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A SPECIFIC ESTRADIOL-17β RECEPTOR IN CELL NUCLEI FROM ANTERIOR HYPOPHYSIS OF IMMATURE MALE RATS

C. LE GUELLEC, M. L. THIEULANT, S. SAMPEREZ and P. JOUAN*
Laboratoire de Neurobiologie Moléculaire (Equipe de Recherche associée au C.N.R.S. no. 567)
Faculté des Sciences Biologiques, Av. du Général Leclerc, 35000 Rennes, France

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SUMMARY

The presence of a specific receptor for estradiol-17β in purified nuclei from prepubertal male rats was demonstrated. Its sedimentation constant was 4–5 *S*, and it was shown to be partly a protein. Estrogens were competitive towards its binding sites whereas androgens were completely inefficient. It was found only when nuclei were incubated at 25°C in the presence of the cytosol receptor. These facts led us to think that it was due to the nuclear translocation of a part of the soluble receptor by a two-step, temperature-dependent mechanism.

INTRODUCTION

Two hours following the injection of tritiated estradiol to male rats. radioactivity was recovered in some regions of the brain, and its amount in tissues was higher than in blood. The highest uptake was found in anterior pituitary [1]. Moreover when unlabelled estradiol was administered simultaneously with tritiated hormone, the pituitary uptake was significantly reduced [1]. These facts were in favour of the presence of receptors for estradiol-17β in the male rat anterior pituitary. In fact, several laboratories demonstrated the presence of specific receptors in the pituitary cytosol from adult or immature, castrated or intact male rats [2–7]. Recently we were able to confirm these findings [8, 9].

The presence of a nuclear receptor for estradiol was first demonstrated by Clark *et al.* [10]. Their technique consisted in incubating immature male rats pituitaries with tritiated estradiol and diethylstilboestrol (DES) followed by ethanol extraction of nuclear radioactivity.

The aim of our work was to find some precise details concerning the nuclear receptor of estradiol in the prepubertal male rats pituitary, and to define its main characteristics.

MATERIALS AND METHODS

Animals. Male rats (34–37 day-old) from Elevage Janvier (53680 Le Genest, France) were used in our experiments. They were sacrificed by decapitation without anaesthesia.

Incubations of whole hypophysis. Eighteen anterior hypophysis were quickly removed and washed in a small vol. of cold Krebs–Ringer solution. Then, they were incubated in 1 ml of the same solution in the presence of 4×10^{-7} M tritiated estradiol. Incubations were carried out at 37°C, for 60 min in an atmosphere of O_2–CO_2 (95–5). At the end of incubation, organs were washed with Krebs–Ringer buffer and purified nuclei were then prepared.

Incubations of cytosol. Twelve anterior pituitaries were homogenized in 500 μl of 0.05 M phosphate buffer pH 7.4, using a Potter homogenizer fitted with a Teflon pestle. Homogenate was first centrifuged at 600 *g* for 10 min, then at 105,000 *g* for 1 h in a SW 50 rotor of a L4 Beckman ultracentrifuge. The supernatant was considered as cytosol. All operations were carried out at 4°C. The pituitary cytosol was then incubated at 0°C for 1 h in the presence of 2×10^{-9} M tritiated estradiol.

Incubations of purified nuclei. Purified nuclei were obtained from 12 pituitaries as previously described [11]. They were then incubated for 1 h either at 0°C or at 25°C in 480 μl of previously labelled cytosol. At the end of incubation nuclei were spun down at 3,500 *g* for 10 min. The nuclear pellet was carefully washed with 0.05 M phosphate buffer and extracted with 1 M NaCl as described below. In some experiments, purified nuclei were directly extracted with 1 M NaCl. This extract was then incubated for 1 h at 25°C with tritiated estradiol (2×10^{-9} M). Bound materials were separated by gel filtration through Sephadex G25 column.

Nuclear materials extraction. Purified nuclei were extracted using 500 μl 1 M NaCl (pH 5.5). Extraction was carried out at 0°C for 2 h with magnetic stirring. Then the homogenate was centrifuged for 20 min at 25,000 *g* and the supernatant was submitted to analysis.

