Application of a Sequence-Specific Radioimmunoassay for the Carboxyl-Terminal Region of Corticotropin

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The carboxyl terminal region of corticotropin (ACTH), ([Ala34-Phe-Pro-Glu-Leu-Phe50]), a region of the hormone conserved during evolution, served as an antigen for the production of a sequence-specific antiserum. In a radioimmunoassay, peptides that extend toward the amino terminal from Ala34, such as [Tyr, Gly]-ACTH34-39, ACTH18-39, and ACTH, had greater affinity for the antibody, which suggests that the antiserum recognizes the peptide bond preceding the alanyl residue. The assay readily detects 30 to 50 pmol of ACTH per liter with an incubation of only 3 h, and the antiserum cross reacts with larger molecular mass forms of the hormone. The amount of immunoreactive ACTH extracted by adsorption onto silicic acid from rat and human plasma was only 0.36 to 0.79 of that detected by a mid-region ACTH assay, which suggests proteolytic degradation at the carboxyl terminus of ACTH.

Additional Keyphrases: corticotropin in plasma and pituitary • peptide hormones

Radioimmunoassays represent a diagnostic tool in the quantitation of polypeptide hormones isolated from biological tissues, including blood (1-3). Although the antisera used in these assays are commonly obtained by the use of the intact hormone as the immunogen, the technique may be used to produce antiserum with different or multiple specificities. In the case of ACTH,4 the antibodies could cross-react with α-MSH and CLIP as well as other regions of the molecule that persist or vary evolutionarily. Thus, the heterogeneity of antibody specificities remains a factor in the reproducible quantitation of hormones by different laboratories (3). One convenient method to alleviate this problem involves the use of small peptides coupled to larger immunogenic proteins (4, 5); in this way, recognition of the hormone is potentially limited to one specific sequence. To obtain antibodies specific for the carboxyl terminal region of ACTH, we used the amino acid sequence of ACTH34-39, an evolutionarily conserved region of the hormone (6), in preparation of antiserum. We present data on the applicability of this antiserum for detecting ACTH in plasma of rats and humans for detecting larger forms of this hormone.

Materials and Methods

Peptides and Chemicals

The synthetic ACTH sequence 1-24 (Cortrosyn) was donated by Organon, Inc., West Orange, NJ 07052; synthetic human ACTH was a gift of Ciba-Geigy Ltd., Basel, Switzerland; ACTH1-10, ACTH11-1, ACTH34-39, and [Tyr, Gly]-ACTH34-39 were from Bachem, Inc., Torrance, CA 90505. J. E. Rivier (The Salk Institute for Biological Sciences, San Diego, CA 92138) synthesized and supplied us with human (h) β-MSH, bovine (b) β-MSH, α-MSH, and hCLIP; C. H. Li (University of California, San Francisco, CA) generously provided hβ-LPH. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, polyethylene glycol 6000, gamma-globulins, and BSA were from Sigma Chemical Co., St. Louis, MO 63178; Freund's complete adjuvant from Calbiochem, San Diego, CA 92112; carrier-free Na1251 from New England Nuclear, Boston, MA 02118 or Amersham, Arlington Heights, IL 60005; and Sephadex LH-20 and G-75 from Pharmacia, Upsala, Sweden. Polypropylene tubes, 12 x 75 mm, were used because ACTH avidly binds to both polystyrene and glass.

Biological Samples

Blood from rats or humans was collected into polypropylene tubes surrounded by crushed ice and containing EDTA to give a concentration of 1 mg/mL (7). Plasma was obtained by centrifugation (1200 x g, 10 min, 4°C). We added 60 mg of silicic acid (Bio-Sil A, 100–200 mesh; Bio-Rad Laboratories, Richmond, CA 94804) per milliliter of plasma, to adsorb ACTH (8), the mixture being allowed to stand for 30 min at 4°C, then washed it with 5 mL of assay diluent and eluted with 5 mL of acetone/water/acetic acid (40/59/1 by vol). The acetone was evaporated in a stream of nitrogen and then either the samples were lyophilized or aliquots were diluted for immediate use in the assay.

We extracted 1-g lots of powdered, lyophilized bovine pituitary glands by homogenization with a Polytron (Brinkmann Instruments, Westbury, NY 11590), using boiling 0.1 mol/L acetic acid containing 5 g of 2-mercaptoethanol per liter, then boiling for an additional 10 min according to Lee and Lee (9).

Procedures

Production of antiserum. The peptide ACTH34-39 was coupled to BSA by the carbodiimide method (10). The coupled peptide was dialyzed for 24 h, first against 4 L of 0.15 mol/L NaCl containing 28 mmol of 2-mercaptoethanol per liter (four changes), then against distilled water (two changes), as described previously (11). Thin-layer chromatography indicated that all the peptide was coupled to BSA. An equal volume of antigen was emulsified with adjuvant, and a total of 100 µg of coupled peptide was injected subcutaneously over 10 sites on the back of New Zealand White rabbits at four- to seven-week intervals.

