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A SPECIFIC TESTOSTERONE RECEPTOR IN THE CYTOSOL OF RAT ANTERIOR HYPOPHYSIS

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SUMMARY

A study was designed to measure the specificity of the testosterone binding in the cytosol of the anterior pituitary of immature male rats (40 day old rats).

The binding at equilibrium was studied after cytosol incubation at 0°C with a constant dose of [^{3}H]-testosterone at various times. The binding was measured after gel filtration on Sephadex G 25 medium. Equilibrium was reached at 5 h and remained constant during 24 h. No significant testosterone metabolism was detected.

The binding specificity was determined using different concentrations of [^{3}H]-testosterone. Cytosols were incubated for 5 h at 0°C with concentrations of [^{3}H]-testosterone ranging from 10^{-9} M to 10^{-6} M. The protein–testosterone complex was isolated by gel filtration on Sephadex G 25 medium. A Scatchard plot of the binding data produced a curved line showing that more than one binding component was involved. This curve was resolved into two linear components by the Rosenthal method. The dissociation constant (K_D) for the specific binding was $2{\cdot}3 \times 10^{-9}$ M and the estimated amount of specific receptor was 8×10^{-11} M. The specific binding capacity was estimated to be 2×10^{-14} moles per mg of protein.

INTRODUCTION

Rat anterior hypophysis is able to take up and to retain androgens to a larger extent than other brain structures including hypothalamus [1–3]. Moreover, in previous papers, we have described a testosterone binding component in the cytosol of the male rat anterior hypophysis and two macromolecular associations for testosterone in the purified nuclei of this gland [2–4]. The present study was designed to search for and to measure the specificity of the cytosol testosterone protein association.

MATERIALS AND METHODS

Steroids

[^{3}H]-testosterone (specific activity 46 Ci per mmol) was supplied by C.E.N., Belgium. It was checked for purity by paper chromatography in the solvent system of Kochakian and Stidworthy[5].

Biological material

Immature male Wistar rats, 40–43 days old, were used. They were killed by decapitation without anaesthesia and anterior hypophysis were promptly removed and homogenized in a phosphate buffer 0·05 M, pH 7·4. The homogenate was centrifuged at 600 g and the 600 g supernatant was spun down at 105,000 g for 1 h in the SW 25 rotor of a Beckman ultracentrifuge (Model L_4). The 105,000 g supernatant (cytosol) was used for binding analysis.

Binding at equilibrium

Six hundred microliters of cytosol fractions with a protein concentration of 1250 μg were incubated with [^{3}H]-testosterone (final concentration 10^{-7} M). The incubations were performed at 0°C from 5 min to 24 h. At various times samples were removed and the bound and unbound testosterone were measured after gel filtration on Sephadex G 25 columns.

Binding analysis

For the binding study, cytosol samples (containing 3 mg protein per ml) were incubated at 0°C with [^{3}H]-testosterone from 10^{-9} M to 10^{-6} M for 5 h. The bound and free testosterone were separated by gel filtration on Sephadex G 25. The binding data were plotted on a curve according to Scatchard[6] and that curve was resolved into two linear components by the Rosenthal method[7].

Gel filtration

Columns (30 × 1·5 cm) were packed with Sephadex G25 (medium) and the gel was equilibrated with phosphate buffer 0·05 M, pH 7·4. Elutions were carried out with the same buffer. 1·5 ml fractions were collected for protein and radioactivity determinations.

Chromatographic procedure

After gel filtration, the fractions containing the radioactivity bound to the molecules excluded on Sephadex G 25 were extracted by the method of Folch *et al.*[8] modified by Bruchosky and Wilson[9]. Androgens were chromatographed on Whatman n° 1 paper cut into 2 cm wide strips in the solvent system of Kochakian and Stidworthy[5]. After a development for 4–5 h at 30°C, the strips of chromatographic paper were divided into 2 × 1 cm segments and these were placed in counting vials.

Radioactivity measurement

The radioactivity of aqueous fractions was counted using the Bray's mixture[10]. The radioactivity of the chromatograms was measured as previously described[3]. Radioactivity measurements were performed using an automatic liquid scintillation system, Nuclear Chicago, Isocap/300.