Sucrose gradient analysis. Linear 5–20% sucrose density gradients were prepared either in 0.05 M

* To whom all correspondence and requests for reprints should be addressed.

phosphate buffer for cytosol analysis, or in 1 M NaCl for nuclear extracts analysis. 500 μl of cytosol or of nuclear extract were layered on the gradients and centrifuged at 130,000 g for 16 h, at 4°C in the SW 50 rotor of a L4 Beckman ultra-centrifuge. Then gradients were fractionated using a "density gradient fractionator" fitted with a U.V. analyzer (UA_2 ISCO). Fractions (6 drops) were collected and radioactivity was measured in every fraction. Sedimentation coefficients were determined according to Martin and Ames [12] using bovine serum albumine as internal standard.

Gel filtration. Cytosols and nuclear extracts were filtered through columns of Sephadex G 25 medium (30 cm × 1.5 cm) equilibrated with either 0.05 M Phosphate buffer or 1 M NaCl. Void vol. was in each case 18 ml. Bound radioactivity was collected by fractions (1.5 ml) and, in every fraction, proteins and radioactivity were measured.

Nuclear extracts were also submitted to gel filtration through a column of Sephadex G 200 (80 cm × 1.5 cm) overlayed with a small amount of Sephadex G 25, and equilibrated with 1 M NaCl. Work pressure was 7 cm in water and flow rate was 7 ml per h. Void vol. was 42 ml. Fractions (2 ml) were collected and submitted to analysis for proteins and radioactivity. All gel filtrations were carried out at 7°C.

Competitions studies. Cytosol fractions (480 μl) were incubated at 0°C for 1 h in the presence of 2×10^{-9} M tritiated estradiol and of 2×10^{-7} M competitor. Then, purified nuclei from 18 pituitaries were added to cytosol fractions and a second incubation was carried out at 25°C during 1 h. Finally nuclei were pelleted and extracted with 1 M NaCl. Nuclear extracts were then submitted to gel filtration through a Sephadex G 25 medium column. Steroids tested as possible competitors were: 17β-estradiol, diethylstilboestrol, testosterone, dihydrotestosterone. Results were expressed as percentage of binding compared to binding in controls.

Enzymes action. Purified nuclei from 24 pituitaries were incubated for 1 h at 25°C in cytosol previously labelled with estradiol (1.3×10^{-9} M). They were then extracted with 1 M NaCl and resulting nuclear extracts were incubated for 30 min at 37°C in the presence of various enzymes. Trypsine, pronase, pancreatic ribonuclease, deoxyribonuclease were dissolved in 1 M NaCl before use (50 μg/(10 μl). Nuclear extracts were then filtered through Sephadex G 25 column. Control samples were incubated under the same conditions but without enzymes.

Analysis of bound steroids. 1 M Nuclear extract was filtered through Sephadex G 25 column and bound steroids were extracted using the method of Folch [13] modified by Bruchowsky [14]. Steroids were then analyzed by paper chromatography in the solvent system of Payne *et al.* [15]: isooctane–toluene–methanol–water (25:75:80:20, by vol.). Unlabelled internal standards (estradiol-17β, estrone, estriol) were used, and migration time was 4 h. Paper strips were then cut into segments, and radioactivity was counted.

Other analytical procedures. Proteins were estimated according to Lowry *et al.* [16]. Radioactivity was measured in the Bray's mixture for phosphate buffer extracts [17] and in Instagel for NaCl extracts. Chromatogram strips were counted in a toluene scintillator system. All measures were made with the help of an Isocap 300 counter (Nuclear Chicago) using the channels-ratio method to correct for quench when necessary.

Steroids and chemicals. [1,2,6,7-^{3}H]-Estradiol (S.A. 90 Ci/mmol) was purchased from the Radiochemical Centre, Amersham (England). Its purity was checked by paper chromatography in several solvent systems, and was 98%. Unlabelled steroids were from Sigma Chemical Co. Enzymes were supplied by Boehringer (Mannheim). Sephadex G-25 and G-200 was from Pharmacia Fine Chemicals (Uppsala).

RESULTS

Extraction of purified nuclei with 1 M NaCl

Under the conditions described above, the degrees of recovery were 93% for DNA, 60% for proteins and 80% for radioactivity [8].

Nuclear binding of tritiated estradiol

When whole hypophysis were incubated with tritiated estradiol (4×10^{-7} M) macromolecular binding of the hormone was found in the 1 M NaCl nuclear extract. Its S.A. was 18,000 d.p.m./mg proteins (136 fmol).

