COMPARISON OF SECRETED AND EXTRACTED FORMS OF RAT PITUITARY PROLACTIN *

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Summary

Prolactin secreted by rat anterior pituitary explants into organ culture medium was purified by salt fractionation and gel filtration. A yield of 22 mg/g was obtained, which clearly represented de novo synthesis and secretion of the hormone. Comparative characterization studies were performed on the secreted prolactin and pituitary extracted rat prolactin obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases. The biological and immunological activity estimates of both forms were comparable, although the specific activities of the secreted prolactin were somewhat lower than those of the pituitary prolactin. The secreted and extracted forms of prolactin appeared to be identical in primary structure as evidenced by similar amino acid compositions and identical NH₂-terminal sequences. Circular dichroism spectra suggested that there may be differences in tertiary structure, since the positive tryptophan band at 292 nm, which was observed with extracted hormone, was absent in the secreted prolactin.

Introduction

In a number of studies that have attempted to measure rat growth hormone and prolactin in serum or organ culture medium, it was found that bioassay and radioimmunoassay activity estimates were markedly divergent [1–4]. However, when purified preparations of rat pituitary prolactin or homogenates of rat pituitary tissue are assayed by bioassay and radioimmunoassay for prolactin and growth hormone, good correspondence between the two assays has been obtained [5–8]. A possible explanation of this discrepancy is that the secreted

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or circulating forms of these hormones differ from the extracted forms. Ellis and Grindeland [1] have reported biological and chemical data with rat and human sera which support this hypothesis in the case of growth hormone. In order to definitively establish the existence of two different forms of a hormone, however, they must be isolated separately and characterized. Fractionation of rat serum would require prohibitively large quantities of blood, due to the very low levels of circulating hormone. An alternative approach to obtaining secreted hormones is provided by pituitary organ culture, at least in the case of prolactin, because large amounts of this hormone are secreted by the pituitary in vitro, in the absence of the hypothalamic inhibitory control. This approach has been employed previously [9,10] to obtain secreted rat prolactin for the development of a radioimmunoassay. To date, however, there has been no report on the chemical characterization of secreted rat prolactin. To this end we have examined prolactin isolated from rat pituitary organ culture medium and compared some of the properties of this material with those of the same hormone isolated from rat pituitaries. Although information on the properties of rat prolactin is available [11] for comparative purposes, in our studies concurrent analyses were carried out with a highly purified preparation of extracted pituitary prolactin obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases (N.I.A.M.D.D.).

Materials and Methods

Hormone preparation. Prolactin isolated from rat pituitaries (AFP-913-B) by Dr. Albert F. Parlow (University of California, Harbor General Hospital, Torrance, Calif.) was generously supplied by the Hormone Distribution Program of the N.I.A.M.D.D. The rat pituitary prolactin was initially extracted as described by Ellis et al. [11]. The crude prolactin fraction (Extract D) was further purified by (NH₄)₂SO₄ fractionation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-cellulose (Parlow, A.F., personal communication).

Prolactin isolated from organ culture medium was prepared as follows. Adenohypophyses from 197 adult Long-Evans rats of both sexes were cultured in Medium 199 for 1 and 3 days as previously described [12] with a change of medium after 24 h of incubation. At termination of the culture, the 24-h and 3-day medium samples were assayed by bioassay and radioimmunoassay. No difference in the bio/radioimmunoassay activity ratio was found. Therefore, both samples were pooled to give a volume of 400 ml, and this was dialyzed and lyophilized. After incubation, the wet weight of the cultured pituitary tissue was 900 mg.

The lyophilized medium was dissolved in 0.05 M NH₄OH and applied to a Sephadex G-25 column (2.4 X 44 cm) equilibrated with the same buffer. Chromatography in this system yielded a single protein peak (98 mg) and removed a significant amount of low molecular weight materials. After dialysis and lyophilization, the protein was dissolved in 22 ml water, the pH was adjusted to 3 and a small amount of insoluble material was discarded. Saturated NaCl solution was added to a final concentration of 6% (0.37 M) and the resulting precipitate was removed by centrifugation. The majority of the
Prolactin was found in the supernatant, which was dialyzed and lyophilized. The prolactin fraction was then applied to a Sephadex G-100 column (2.5 × 92 cm), equilibrated with 0.05 M NH₄HCO₃. The fraction eluting with a $V_e/V_o$ of 2.1 was used for characterization studies. The yield of the prolactin in this fraction was 19.6 mg, which represents 21.8 mg/g calculated on the basis of the wet weight of the cultured adenohypophyses.

Assay. Prolactin bioactivity was determined by the local pigeon cropsac assay [13], with NIH-P-S10, 26 (22–31) I.U./mg [14] employed as a standard. Prolactin immunoactivity was measured using the RP-I-1 and the RP-1 preparations of the N.I.A.M.D.D. rat prolactin radioimmunoassay kit for iodination and as standard, respectively. The antiserum used was anti-rat prolactin, kindly provided by Dr. J. Neill (Emory University). The biopotency of the RP-1 rat prolactin standard of the radioimmunoassay was calibrated against the bioassay standard (NIH-P-S10), and the unweighted mean potency estimate of five determinations was found to be 10.4 I.U./mg ± 0.6 (S.E.). Because different standards were employed in the two assays, the values have been converted to I.U./mg of the respective standards to facilitate comparisons.

