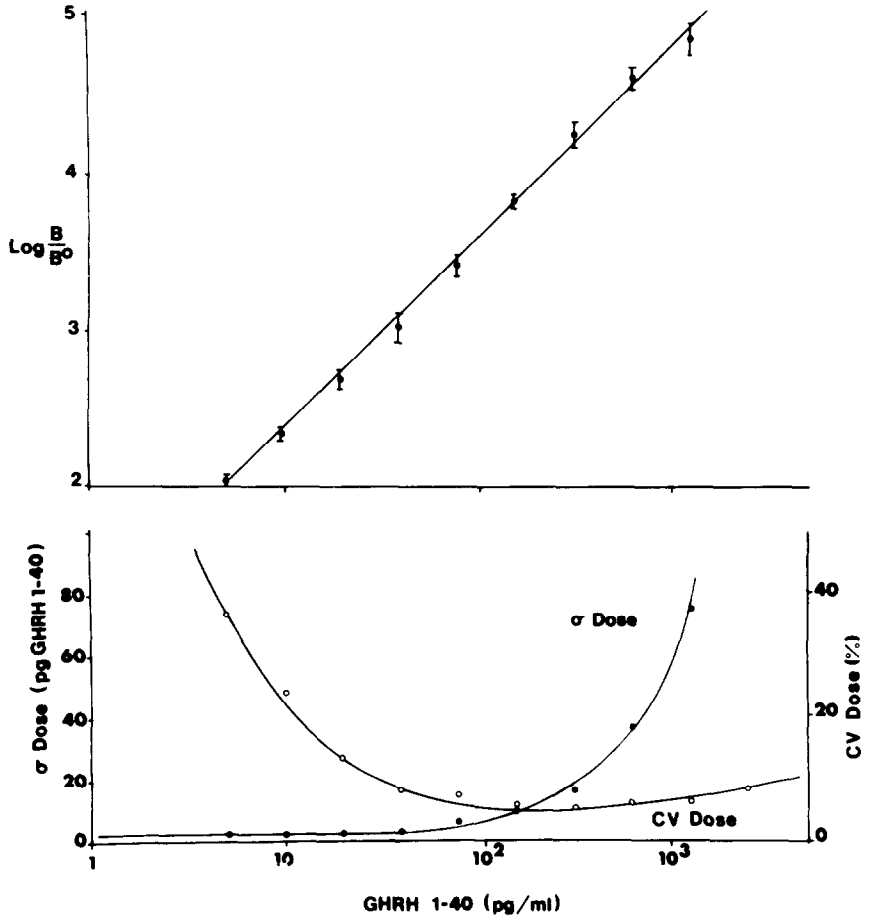

Fig. 2. Mean standard curve and precision profiles for the GHRH 1-40 ICMA.

solute sensitivity of the 1-44 and 1-40 ICMAs, corresponding to the intersection of the assymptote of the σ precision profile (the standard deviation of the dose) with the ordinate axis, were 2.6 and 2.2 pg/ml respectively, with working ranges (coefficient of variation $< 15\%$) of 29.5–4850 and 18–4500 pg/ml, respectively. Recovery experiments using 40–400 pg/ml added peptide gave satisfactory values, ranging from 91 to 104% recovery.

Cross reactivity

The results of cross reactivity experiments are presented in Table I. In addition to GHRH derivatives, a variety of hypothalamic and gastro-entero-pancreatic peptides were assessed for their ability to react in the two assays at a wide range of doses. It can be seen that both assays are specific for their respective C-termini, though GHRH 3-44 NH_2 is fully cross-reactive in the 1-44 ICMA. There was no significant binding of any other peptide in either assay.

Extraction and HPLC experiments

GHRH 1-44 NH_2 and 1-40 OH concentrations (mean of 2) in the extracts of human median eminence and pituitary stalk were 35.2 and 23.8 ng/mg protein, respectively which represents an almost equimolar ratio (1-44:1-40 = 0.74).