Idination. We iodinated the peptide [Tyr, Gly]-ACTH34-39 by using Chloramine T as the oxidant (12) at a molar ratio of
peptide/iodine/Chloramine T of 1/2/50 and purified it on 0.7 x 4 cm Sephadex LH-20 columns in n-butanol/acetic acid/water (10/2/1 by vol) (13). About 80% of the radiolabeled peptide could be precipitated with an excess of antiserum. ACTH1-24 was iodinated under similar conditions and purified by adsorption on silicic acid (14).

Radioimmunoassay. Assays were done in duplicate or triplicate samples in a total reaction volume of 100 μL. The final concentration of constituents of the assay diluent were, per liter, 50 mmol of potassium phosphate (pH 7.6), 25 mmol of NaCl, 30 mmol of 2-mercaptoethanol, 400 mg of aprotinin (an enzyme inhibitor), 2 g of BSA, and 1 g of gamma-globulins. To the 100-μL mix we added 10 000 cpm of [125I]-labeled [Tyr,Gly]-ACTH34-39 and the carboxy-terminal region antiserum (B-XII-161-3, 1000-fold final dilution). Standard peptides (ACTH and [Tyr,Gly]-ACTH34-39), plasma, or extracts were diluted with this assay diluent. The tubes were incubated either at 22 °C for 3 h or overnight (16 to 18 h) at 4 °C, and the reaction was terminated by adding 0.1 mL of out-dated human plasma followed by 1 mL of a 156 g/L solution of polyethylene glycol in phosphate buffer (50 mmol/L, pH 7.6). We thoroughly mixed and centrifuged (4000 x g, 10 min, 4 °C) the samples, aspirated the supernate, and counted the radioactivity in the pellet.

In assaying for the mid-region of ACTH (specific for residues 19 through 24) we used antisera A-7 (40 000-fold final dilution) under similar conditions (Khan and DiAugustine, unpublished data).

Column chromatography. Sephadex G-75 columns, 1.5 x 98 cm, were equilibrated in and eluted with 0.1 mol/L acetic acid containing 1 mmol of EDTA per liter. A 1.0-mL solution of extract clarified by centrifugation (9000 x g, 5 min, 4 °C) was applied to the column, and 0.96-mL fractions were collected at a flow rate of 30 mL/h. These fractions were lyophilized and redissolved in diluent before assay.

Results

Specificity of the Radioimmunoassay

The absence of a tyrosyl residue in ACTH34-39 prompted us to use the analog [Tyr,Gly]-ACTH34-39 as the radioligand. In contrast to iodinated ACTH, this peptide did not appreciably adsorb onto glass or plastic surfaces; mean nonspecific radioactivity in 24 samples without antiserum or antiserum in the presence of 100 ng of peptide was 2.67% (SD 0.48%) of the total radioactivity. With peptide more than a year old, this background increased to 5.53% (SD 0.84%) (n = 11). As shown in Figure 1, a series of parallel displacement curves was obtained with ACTH, CLIP, [Tyr,Gly]-ACTH34-39, and ACTH18-39, with 50% displacement for each peptide at 80, 80, 160, and 600 pmol/L, respectively. No other ACTH-related peptides lacking the carboxyl-terminal hexapeptide (including α-MSH, β-MSH, and β-LPH at concentrations up to 10 μmol/L) competed with the antibody in this assay. The least amount of ACTH detected with the antibody was 1 to 3 pg per tube. The within- and between-assay coefficients of variation (n = 7) for ACTH were 11 and 16% at 23 pg, and 8 and 12% at 41 pg, respectively.

A proteolytic inhibitor, such as aprotinin (Trasylol), is needed in the diluent because of the relatively low titer of the antiserum. However, if other inhibitors (e.g., bacitracin, sodium azide, and thimerosal) are simultaneously present in the diluent, the binding of peptide to antibody is greatly reduced.

Within three to six months we obtained large quantities of antiserum for use at 1000-fold final dilution. Continued reimmunization of the seven rabbits during six to 10 months failed to increase the titer of the antiserum, but rather led to an overall decrease in titer and sensitivity (i.e., the 50% displacement value) in all antiseras.

Quantitation of Plasma ACTH

Table 1 summarizes the results of the immunoassay of ACTH in rat and human plasma with use of antiserum to the carboxy-terminal region and antiserum to the mid-region of ACTH. The difference in the amount of ACTH found by the two assays cannot be attributed to loss of ACTH during extraction from plasma; the recovery of hACTH extraneously added to outdated human plasma or to plasma from hypophysectomized rats was the same by both radioimmunoassays. Even in the case of ether- or methoxyflurane-exposed rats or adrenalectomized animals, in which plasma ACTH concentrations are significantly greater than in the controls, the carboxy-terminal region assay detected only 0.36 to 0.79 as much as the mid-region assay. This also was true for assay results for four plasma samples from the authors, taken between 1500 and 1600 hours: the ratio between the two ACTH immunoassays ranged from 0.68 to 0.72. A 36-year-old woman (M.C.) who had undergone bilateral adrenalectomy for treatment of Cushings disease and who was on glucocorticoid replacement therapy (30 mg of hydrocortisone and 0.06 mg of 9α-fluorocortisol per day) had above-normal ACTH concentrations by both assays, but in this case the ratio between carboxy-terminal and mid-region assays was 1.3. A male patient (K.M.) with small-cell carcinoma of the lung and Cushings syndrome exhibited a fivefold increase in the mid-region assay, while the value as determined by the carboxy-terminal region assay remained within the normal range (20–50 ng/L). These findings agree with a previous report of similar endocrine disorders in which concentrations of immunoreactive ACTH in individual plasma samples varied considerably with different region-specific assays (3).

