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DIHYDROTESTOSTERONE-PROTEIN BINDING IN THE CYTOSOL OF RAT ANTERIOR HYPOPHYSIS *IN VITRO*: EVIDENCE FOR A SPECIFIC RECEPTOR

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SUMMARY

A study was designed to measure the specificity of the dihydrotestosterone binding in the cytosol of the anterior pituitary of immature male rats (40-day-old rats). The binding at equilibrium was studied after incubation of cytosol at 0°C with a constant dose of [^{3}H]-dihydrotestosterone (DHT) for various times. The binding was measured after gel filtration on Sephadex G 25 medium. Equilibrium was reached at 6 h and remained constant for 24 h. No significant dihydrotestosterone metabolism was detected. The binding specificity was determined using different concentrations of [^{3}H]-dihydrotestosterone. Cytosols were incubated for 6 h at 0°C with concentrations of [^{3}H]-dihydrotestosterone ranging from 10^{-10} M to 10^{-7} M. The protein-dihydrotestosterone 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 $7{\cdot}8 \times 10^{-10}$ M and the estimated amount of specific receptor was $1{\cdot}4 \times 10^{-11}$ M, which represents $4{\cdot}7 \times 10^{-15}$ mol per mg of proteins. Unlabelled dihydrotestosterone and testosterone were potent and specific inhibitors of the [^{3}H]-DHT binding. Other androgens, estrogens and progestagens were less potent competitors. 4-Androstenedione, corticosterone and estradiol-17α were without effect on the [^{3}H]-DHT-binding.

INTRODUCTION

The anterior pituitary gland from the male rat is able to concentrate and to retain radioactive testosterone preferentially [1]. Moreover, in this gland, testosterone is partially metabolized into 5α-dihydrotestosterone and 3α-androstanediol [2, 3] and these androgens are partly free and partly bound to macromolecules [4–6]. This binding appears to occur in a two-step mechanism in which androgens first interact with cytoplasmic receptors and then are translocated to the nuclear fraction [5, 6]. In the nucleus, testosterone and dihydrotestosterone are bound to non-histone proteins [5]. Thus, we demonstrated that the male rat pituitary gland contained a testosterone and a dihydrotestosterone binding system. It is important to demonstrate the existence of specific testosterone and 5α-dihydrotestosterone receptors in the rat pituitary cytosolic fraction. Indeed, identification and characterization of androgens receptors in the anterior pituitary are an important issue in the area of reproductive physiology. It is now established that androgenic feedback control over gonadal function in the rat is located within the hypothalamus and the pituitary and receptors are generally thought to be involved in the mechanism of androgen action. Consequently, assessment of the role of cytosol receptor interactions in modification of reproductive function requires a study of the kinetics of binding of androgens by these receptors.

In recent papers, we have demonstrated the presence of a specific testosterone receptor in this gland [7, 8]. The present study was designed to measure the specificity of the cytoplasmic dihydrotestosterone receptor from immature male rat pituitary gland.

MATERIALS AND METHODS

Steroids. [1,2-^{3}H]-dihydrotestosterone (43 Ci/mmol) was obtained from C.E.N. (Belgium). Its radiochemical purity was checked by paper chromatography using the solvent system of Kochakian and Stidworthy[12].

Sample preparation. Immature Wistar rats, 38–40 days old, were used in this study. The animals were decapitated and anterior hypophysis were combined in cold 0·05 M phosphate buffer, pH 7·4. They were homogenized in the same buffer. The homogenate was

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Trivial and systematic names of steroids used in this paper: Testosterone, 17β-hydroxy-4-androsten-3-one. Dihydrotestosterone, DHT, 17β-hydroxy-5α-androstan-3-one. 3α-Androstanediol, 5α-androstane,-3α, 17β-diol. 4-Androstenedione, 4-androstene-3,17-dione. Progesterone, 4-Pregnene-3,20-dione. estradiol-17β,: 1,3,5 (10)-Estratriene-3,17α-diol. Corticosterone, 11β, 21-Dihydroxy-4-pregnene-3,20 dione.

centrifuged for 6 min at 1000 g and the supernatant was re-centrifuged at 105,000 g for 60 min at 4°C in a Spinco L_4 ultracentrifuge. The 105,000 g supernatant (cytosol) was diluted with phosphate buffer to give an appropriate protein concentration. (2920 μg/ml).

Binding at equilibrium. Cytosol (1·2 ml) was incubated at 0°C with [^{3}H]-dihydrotestosterone ($2·4 \times 10^{-8}$ M) for various time periods (60 min to 24 h). The bound and unbound dihydrotestosterone was measured by gel filtration.

Steroid binding experiments. For the binding study, cytosol was adjusted to a protein concentration of 2920 μg/ml. Samples of 150 or 300 μl were incubated at 0°C with 10^{-10} M to 10^{-7} M [^{3}H]-dihydrotestosterone (6 h at 0°C) and without addition of non-labelled steroid. The analysis of binding components was obtained by gel-exclusion chromatography.