Chemical characterization. All fractions were examined by polyacrylamide disc gel electrophoresis at pH 8.3 [15] in 7.5% gels stained with Amido Schwartz. NH₂-terminal amino acid analyses were performed by the dansyl technique [16,17] and a short segment of the amino-terminal sequence of the rat prolactin was determined by the dansyl-Edman procedure [18]. C-terminal amino acid determinations were made by the hydrazinolysis reaction [19]. Amino acid analyses of hydrolyzed samples of the pure materials were performed by the method of Spackman et al. [20] in a Beckman automatic analyzer. Circular dichroism (CD) spectra were taken in a Cary Model 60 spectropolarimeter equipped with a Model 6002 circular dichroism attachment according to procedures previously described [21]. Protein concentrations were determined spectrophotometrically using the relation $E_{1%}^{1cm,277nm} = 9.09$ reported previously for ovine and porcine prolactin [22].

Results

Purification of prolactin from culture medium

The prolactin content of each fraction obtained during the purification procedures was determined by bioassay and radioimmunoassay. In addition, the prolactin, albumin and growth hormone present in the medium were readily identifiable by disc electrophoresis. The growth hormone was present in such small quantity that it could not be concluded that this material was secreted nor could the same kinds of analyses be performed as were done with the prolactin. In the first purification step, the majority of the salts, amino acids, and other low molecular weight compounds contained in the culture medium were removed by dialysis and chromatography on Sephadex G-25. The protein concentrate obtained at this step contained prolactin, growth hormone, and albumin; the disc electrophoresis pattern is shown in Fig. 1. At the next purification step, the growth hormone was precipitated with 6% NaCl at pH 3; prolactin and albumin remained in the supernatant. The solubility of rat prolactin at this step contrasts with that of ovine prolactin, which is precipitated at
6% NaCl, pH 3 [23]. The gel filtration pattern of the NaCl supernatant fraction is shown in Fig. 2. A high molecular weight material (peak A) and albumin (peak B) were separated from prolactin (peak C). The purity of the prolactin was demonstrated by the absence of contaminating bands in disc electrophoresis (Fig. 1) and the single N-terminal amino acid residue, leucine. The growth hormone contamination by radioimmunoassay determination is 1.6% (0.016 × N.I.A.M.D.D. rat growth hormone I-2), compared with a value of 0.09% (0.009 × N.I.A.M.D.D. rat growth hormone I-2) for the pituitary prolactin *

Comparison of pituitary and medium prolactin

The biological potency of the medium prolactin was 15.3 ± 3.2 (S.E.) I.U./mg and the immunoactivity was 14.7 ± 0.4 I.U./mg. These values represent the unweighted mean potency averaged from four and three determinations, respectively. Both the bioactivity and the immunoactivity of the pituitary prolactin were higher, 20.1 ± 0.9 and 22.8 ± 0.1 I.U./mg, respectively. These values are the unweighted mean potencies averaged from four determinations each.

Disc gel electrophoresis patterns of the protein mixture isolated from the incubation medium after Sephadex G-25 and the purified hormones are presented in Fig. 1. After purification, the medium prolactin had an identical pattern and mobility compared to pituitary prolactin. In both cases, the additional bands are characteristic and believed to be deamidated forms of the hor-

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* These determinations were made by Dr. A.F. Parlow, University of California, Harbor General Hospital, Torrance, California.
Fig. 2. Gel filtration of the 0.37 M NaCl supernatant fraction on a 450 ml column of Sephadex G-100 (2.5 × 92 cm) equilibrated with 0.05 M NH₄HCO₃, 24°C; 32 mg applied; 4 ml/tube; A, unidentified material, 5 mg; B, rat albumin, 7 mg; C, rat prolactin, 19.6 mg.

mones and not impurities [24]. The secreted hormone stained less intensely than the pituitary material when initially tested on electrophoresis; however, subsequent electrophoresis with the two prolactin preparations revealed no differences in stainability.

The results of amino acid analyses of pituitary and medium prolactin are pre-

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a Taken from Ellis et al. [11].

b Determined by methane sulfonic acid hydrolysis [25] average of two determinations.

c Determined by spectrophotometric measurement [26].
The composition of both forms of prolactin are in close agreement with previously published analyses of this hormone [11] and there are no major differences between the pituitary- and medium-isolated hormones. The first three amino acids at the N-terminal of prolactins were determined and in both cases the sequence NH₂-Leu-Pro-Val- was found. Hydrazinolysis revealed no C-terminal residue for the prolactins, which would be expected if rat prolactin contains a carboxyl-terminal disulfide loop as does ovine prolactin [27].