Analytical procedure

The method of Lowry *et al.*[11] was used for protein determination with bovine serum albumin as standard. (Bovine albumin, Fr V, National Biochemicals Corporation, Cleveland, Ohio).

RESULTS

Binding at equilibrium

The [^{3}H]-testosterone binding to cytosol proteins was measured at various incubation periods at 0°C. The amount of testosterone bound increased up to 5–6 h and remained constant during 24 h (Fig. 1). After a 5 h incubation time, equilibrium was obtained and for this reason, we have chosen 5 h as the incubation time for the binding analysis.

During prolonged incubation at 0°C, no testosterone metabolism was observed. The chromatographic analysis of androgen bound to cytosol proteins showed a single peak identified as testosterone (Fig. 2). A minor peak was also detected on the chromatogram. It is an apolar, unidentified steroid (steroid X).

Binding analysis

The testosterone binding specificity was analyzed using a 5 h incubation period of cytosol with [^{3}H]-testosterone concentrations from 10^{-9} M to 10^{-6} M. Bound and unbound testosterone were separated by gel filtration on Sephadex G 25 (medium) (Fig. 3). Figure 4 shows a Scatchard plot of the binding data. This Scatchard plot produced a curved line confirming that more than one binding component was involved. This curve was resolved into two linear components by Rosenthal's method. (Fig. 4). The dissociation constant for the specific binding is: $2{\cdot}3 \times 10^{-9}$ M. The intercept of the X-axis provided an estimate of the amount of the specific testosterone receptor, 8×10^{-11} M, which represents 0·027 pmol testosterone per mg of protein.

It was necessary to measure an eventual loss of binding during gel filtration. For that experiment we used 3 columns, 1·5 cm dia and 27, 20 and 13·5 cm high containing respectively 9·5, 7 and 4·7 g of Sephadex G 25 (medium). Cytosol was incubated as previously described in the presence of [^{3}H]-testosterone 1×10^{-9} M and was divided into 3 parts which were submitted to gel filtration on the 3 columns. The specific activities of the testosterone–protein associations were respectively: 0·0085, 0·0092 and 0·0097 pmol per mg protein (Fig. 5). Its real value obtained at the Y-axis intercept was 0·0106. Thus, the real value of the specific binding capacity was superior by about 20% to that obtained in our experiments and was exactly 0·028 pmol per mg protein.

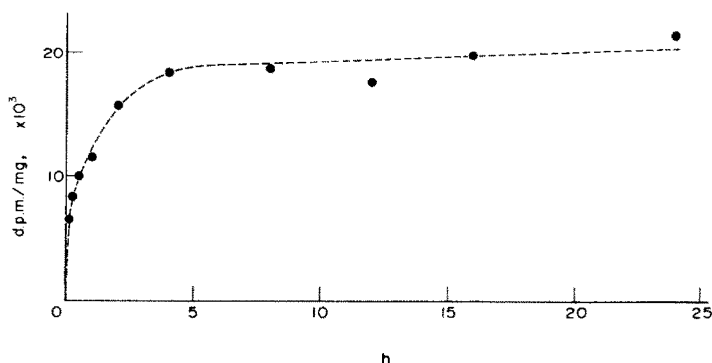

Fig. 1. Binding at equilibrium of testosterone by cytosol proteins of rat anterior hypophysis.

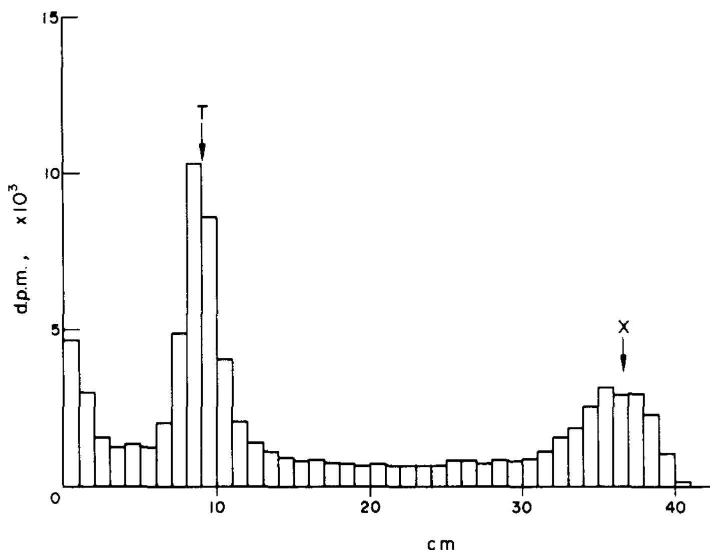

Fig. 2. Analysis by paper chromatography of the bound radioactivity at equilibrium after a 16 h incubation period at 0°C.

