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ANDROGENS BINDING PROTEINS IN THE CYTOSOL FROM PREPUBERTAL MALE RAT HYPOTHALAMUS, PREOPTIC AREA AND BRAIN CORTEX

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SUMMARY

The cytosol from hypothalamus and preoptic area of prepubertal male rats contains binding proteins for androgens, whereas the cytosol from brain frontal cortex does not. By Scatchard analysis and gel filtration through Sephadex G 200 column, it appeared that there were two classes of binding sites: one with high affinity for androgens and low binding capacity, the other non specific. The dissociation constants at equilibrium (K_D) were: 7×10^{-10} M for testosterone and 7×10^{-11} M for dihydrotestosterone. Specific binding capacities were respectively 6×10^{-15} and 1.5×10^{-15} mol/mg of cytosol proteins. The steroid specificity of these binding proteins was checked by competition assays. It appeared that there was no strict specificity neither for testosterone nor for dihydrotestosterone. Taking this fact into account the problem is to know whether these proteins are true receptors and what is their physiological role.

INTRODUCTION

It is now accepted that androgens in the male regulate gonadotropin secretion by their action on the hypothalamus and hypophysis. Furthermore, it is established that the androgenic action is mediated by the presence in target organs of specific receptors. The presence of such receptors for testosterone and for dihydrotestosterone in the male rat anterior pituitary was demonstrated [1–4]. However, the specific binding of androgens in the hypothalamic cytosol remains a debated question. Thus, Korach and Muldoon failed to find a specific receptor for testosterone or dihydrotestosterone in the hypothalamic cytosol from male rats [5]. Whereas, Kato and Onouchi[6], Naess[7], Gustafsson *et al.*[8] demonstrated the presence of such a receptor in the same region.

In 1971, we showed that there were in the hypothalamic cytosol, some macromolecules able to bind androgens and we defined the chemical nature of bound hormones [9].

The purpose of our present work is to make our previous investigation more complete, and to determine some characteristics of the binding macromolecules found in the cytosol from male rat hypothalamus and preoptic area.

MATERIALS AND METHODS

Chemicals. [1,2-^{3}H]-testosterone (S.A. 46 Ci/mmol) and [1,2-^{3}H]-dihydrotestosterone (S.A. 43 Ci/mmol) were obtained from C.E.N. (Belgium). The original solution was evaporated to dryness under vacuum, and the dry residue was dissolved in ethanol. This solution was diluted with 0.05 M phosphate buffer (pH 7.4) before use. The radiochemical purity of labelled steroids was checked by paper chromatography using the Kochakian and Stidworthy solvent system [10]. Unlabelled steroids were obtained from Sigma Company and were used without further purification.

Samples preparation. Immature Wistar male rats (38 day-old) were used. They were sacrificed by decapitation without anaesthesia. Brains were quickly removed and dissected. The hypothalamus was cut as a block limited anteriorly by the optic chiasma, laterally by the hypothalamic sulci, posteriorly by the mammillary bodies and horizontally 2–3 mm under tha basal surface. The rostral border of the preoptic area was 2 mm anterior to the cut through the optic chiasma; the depth was 2–3 mm. Cortical tissue was excised from the frontal lobe. 9 Hypothalami, or the same weight of cortical tissue, or 30 preoptic areas were homogenized in 1 ml of cold phosphate buffer (0.05 M at pH 7.4). The homogenate was centrifuged at 800 *g* for 6 min and the supernatant was spun down at 105,000 *g* for 1 h (Spinco L_4 centrifuge). The 105,000 *g* supernatant (cytosol) was diluted with phosphate buffer in order to obtain an appropriate protein concentration (2.9 mg per ml). This protein concentration was used throughout our experiment. All operations were carried out at 0–4°C.

Binding at equilibrium. Cytosol samples (300 μl, 876 μg proteins) were incubated at 0°C with [^{3}H]-testosterone (2.4×10^{-8} M) or with [^{3}H]-dihydrotestosterone (1.2×10^{-9} M) for periods varying from

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30 min to 24 h in order to define the time at which equilibrium was reached.