Specificity for binding of the steroids. The cytosols prepared from 30 rat anterior pituitaries were diluted to obtain a suitable protein concentration and were then incubated 6 h at 0°C with $2·2 \times 10^{-9}$ M [^{3}H]-dihydrotestosterone in the absence or in the presence of competing unlabelled steroid ($2·2 \times 10^{-7}$ M). Bound radioactivity was measured after passage through a column of Sephadex G 25 medium. The results are calculated according to the following equation [9].

$$\frac{\text{bound } [^3\text{H}].\text{DHT S.A.} - (\text{bound } [^3\text{H}].\text{DHT} + \text{unlabelled steroid})}{\text{bound } [^3\text{H}].\text{DHT S.A.} - (\text{bound } [^3\text{H}].\text{DHT} + \text{unlabelled DHT})} \times 100$$

Gel-filtration. Samples of labelled cytosol were chromatographed in 27 × 1·5 cm columns of Sephadex G 25 medium equilibrated with 0·05 M phosphate buffer, pH 7·4. The column was eluted with the same buffer and fractions of 1·5 ml were collected for protein and radioactivity determinations. Gel filtrations were performed in a cold room.

Chromatographic procedure. The bound fraction recovered after gel-filtration was extracted according to the method of Folch *et al.*[10] modified by Bruchovsky and Wilson[11]. The steroids were separated by paper chromatography in the solvent system of Kochakian and Stidworthy[12]. After developing for 4–5 h at 30°C, the chromatographic paper was divided into 2 × 1 cm segments which were put in counting vials.

Analytical procedures. The radioactivity of chromatograms was measured as previously described [5]. The radioactivity of aqueous eluates from gel filtration was measured by liquid scintillation in Bray's mixture [13]. The measurements were made with the help of an Isocap 300 counter (Nuclear Chicago). External standardization was used to correct for quenching if necessary. The method of Lowry *et al.*[14] was used for protein determination with bovine serum albumin as standard (bovine albumin, Fr V, National Biochemical Corporation).

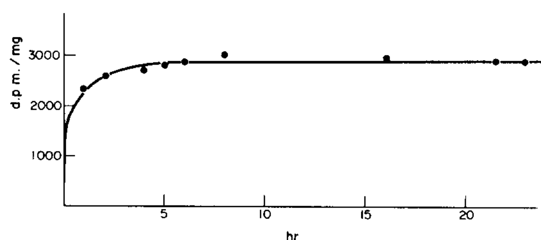

Fig. 1. Binding at equilibrium of dihydrotestosterone by cytosol receptor of rat anterior hypophysis.

RESULTS

Binding at equilibrium. As shown in Fig. 1, the concentration of specific and non-specific bindings of dihydrotestosterone with cytosol proteins increased between 60 min and 5–6 h of incubation at 0°C. A maximum value was reached at 5–6 h and remained constant up to 24 h. Thus, a 6 h incubation time was chosen for the dihydrotestosterone binding experiments. Steroid bound at equilibrium was subjected to chromatographic analysis and main steroid detected on the chromatogram was dihydrotestosterone (Fig. 2). This result indicates that no significant dihydrotestosterone metabolism occurs at 0°C even after a prolonged incubation time.

Evaluation of receptor site concentration and equilibrium constants. The binding specificity was determined using different concentrations of [^{3}H]-dihydrotestosterone. Cytosols were incubated for 6 h at 0°C with concentrations of [^{3}H]-dihydrotestosterone ranging from 10^{-10} M to 10^{-7} M and the protein–androgen complex was isolated by gel filtration. A Scatchard plot [15] of the binding data produced a curved line showing that more than one binding component was involved (Fig. 3). This curve was resolved into two linear components according to the method of Rosenthal[16] (Fig. 3). The dissociation constant (K_d) for the specific binding is $7·8 \times 10^{-10}$ M and the estimated amount of specific receptor is $1·4 \times 10^{-11}$ M, which represents $4·7 \times 10^{-15}$ mol per mg of protein.

Comparative hormonal specificity of [^3H]-dihydrotestosterone receptor. The receptor specificity was studied incubating the cytosol either with 3H-DHT alone or with 3H-DHT and an excess (100-fold) of

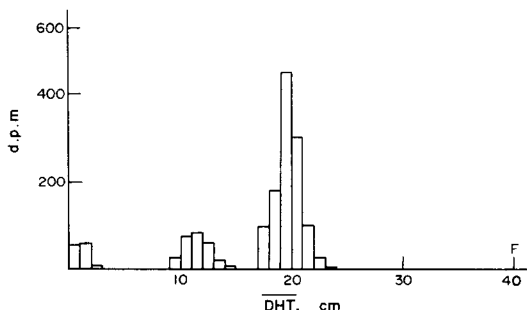

Fig. 2. Analysis of chloroform–methanol-extractable radioactivity from cytosol receptor by paper chromatography, after binding at equilibrium (16 h).