DISCUSSION

During the last years, we have found a macromolecular association of testosterone in the cytoplasm of male rat anterior hypophysis and two macromolecular bindings in the cellular nuclei of the same gland [2, 3]. The experiments described in this paper were undertaken to measure the specificity of the testosterone binding in the cytosal fraction of the immature male rat pituitary gland. The results show two binding components. Thus, the cytosol of the immature rat anterior hypophysis contains a specific receptor for testosterone and some specific associations.

The anterior hypophysis is constituted by a variety of cells. It is possible to think that the aspecific associations would be present in all the cells and that the specific testosterone receptor would be exclusively in the gonadotrophs. Thus, the gonadotrophs would be likely target cells for testosterone. Recent observations described by Sar and Stumpf after autoradiographic studies with [^{3}H]-testosterone showed a preferential

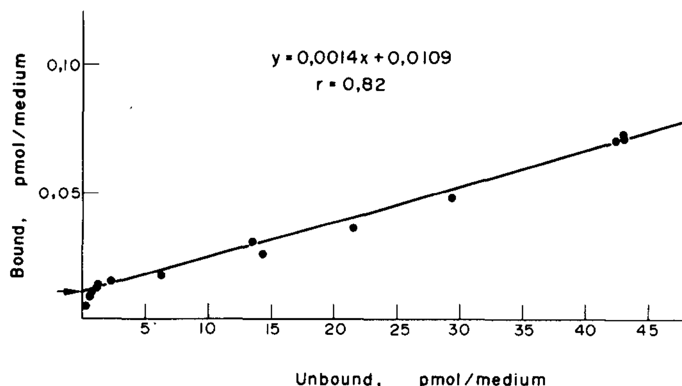

Fig. 3. Bound and free testosterone in the cytosol pituitary after a 5 h incubation time at 0°C. The specific binding capacity was obtained from the Y intercept (arrow).

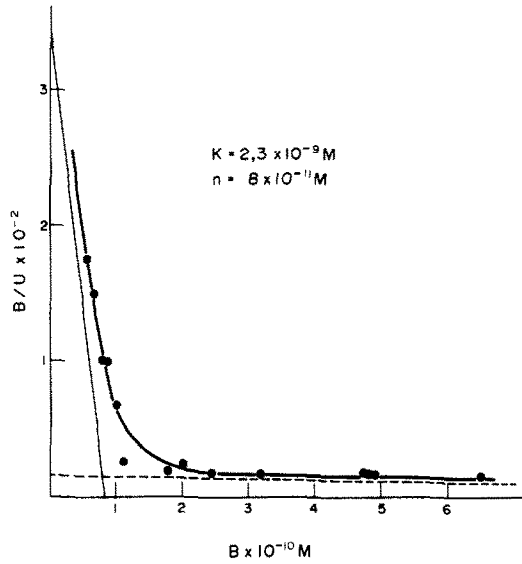

Fig. 4. Scatchard plot of total binding data. The specific and non-specific bindings were obtained according to the Rosenthal method[6].

androgen concentration in gonadotrophs while thyrotrophs, acidophils and chromophobs did not concentrate and retain radioactivity [12, 13].

The dissociation constant (K_D) for the specific component is 2×10^{-9} M. It is of the same order of magnitude as the dissociation constant for estradiol and testosterone in the female and male genital tracts [14]. Moreover, our results are similar to that of Leawit *et al.*[15] and Notides[16], concerning the specific estradiol receptor in the female rat anterior hypophysis.

Thus, the presence of a soluble and specific receptor for estradiol and testosterone in the female and the male rat anterior hypophysis seems to be well established.