Steroid binding analysis. Cytosol samples (300 μl, 876 μg proteins) were incubated at 0°C during 6 h in the presence of tritiated testosterone or dihydrotestosterone at varying concentrations (from 10^{-10} M to 10^{-7} M). Incubations were carried out in the presence or in the absence of an excess of the corresponding unlabelled steroid. The determination of bound radioactivity was achieved using simultaneously two methods: (1) Gel filtration through a column of Sephadex G 25 medium as previously described [4]. Specific binding was expressed as the difference between the values obtained with labelled steroid alone (total binding) and those obtained with both labelled and unlabelled steroid (non specific binding). (2) Protamine precipitation of the steroid protein complex according to the method of King *et al.*[11] with slight modifications: cytosol samples (300 μl) were added to 300 μl of an aqueous protamine sulphate solution (1.5 mg per ml), mixed with a Vortex apparatus and kept at 0°C for 15 min. Then, they were layered on glass fibre filters (GF/C 2.5 Whatman), and washed with 10 ml × 4 of 0.05 M phosphate buffer under a vacuum of 6–8 mm Hg (Samples collector 1225 Millipore). Finally, glass fibre papers were put into scintillation vials for radioactivity counting. Blanks containing the same amount of radioactivity and phosphate buffer, but no cytosol, were made for every assay. Dissociation constants and number of binding sites were determined according to Scatchard[12].

Sometimes, cytosol samples were submitted to gel filtration through a Sephadex G 200 column (1.5 cm × 83 cm) equilibrated with 0.05 M phosphate buffer. Column was calibrated using Dextran blue, beef liver catalase (Sigma. M.W. 250,000), bovine serum albumin Fr. V (Nutr. Biochem. Corp. M.W. 67,000) and myoglobin (Koch Light. M.W. 17,800).

Effect of enzymes on androgen binding. Cytosol samples were incubated at 0°C and at equilibrium in the presence of tritiated testosterone or dihydrotestosterone (2.5×10^{-9} M). Then, enzymes were added to the samples: pancreatic chymotrypsin (237 μg/mg of proteins), pancreatic trypsin (237 μg/mg of proteins), pronase (from streptomyces griseus 95 μg/mg of proteins), pancreatic ribonuclease A (237 μg/mg of proteins) and ribonuclease T_1 (from *Aspergillus orizae* 38 μg/mg of proteins). All enzymes were from Boehringer. Samples were incubated at 37°C for 30 min and submitted to gel filtration. Reference samples were treated in the same conditions (i.e. incubations at 0°C and at 37°C without added enzymes) in order to eliminate a loss of binding activity due only to increased temperature.

Competition assays. Cytosol samples were incubated at 0°C for 6 h in the presence of labelled hormones in saturating concentrations ($T = 1 \times 10^{-9}$ M, $DHT = 3 \times 10^{-10}$ M). Simultaneously unlabelled steroids were added in a 500-fold excess for testosterone receptor, and in a 50-fold excess for dihydrotestosterone receptor. Bound radioactivity was obtained by gel filtration or by protamine precipitation, and results were expressed as competition efficiency according to Atger's equation [13]. In this case, the displacement of labelled hormone by the same unlabelled compound is considered as 100% competition efficiency. Displacements by other steroids are referred to this maximal efficiency.

Protein binding of androgens in rat serum. In some experiments, blood was collected and an aliquot of serum was diluted in order to obtain a suitable concentration of proteins (2.9 mg/ml). Binding studies were carried out *in vitro* under the same experimental conditions as were used in binding studies with cytosol. Bound radioactivity was separated by protamine precipitation.

Other analytical procedures. For chromatographic analysis, steroids were extracted from bound fraction by the method of Folch *et al.*[17] modified by Bruchowsky[15]. They were identified by paper chromatography as described before [4].

Radioactivity was measured in a liquid scintillation spectrometer (Isocap 300, Nuclear Chicago) using the channels ratio method to correct for quenching if necessary. Aqueous samples and glass fibre filters were counted in the Bray's mixture [16], and chromatogram strips in a toluene system.

Proteins were determined by the method of Lowry *et al.*[17] using bovine serum albumin as standard.

RESULTS

As shown in Fig. 1 binding equilibrium was reached after 5 h and remained stable up to 24 h. Thus, 6 h seemed to be an appropriate time for our experiments. The steroids bound at equilibrium were submitted to chromatographic analysis. It was pointed out that no significant metabolism of testosterone or dihydrotestosterone occurred at 0°C even following an extensive period.