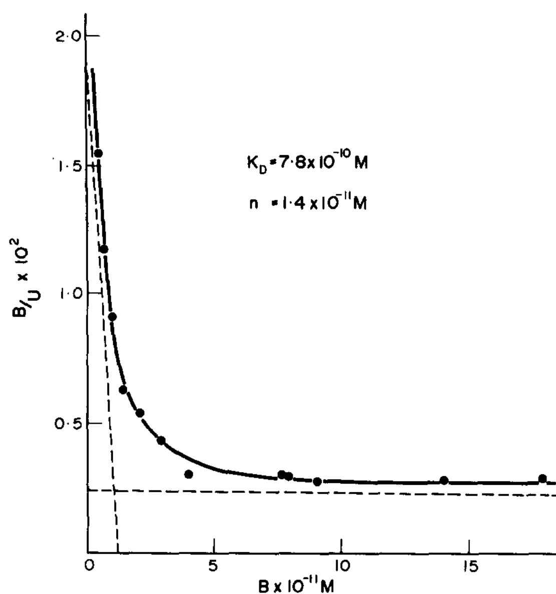

Fig. 3. Scatchard plot of total binding data. The specific and non-specific bindings were obtained according to the method of Rosenthal.

unlabelled steroid. As seen in Table 1, it is apparent that nonradioactive DHT and testosterone compete with radioactive [^{3}H]-dihydrotestosterone for binding to the DHT-receptor. Competition by 5α-androstane, 3α,17β-diol, 5α-androstane, 3β,17β-diol, estradiol-17β and progesterone was also observed to a lesser extent. On the other hand, 4-androstenedione, corticosterone, and 17α-estradiol do not affect the amount of [^{3}H]-DHT bound to the receptor.

DISCUSSION

This study shows the presence of a specific dihydrotestosterone receptor in the anterior pituitary of prepuberal male rat. Its dissociation constant (K_d = $7{\cdot}8 \times 10^{-10}$ M) is of the same order of magnitude as the dissociation constant (K_d) obtained for estradiol and androgen receptors in the female and male reproductive tracts respectively. (For a review see ref. 17). The value obtained in this experiment is identical to that reported by Leavitt *et al.* for the specific estradiol receptor in the pituitary cytosol fraction from mature and ovariectomized female rat [18] and by Notides[19].

Table 1. Hormonal specificity of dihydrotestosterone receptor from the rat anterior hypophysis

Unlabelled steroid	Competing efficiency for [^{3}H]-dihydrotestosterone binding to dihydrotestosterone receptor
5α-Dihydrotestosterone	100
Testosterone	100
5α-Androstane, 3α,17β-diol	66
5α-Androstane, 3β,17β-diol	56
4-Andostène,3,17-dione	10
Estradiol-17α	5
Estradiol-17β	47
Progesterone	63
Corticosterone	10

The DHT-receptor has a high degree of specificity for dihydrotestosterone, when cytosol was incubated with [^{3}H]-dihydrotestosterone in the presence of unlabelled steroids, only unlabelled 5α-DHT and testosterone competed with [^{3}H]-DHT for the binding sites. Other steroids *e.g.* progesterone, estradiol-17β and 3α-androstanediol are relatively poorly bound *in vitro*, whereas 4-androstenedione, corticosterone and estradiol-17α are not bound by the DHT-receptor. Similar results were obtained by Fang *et al.*, with the DHT-receptor from the rat ventral prostate cytosol [20]. Testosterone and cyproterone competed strongly for binding sites, oestrogens and progestagens to a lesser extent but cortisol did not compete. In rat epididymis cytosol, appreciable competition for DHT binding sites was exhibited by testosterone and estradiol-17β [21]. Similar results were obtained by Danzo *et al.* studying the specificity of the cytoplasmic receptor for DHT in the caput epididymis of intact rabbits [22]. Thus, some steroids (androgens and estrogens) have a relative affinity for DHT-receptors.

Our results are also similar to those obtained recently by Kato *et al.*, in a preliminary note [23].

The anterior pituitary of male rats contains also non specific components. It is possible that these associations would be present in a variety of pituitary cells other than gonadotrophs. On the other hand, it is to be expected that the specific dihydrotestosterone association would be present only in the gonadotrophs, and that these cells are target cells for androgens. Autoradiographic analysis substantiates this view. Sar and Stumpf have studied the autoradiographic localization of radioactivity in the male rat pituitary after injection of [^{3}H]-dihydrotestosterone. Their results clearly demonstrate that labelling of anterior pituitary cells is confined to gonadotrophs [24, 25].

Whether the cytosol of rat anterior hypophysis contains two receptors, one for each androgen, or a single receptor for these two androgens remains to be determined.

Thus, the demonstration of a specific dihydrotestosterone receptor with high affinity and low capacity in the pituitary cytosol fraction of the prepubertal male rat, suggests that this gland contains target cells for androgen which may directly regulate anterior pituitary function.

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