Circular dichroism spectra of pituitary and medium prolactin, in the region of side chain absorption and at slightly alkaline pH, are shown in Fig. 3. The hormone isolated from pituitary glands exhibits a positive asymmetric band centered near 292 nm and four negative maxima appearing at 262, 270, 279, and 286 nm. In contrast, the protein isolated from culture medium shows a complete absence of the positive band seen in the pituitary material above 290 nm. The medium prolactin does exhibit four negative bands which correspond very closely to those observed with the pituitary hormone, although the entire dichroism envelope of the medium prolactin appears at more negative ellipticity values than pituitary prolactin. When titrated from pH 8.2 to pH 3.6, both of these materials exhibit altered side-chain CD spectra indicating a limited change in tertiary structure similar to that previously reported for ovine and porcine prolactin [22]. Under these acidic conditions the CD spectra of both forms of rat prolactin appear identical. However, on dialysis of the treated samples back to pH 8.2, the original spectra return with the medium prolactin still lacking the positive band above 290 nm.

The possibility that this difference in CD spectra is a function of the difference in the particular purification techniques employed with the medium versus the pituitary prolactin was tested. Purified rat pituitary prolactin was dissolved in Medium 199, dialyzed, lyophilized and re-purified by the same techniques employed with the medium isolated prolactin. CD analysis of this material showed no differences when compared with the starting material; the positive band was still present at 292 nm.

The far ultraviolet CD spectra of these proteins (not shown) both contain
the two intense, negative bands at 221 and 209 nm, characteristic of α-helical proteins. Both prolactins exhibit essentially the same spectrum in this region, indicating an α-helix content of approx. 45–50%. The far ultraviolet spectra are not significantly effected by titration to pH 3.6.

Discussion

We have described a simple method for the isolation and purification of rat prolactin from organ culture medium and compared this material with the same hormone isolated from rat pituitaries. The purification techniques differ from those of Gala [9], who isolated rat prolactin from medium by preparative gel electrophoresis, but our results are in good agreement. The methods described in the present study yield 22 mg prolactin/g of cultured pituitaries, compared with 1 mg/g which has been obtained when pituitaries have been directly extracted [11]. The 22-fold greater yield of secreted prolactin clearly represents de novo synthesis of the hormone and thus the purified medium prolactin described is a secreted form of prolactin. The observation that labelled amino acids are actively incorporated into secreted prolactin in organ culture [28] supports this conclusion.

The results obtained in this study indicate that the form of secreted prolactin that we have isolated from culture medium does not differ in primary structure from the pituitary hormone. The data that support this conclusion are the virtually identical amino acid compositions, the identical NH$_2$-terminal sequences, and the implied identity of the COOH-terminal residues. A possible mechanism for the generation of a unique secreted form of prolactin that differs from the intraglandular form might be the removal of a small peptide by a plasmin-like enzyme. Experiments with plasmin digestion of rat growth hormone have resulted in decreases in immunoactivity (complement fixation) without affecting bioactivity [29]. These results correlate well with the high bio/immunoactivity ratio of growth hormone in rat serum observed by Ellis and Grindeland [1]. Our results, however, indicate that the form of rat prolactin that we isolated from culture medium has not undergone such a transformation.

Although we found no evidence for a difference in the primary structure between secreted and pituitary prolactin, the results of circular dichroism studies suggest that there may be a difference in tertiary structure between these forms of prolactin. The circular dichroism spectrum of rat pituitary prolactin shown in Fig. 3 is very similar to the spectra of both human growth hormone and ovine prolactin under similar conditions [30]. By analogy with these previously reported spectra, the positive band at 292 nm may be assigned to tryptophan. The medium prolactin shows no evidence of any indole dichroism above 290 nm, suggesting a significant difference in the conformations and local environments of the tryptophan residues in these two proteins. Moreover, this difference re-establishes itself after a limited, reversible denaturation of the tertiary structure, further suggesting that this conformational difference is an intrinsic property of each substance. Whether the absence of indole dichroism in the medium prolactin is due to a lack of steric restrictions on this chromophore, or due to cancellation by two chromophores of opposite sign is not presently...
known. It also remains to be determined if the circular dichroism difference observed with the secreted prolactin relates to the process of secretion, the effects of 3 days in organ culture, or to differences in the purification procedure employed with the pituitary prolactin. Our results did show, however, that the difference observed in the circular dichroism spectrum was not due to the purification techniques employed with the secreted prolactin.

No significant differences were found in the biological/immunological activity ratios of the pituitary or secreted prolactins, although both biological and immunological activities of the secreted prolactin were somewhat lower than those of rat pituitary prolactin. The circular dichroism studies suggesting a difference in tertiary structure between the two forms of prolactin possibly explains the difference in immunopotency, noted by both Gala [9] and ourselves. The significance of the difference in bioactivity cannot be evaluated because of considerable variation in the biopotency estimates for the secreted prolactin.

Whether the secreted form of rat prolactin differs from the circulating form remains to be determined. Ben-David et al. [31] have compared forms of human prolactin isolated from the pituitary, amniotic fluid, and plasma by electrophoresis and isoelectric focusing in polyacrylamide gel. They found that the prolactins isolated from these three sources were indistinguishable on the basis of molecular size, net charge, and isoelectric point. It will be of interest to determine how the differences we have observed between pituitary and secreted rat prolactin relate to the properties of circulating rat prolactin.

Acknowledgements

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