Analysis of the binding data according to Scatchard[12] indicated the presence of a high affinity and low capacity binding macromolecule for testosterone and dihydrotestosterone in cytosols from hypothalamus and preoptic area. Such a macromolecule was not found in the cytosol from frontal cortex (Table 1). (Figs. 2 and 3).

From competition experiments it could be ascertained that testosterone and dihydrotestosterone had the same competition efficiency towards the binding sites for both ligands. However, it should be taken into account that a ten-fold less concentration of dihydrotestosterone than of testosterone was used in these experiments. In these conditions, their equal competition efficiency corroborates the different affinities found for these two steroids. Some other hormones, including estrogens, were also competitive

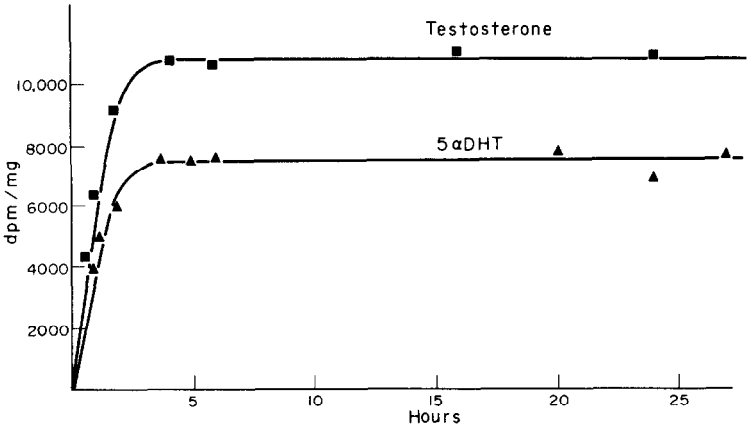

Fig. 1. Study of the binding of [^{3}H]-testosterone and [^{3}H]-dihydrotestosterone in terms of incubation times, in the cytosol from hypothalamus of prepubertal male rats. Cytosol samples were incubated at 0°C with [^{3}H]-Testosterone (2.4×10^{-8} M) or [^{3}H]-dihydrotestosterone (1.2×10^{-9} M).

Table 1. Some characteristics of binding proteins for androgens in hypothalamus and preoptic area from prepubertal male rats; results were the same whether gel filtration or protamine precipitation was used to obtain bound fraction

Brain region and ligand	K_D	Number of binding sites (mol per mg of proteins)
Hypothalamus		
Testosterone	7×10^{-10} M	6×10^{-15}
Dihydrotestosterone	7×10^{-11} M	1×10^{-15}
Preoptic area		
Dihydrotestosterone	6.2×10^{-11} M	2×10^{-15}

to a great extent. Only corticosterone and androstenedione could be considered as inefficient (Table 2).

When hypothalamic cytosol incubated only in the presence of labelled hormone was filtered through a Sephadex G 200 column, three distinct radioactive peaks were found. The first one was excluded, the second represented slightly retained materials, and the third was free radioactivity (Fig. 4). These two kinds of binding sites are thought to represent specific and non specific components and could be also calculated