HPLC separation of GHRH peptides using a 250-mm C4 column and isocratic elution with 29% acetonitrile in 0.013 M TFA gave satisfactory resolution. GHRH 1-44 NH_2, 1-40 OH and 1-37 OH eluted around 18, 22 and 28 min, respectively. For GHRH 3-44 NH_2, however, removal of the first two residues, tyrosine and alanine reduced the retention time to around 12 min, not surprising since tyrosine is

TABLE I

Cross-reactivities of different peptides in the GHRH 1-44 and 1-40 ICMAs (GHRH 3-40) could not be obtained

Peptide	Cross-reactivity (%)	
	1-44 ICMA	1-40 ICMA
GHRH 1-44 NH_2	100	<0.1
3-44 NH_2	100	<0.1
1-40 OH	<0.1	100
3-40 OH	?	?
1-37 OH	<0.1	<0.1
1-29 NH_2	<0.01	<0.01
Glucagon, Secretin PHI, VIP, Somatostatin 14 and 28, LHRH, TRH, CRH,	<0.01	<0.01

hydrophobic. Oxidised peptides all eluted earlier than their parent peptide, 5, 9 and 13 min for 27 Met(O)GHRH 3-44 NH_2, 1-44 NH_2 and 1-40 OH, respectively.

HPLC and assay of the hypothalamic extract demonstrated that most of the immunoreactivity resided in the GHRH 1-44 NH_2 and 1-40 OH peaks (Fig. 3). These were specifically recognised only by the appropriate ICMA, though both were recognised by the CIA. Despite the delay between death and post-mortem (>24 h), there was only a small peak representing GHRH 3-44 NH_2. It is possible that the small peak at 17 min in the 1-40 ICMA is GHRH 3-40 OH. A significant quantity

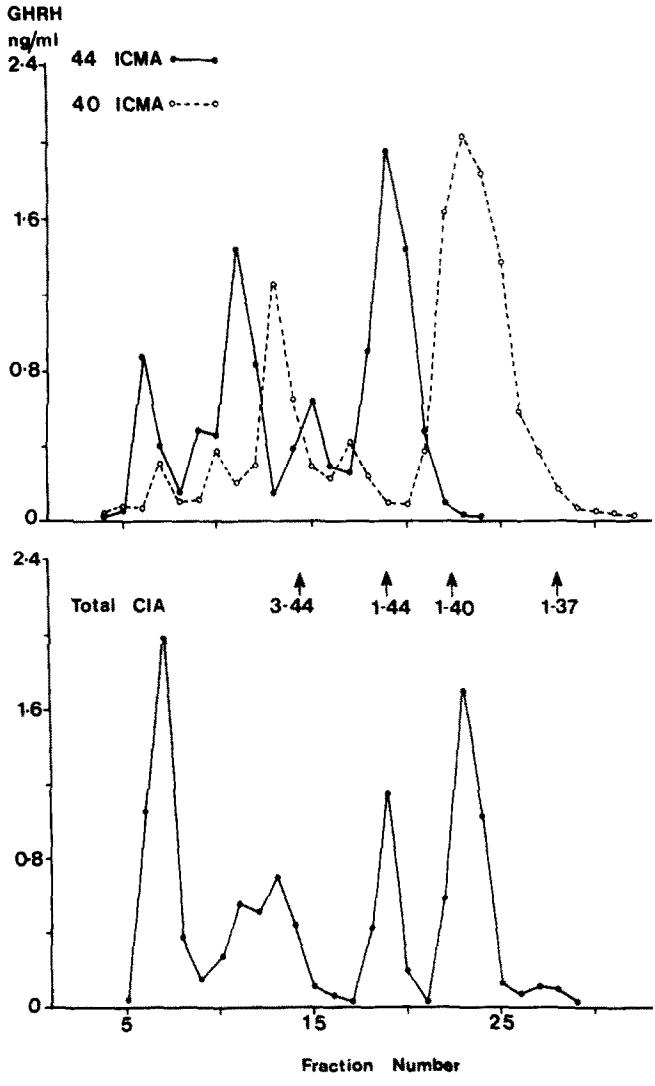

Fig. 3. HPLC of hypothalamic extract. Measurement in the GHRH 1-44 (●) and 1-40 (○) ICMAs (upper panel) and the GHRH CIA (lower panel).

of material elutes early as various peaks in the oxidised region and is present in all three assays. There was only a small amount of immunoreactivity in the region where GHRH 1-37 OH was expected to elute, seen in the CIA and this could simply represent carry over from the 1-40 peak.