Fractionation

The carboxy-terminal region assay cross reacts with ACTH from bovine pituitary (Figure 2). An immunoreactive peak representing material of relative molecular mass of 31 000 to 34 000, probably the pro-ACTH/β-LPH precursor (15), was readily detected in the column eluates. Furthermore, the pooled peak areas I (fractions 60–69), II (fractions 90–97), and III (fractions 111–120)—representing the pro-hormone, an intermediate fragment of the pro-hormone, and ACTH, respectively—all cross reacted in a manner parallel to the cross
reactivity of [Tyr,Gly]-ACTH$^{34-39}$ (Figure 3). The crude extract also exhibited parallel displacement.

**Discussion**

Accurate quantitation of the total concentration of a peptide hormone in biological fluids or tissues by use of a sequence-specific radioimmunoassay requires that the antibody cross react with the intact hormone, a subunit, a fragment containing that sequence, and the precursor form of the hormone. Our immunoassay meets all these criteria. Thus, it is evident that (a) only the specific sequence ACTH$^{34-39}$ cross reacts with the antiserum, and (b) peptides with sequences which extend from the amino-terminal residue Ala$^{34}$ of that peptide exhibit enhanced displacement of the label. Because the peptide is coupled to BSA through the free amino group of Ala$^{34}$, the antibody probably recognizes the peptide bond between Ala$^{34}$ and a carboxyl group in BSA. If the antibody recognized a peptide coupled through the carboxy terminus Phe$^{39}$, we would not observe the enhancement in displacing the radioligand by ACTH or [Tyr,Gly]-ACTH$^{34-39}$.

The range of values we found for immunoreactive ACTH in plasma from normal subjects is in accord with those from other clinical or laboratory studies (2, 3, 7, 14, 16). However, in plasma from patients with Cushing's syndrome (ectopic ACTH syndrome), Orth et al. (3) found that their “NH$_2$-terminal” ACTH assay (with a specificity similar to our mid-region assay) gave lower values for ACTH when compared with their assay of the “extreme” carboxyl terminal (of unknown specificity, but estimated to be somewhere in the region of residues 25 to 39). Thus, a comparison between the values quantitated for ACTH among different laboratories in which the precise specificity of the antisera is suspect must be interpreted with caution. This is particularly important in evaluating the degradation of ACTH in blood or other tissues because major proteolytic cleavage sites seem to be both the amino- and carboxyl-termini (17).

<table>
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<tr>
<th>Table 1. Quantitation of ACTH in Rat and Human Plasma</th>
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<td><strong>Plasma</strong></td>
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<td>M.C. (Cushing's disease)</td>
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* Analytical recovery of synthetic human ACTH (100 ng/L) added to out-dated human plasma or plasma from hypophysectomized rats ranged from 69 to 81% and 72 to 79%, respectively, by either immunoassay. Loss of ACTH reflects a variation in its adsorption and elution from the silicic acid and its adhesion even to plastic pipettes and tubes.

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**Fig. 2. Chromatogram of Sephadex G-75 of extracts of bovine pituitary gland extracts**

The 1.5 X 98 cm column was eluted with 0.1 mol/L acetic acid containing EDTA (1 mmol/L). The extract (1.0 mL) was applied and 0.96-mL fractions were collected at a flow rate of 30 mL/h. The arrows indicate relative molecular mass markers: 1, Blue Dextran (marks void volume); 2, ovalbumin (M, 45,000); 3, cytochrome c (M, 12,400); 4, bovine insulin (M, 6612); and 5, vitamin B$_{12}$ (M, 1355).

**Fig. 3. Cross reactivity of the extracts of bovine pituitary and regions from the Sephadex G-75 column fractionation (Fig. 2)**

Immunoreactive peptides: O, the original acetic acid extract; X, pooled area I (column fractions 63–89, representing the Pro-ACTH/β-LPH region); W, pooled area II (column fractions 90–97 are the intermediate A4 fragments of the pro-ACTH/β-LPH molecule); A, pooled area III (column fragments 111–120 are ACTH); and ● [Tyr,Gly]-ACTH$^{34-39}$ standard.

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Our sequence-specific radioimmunoassay offers both the investigator and clinician the knowledge that the material recognized in the assay is strictly limited to the 34–39 region of ACTH. Furthermore, the relatively short time required for this assay (3 h) makes practicable its use in routine clinical analyses of plasma samples for diagnosis of pituitary function or ectopic ACTH-producing tumors.

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References