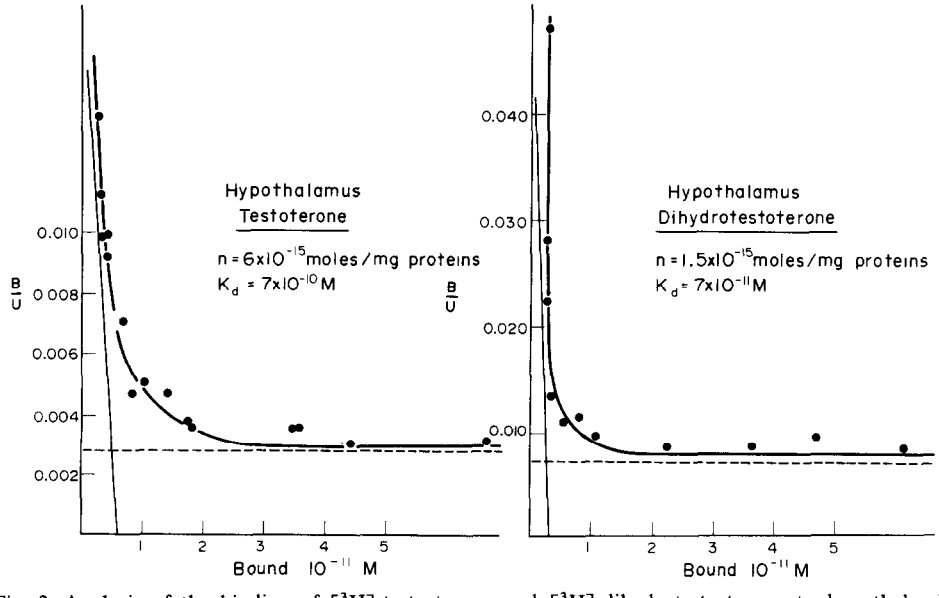

Fig. 2. Analysis of the binding of [^{3}H]-testosterone and [^{3}H]-dihydrotestosterone to hypothalamic receptors by Scatchard plot. Specific binding was calculated using the Rosenthal method [18].

Table 2. Effects of some unlabelled steroids on the specific binding of [^{3}H]-testosterone or [^{3}H]-dihydrotestosterone in the cytosol from prepubertal male rats hypothalamus; cytosol samples were incubated in the presence of 1×10^{-9} M tritiated testosterone and 5×10^{-7} M unlabelled steroids, and of 3×10^{-10} M tritiated dihydrotestosterone and 1.5×10^{-8} M unlabelled steroids respectively. Results are expressed as competition efficiency according to Atger[13]

Unlabelled steroid	Bound testosterone %	Bound dihydrotestosterone %
Testosterone	100	135
Dihydrotestosterone	97	100
5α-Androstan-3β,17α-diol	92	80
5α-Androstan-3α,17α-diol	67	42
Androstenedione	29	19
Diethylstilboestrol	60	37
17β-Estradiol	97	96
Corticosterone	18	5
Progesterone	87	78

by Scatchard analysis of the numerical data obtained from cytosol incubations without unlabelled ligand, using the Rosenthal method [18]. The study of the effects of enzymes showed that this macromolecule was protein in nature (Table 3).

It could also be demonstrated that hypothalamic binding proteins were not of blood origin. Indeed, when serum was incubated in the presence of labelled androgens and then submitted to protamine precipitation no radioactivity was retained by filters. Also, when it was filtered through Sephadex G 200, the K_{AV} of its binding proteins was different from that of the cytosol binding proteins.

DISCUSSION

We found in the cytosol from hypothalamus and preoptic area of prepubertal male rats specific binding proteins for androgens. Both testosterone and dihyd-

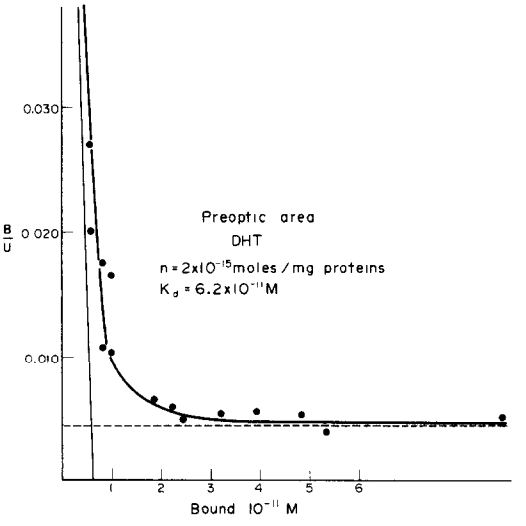

Fig. 3. Scatchard plot of the binding data for [^{3}H]-dihydrotestosterone in preoptic area. Specific binding was calculated using the Rosenthal method [18].

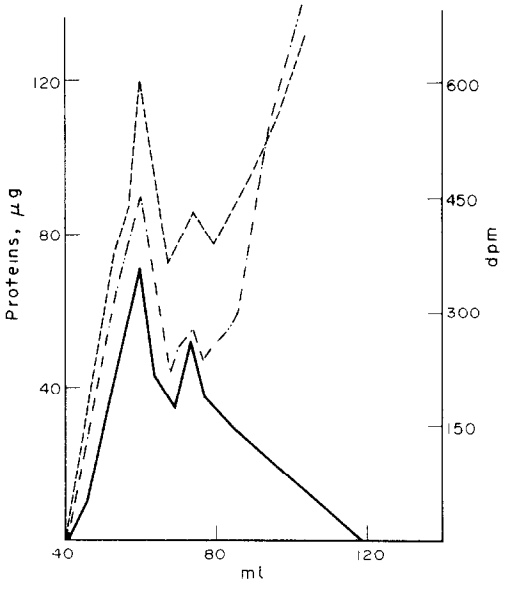

Fig. 4. Gel filtration on Sephadex G 200 of the cytosol fraction of hypothalamus after incubation at 0°C and at equilibrium with tritiated testosterone or dihydrotestosterone. —— Proteins elution. – – – – Elution of radioactivity after incubation with [^{3}H]-testosterone. ——·····—— Elution of radioactivity after incubation with ^{3}H-dihydrotestosterone.