GHRH concentrations in normal subjects and acromegalics

In the total GHRH CIA, samples from 14 normal subjects (10 male, 4 female, aged 19–40) and 11 acromegalics (8 male, 3 female, aged 33–71) were measured. In the GHRH 1-44 and 1-40 ICMAs samples from 85 normal subjects (69 male, 16 female, aged 19–71) and 25 acromegalics (10 male, 15 female, aged 26–72) were measured. The results are shown in Fig. 4. The median GHRH concentration in the total CIA in the 14 normal subjects was 56 pg/ml (range 6–290) and in the 11 acromegalics was 94 pg/ml (range 18–260), P = NS by the Mann-Whitney test. In the 1-44 ICMA the median concentration in normal subjects was 23 pg/ml, (range 2–200) and in the 25 acromegalics was 56 pg/ml (range 15–113), $P < 0.001$. In the GHRH 1-40 ICMA the median concentration in normal subjects was 30 pg/ml, (range 3–134) and in the 25 acromegalics was 52 pg/ml (range 4–98), $P < 0.001$. There was no evidence of age or sex related differences in GHRH concentrations.

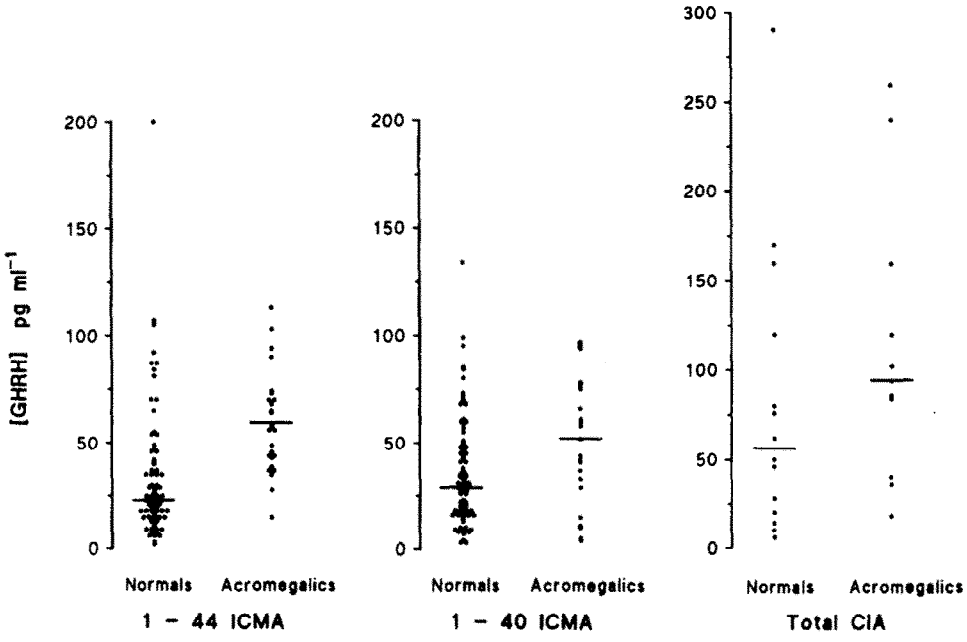

Fig. 4. Fasting GHRH immunoreactivity in the GHRH CIA and the 1-44 and 1-40 ICMAs. Comparison between normal and acromegalic subjects.

Discussion

Previously reported radioimmunoassays have been unable to specifically identify different GHRH peptides and have required extraction of large sample volumes to achieve adequate sensitivity. It has been shown that GHRH concentrations rise after food in normal subjects [11,22] and patients with GH deficiency due to hypothalamic pathology [23] suggesting a gastrointestinal source. In contrast, hypothalamic GHRH may be responsible for the observed rise preceding the GH response to L-dopa [9] which is absent in patients with hypothalamic GH deficiency [10].