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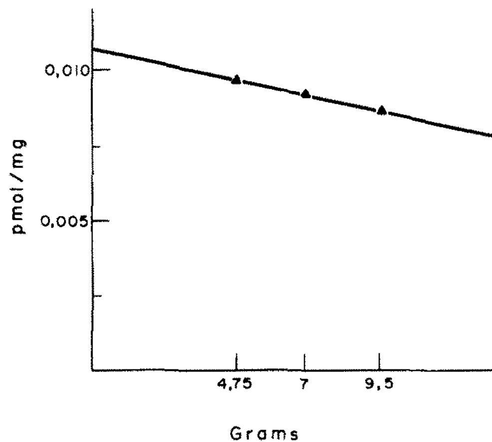

Fig. 5. Determination of the testosterone binding dissociation during gel filtration. (Y-axis: specific activities of the testosterone protein associations in pmol per mg of proteins, X-axis: grams of Sephadex G 25 medium used for the gel filtrations.

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DISCUSSION

Martini:

I wonder whether you have tried to bind other steroids to this protein, like dihydrotestosterone or the androstanediols.

Samperez:

No, but this research is in progress in our laboratory.

Bertrand:

I can answer your question. In our laboratory Dr. Loras studied the evolution of both cytoplasmic and nuclei receptors from hypophysis of rats before puberty and during puberty. He studied the steroids which were bound to these receptors. It was found that not only dihydrotestosterone but also 3α,5α-androstanediol was bound. So we think that in the hypophysis, androstanediol was a very good androgen.

Martini:

Did you find any change in the affinity constants of this protein before puberty or during sexual maturation?

Bertrand:

I cannot answer you. The only thing that I can say is that the percent of 3α,5α-androstanediol bound to the receptor increases with puberty. That is to say that a higher percentage of androstanediol was bound to the cytoplasmic or nuclear receptors during puberty than before puberty.

Martini:

The reason for asking this question was that it has been shown in my laboratory that the 5α-reductase activity of the pituitary shows a marked decrease after puberty has occurred (Massa, Stupnicka, Villa and Martini—*53rd Meeting of the Endocrine Society*, 1971, p. A229).

I have another question. As you know there is a sex steroid binding globulin which binds both testosterone and estrogens. There are several people who have shown that the pituitary and the hypothalamus may aromatize androgens and convert them into estrogens. Now my question is whether the same receptor protein which binds androgens in the pituitary is also able to bind estrogens. This would be crucial to answer the question of whether testosterone has a direct effect in the feedback mechanisms, or whether these are mediated via formation of estrogens or of "better" androgens (e.g., DHT, 3α-diol).

Samperez:

We have not looked for a receptor of estrogens and I cannot say if it is the same or not.

Hansson:

First one question, does your 7S complex dissociate into a 4S complex at high salt?

Samperez:

Yes, there are some differences due to incubation temperature or ionic strength. You have more 8S or more 4S according to the variations but we don't have a complete study of this problem.

Hansson:

I would like to stress one point about androgen receptors. After our paper on testicular androgen binding protein (ABP) presented by Dr. Ritzen earlier today, I think there are excellent reasons for separating androgen "receptors", and other specific androgen binding proteins (like ABP). We should not consider all binding proteins for androgens, showing high affinity and limited capacity for androgens a "receptor". First of all, a "receptor" should be located intracellularly. It must be a protein, and it has to bind androgens with high biological activity. Furthermore, androgen "receptors" demonstrated so far (prostate, seminal vesicles, epididymis, testis) all are similar by size (gel filtration), electrophoretic mobility, and sedimentation in sucrose gradients, and all androgen "receptors" are thermally very unstable (destroyed at 50°C) and easily destroyed by sulfhydryl reagents. All of them can also, in complex with the steroid, be translocated into target cell nuclei. Perhaps the most striking characteristic of androgen-"receptor" complexes is their very slow rate of dissociation at 0°C ($T^{1/2} > 2$ days). I think you have *some* of these data reported here but still you are lacking other important criteria in order to say that this is a "receptor". I would like to mention that Dr. Naess in our laboratory in Oslo has done extensive studies on a cytoplasmic binding protein for testosterone and dihydrotestosterone in the pituitary and various areas of the central nervous system. In most respects this protein behaves just like an androgen "receptor".