Table 3. Effects of various enzymes on testosterone or dihydrotestosterone binding. Results are expressed as % decrease of bound radioactivity compared with reference samples; all experiments were made in triplicate

Enzymes	Testosterone %	Dihydrotestosterone %
Pronase	66	47
Trypsine	66	20
Chymotrypsine	66	27
RNAase A	14	0
RNAase T_1	9	4

rotestosterone were bound and affinity was higher for dihydrotestosterone than for testosterone (see constants in Table 1). On the other hand, we failed to find an androgen receptor in the cytosol from cerebral cortex. Our results are in agreement with those of Kato who did not find any cortical receptor for androgens [20]. This author also found a receptor for dihydrotestosterone in the cytosol from hypothalamus of mature and immature, intact and castrated rats. Its K_D was 3 to 7 $\times 10^{-10}$ M and its binding capacity 3.8 to 9 $\times 10^{-15}$ mol per mg of proteins [20]. In 1973 and 1975 he failed to find a receptor for testosterone in the same cytosol [6, 20]. However in 1976 he succeeded in finding such a macromolecule, the K_D of which was 2.3 $\times 10^{-9}$ M and number of binding sites 2 $\times 10^{-15}$ mol/mg proteins [22].

On the other hand, Gustafsson *et al.* demonstrated the occurrence of a receptor for testosterone in hypothalamus, cerebral cortex and pineal gland from mature and immature rats ($K_D = 1 \times 10^{-10}$ M, number of binding sites = from 0.1 to 1 $\times 10^{-15}$ mol/mg proteins according to the age of animals) [8]. Also Naess found a very specific receptor for testosterone in hypothalamus preoptic area and cerebral cortex of adult castrated rats. K_D were respectively 3.4, 4.3, 2.6 $\times 10^{-10}$ M and binding capacities 3.7, 3.5 and 1.8 $\times 10^{-15}$ mol/mg proteins [7]. Barley *et al.* found a receptor for dihydrotestosterone in rat hypothalamus ($K_D = 3 \times 10^{-10}$ M) [19]. Contrarily, some authors failed to find androgen receptors in hypothalamic cytosol [5, 21].

The specificity of the hypothalamic androgen receptor is a debated question. Naess showed that neither androgens nor estrogens were competitive towards the binding sites for testosterone [2]. On the other hand, Kato found that unlabelled androstenedione, dehydroepiandrosterone, epiandrosterone, cortisol and diethylstilboestrol were without competitive effects, whereas estradiol-17β was efficient [6, 20]. Taking into account that diethylstilboestrol exhibits potent estrogenic properties, it is surprising that a competitive action of estradiol and not of diethylstilboestrol could be found in hypothalamic cytosol, inasmuch as these two compounds had the same competitive efficiency towards the dihydrotestosterone receptor in prostate. For us, we found that both estradiol and diethylstilboestrol partially inhibited the binding of androgens in hypothalamic cytosol. Our results are in good agreement with those of Barley *et al.* [19] and it seemed that there were three groups of steroids: 1. Those which inhibited the androgen binding beyond 85%, such as testosterone, dihydrotestosterone, 5α-androstan-3β,17β-diol, 17β-oestradiol. 2. Those which inhibited it beyond 50%: 5α-androstan-3α,17β-diol, progesterone. 3. Those which were without any significant effect: corticosterone and androstenedione.

Finally our results show that there are in the hypothalamic cytosol from immature rats some proteins which agree with three criteria of a receptor out of four: high affinity, low capacity, tissue specificity. Whether there are true receptors or only proteins able to bind both androgens and estrogens is the first problem. The second is the low level of radioactivity bound to the proteins. Is this fact due to the partial filling of binding sites by andogenous hormones, or due to the occurrence of binding proteins only in a few hypothalamic cells? The third problem but not the least is concerned with the physiological role of such binding proteins towards the regulation of releasing hormone secretion.

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