Using these assays, HPLC separation of pooled serum is able to give information about the contribution of different GHRH peptides to total immunoreactivity. However, the low concentrations present in single samples precludes direct study of the relative physiology of GHRH 1-44 NH_2 and GHRH 1-40 OH by this method. It was for this reason that we set out to develop more sensitive and specific assays for GHRH. Immunometric assays are inherently more precise, sensitive and faster than radioimmunoassays [24]. Two-site immunometric assays combine these benefits with increased specificity conferred by using antibodies to two epitopes on the same molecule. Recently chemiluminescent molecules have been employed as labels in two-site immunometric assays for alpha-fetoprotein, thyrotrophin and parathyroid hormone [14,25,26]. These labels have advantages of stability, safety and sensitivity over the more commonly used radiolabels. Using affinity purified, chemiluminescence-labelled polyclonal N-terminal antibodies and solid phase, C-terminal specific monoclonal antibodies, we now have reliable assays, capable of measuring GHRH 1-44 NH_2 and GHRH 1-40 OH in unextracted plasma samples. The tracer antibody must be directed at an epitope between residues 3-29 since GHRH 3-44 NH_2 is fully cross-reactive in the GHRH 1-44 NH_2 assay. The specificity of these assays is therefore for the C- rather than the N-terminal.

The GHRH gene is present on chromosome 20 and is translated into two polypeptide precursors, pre-pro-GHRH 107 and 108 the former lacking a serine residue at position 103 [27,28]. There is only one copy of GHRH 1-44, contained between residues 32 and 75 flanked by processing sites consisting of basic arginine residues. Glycine at position 76 mediates amidation. Arginine residues at positions 41 and 38 could result in processing to GHRH 1-40 OH or 1-37 OH of either pre-pro-GHRH or GHRH 1-44 NH_2. Like Bohlen et al. [6] we have demonstrated GHRH 1-44 NH_2 and 1-40 OH, but failed to find significant quantities of GHRH 1-37 OH in the hypothalamus, but cannot comment on the actual pathway to synthesis of GHRH 1-40 OH.

It is not known why GHRH 1-44 NH_2 and GHRH 1-40 OH co-exist in humans. In all other species investigated thus far, only one GHRH peptide has been identified [29]. In addition to the hypothalamus, GHRH immunoreactivity has also been demonstrated in the pancreas and mucosa of the gastrointestinal tract by immunocytochemistry and extraction procedures [30]. There are isolated reports of effects of GHRH on feeding behaviour and exocrine pancreatic secretion in animals [31,32]. To date, however, the only clearly defined action for GHRH is stimulation of GH synthesis and secretion from the pituitary and in this regard, GHRH 1-44

NH_2 and GHRH 1-40 OH are equipotent. Nevertheless it remains possible that in addition to the neurohypophysiotropic actions, there are neurotransmitter, neuromodulatory and gastrointestinal actions, in which GHRH 1-44 NH_2 and GHRH 1-40 OH may differ.

It is known that GHRH 1-44 NH_2 is rapidly degraded in plasma by N-terminal peptidolysis to GHRH 3-44 NH_2 which is biologically inactive [33]. We have evidence that this process is very slow in neural tissue since only small amounts of GHRH 3-44 NH_2 were present in hypothalamic extracts >24 h post-mortem. Whether GHRH 1-40 OH is similarly degraded is not known, but may now be studied using the GHRH 1-40 OH assay.

It is clear that acromegalics have GHRH 1-44 NH_2 and 1-40 OH concentrations in the normal range though mean values are slightly higher. Summation of immunoreactivity present in the two assays equates with total GHRH immunoreactivity suggesting that the contribution from other GHRH peptides is insignificant.

In conclusion, rapid, reliable, specific and sensitive assays have been developed for GHRH 1-44 NH_2 and GHRH 1-40 OH. Using these assays it will be possible to study the relative physiology of these two peptides, their changes during GH stimulation tests and in conditions of pathological GH secretion such as acromegaly, diabetes mellitus and carcinoid syndrome.

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