Translocation of estradiol-receptor from cytosol to nucleus

Some conclusions could be drawn from these experiments: 1. When 1 M NaCl nuclear extracts were directly incubated with tritiated estradiol, no bound radioactivity could be found. 2. When nuclei were incubated for 1 h at 0°C in previously labeled cytosol, no binding occurred in 1 M NaCl extracts. 3. When nuclei were incubated for 1 h at 25°C in prelabelled cytosol, then bound radioactivity could be found in nuclear extracts. Besides, the amount of bound hormone missing in cytosol was recovered from nuclear extracts in a bound form (Table 1).

These results seemed to indicate that a nuclear translocation of a part of the estradiol soluble receptor occurred and that this process was dependent on temperature.

Properties of the nuclear macromolecular association of [^{3}H]-estradiol

1. *Analysis of bound hormone.* Bound radioactivity in nuclear extracts was isolated by gel filtration. Steroids were extracted and submitted to paper chromatography as described in Materials and Methods. Results showed that at least 95% of bound radioactivity had the same mobility as pure estradiol.

Table 1. Distribution of bound radioactivity between cytosol and nuclei

	Cytosol incubated alone at 0°C	Cytosol incubated in the presence of nuclei	1 M NaCl nuclear extract
Whole bound [^{3}H]-estradiol(fmol)	51	35	17
S.A. (fmol/mg protein)	82.7	53.5	62.7

Purified nuclei were incubated for 1 h at 25°C in cytosol previously labelled with [^{3}H]-estradiol (2×10^{-9} M). (For technical details see Materials and Methods).

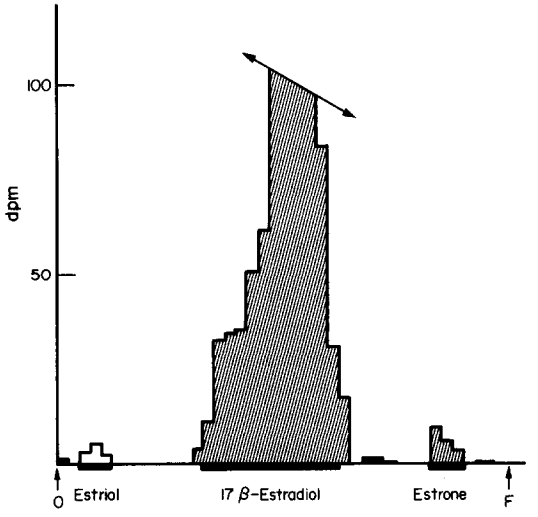

Fig. 1. Paper chromatography of estrogens bound in nuclear extracts. Following incubation in labelled cytosol, nuclei were extracted with 1 M NaCl. The extract was filtered through a Sephadex G 25 column and steroids from bound fractions were extracted and analyzed as described in Materials and Methods. Horizontal bars indicate the migration of unlabelled internal standards.

Thus the estrogen bound to nuclear receptor appeared to be estradiol (Fig. 1).

2. *Specificity of binding.* Cytosol fractions were incubated with [^{3}H]-estradiol (2×10^{-9} M) in the presence of steroid competitors (2×10^{-7} M). Purified nuclei were then incubated in these samples and bound radioactivity in 1 M NaCl nuclear extracts was isolated by gel filtration. Results are shown in Fig. 2. They demonstrated that only estrogens i.e. estradiol-17β, estrone and DES were competitive towards the binding sites of estradiol. Androgens (testosterone and dihydrotestosterone) were completely inefficient.

3. *Chemical nature of the estradiol receptor.* Labelled nuclear extracts were submitted to the action of several enzymes. It was found that pronase entirely destroyed the macromolecular binding of estradiol whereas trypsin was less efficient. On the other hand it appeared that nucleases were partially active. Thus it seemed that the nuclear macromolecule bound to estradiol was only in part of protein nature (Table 2).

4. *Ultra-centrifugation on sucrose density gradients.* Fractions of cytosol incubated with [^{3}H]-estradiol (2×10^{-10} M) alone or in the presence of purified nuclei, and nuclear NaCl extracts were analyzed on linear sucrose gradients (5–20%). Results showed that the sedimentation constant of the cytosol receptor obtained at 0°C was 8–9 *S*. When cytosol was incubated at 25°C alone or in the presence of nuclei, it became 4–5 *S*. The sedimentation constant of the nuclear receptor extracted by M NaCl was also 4–5 *S* (Fig. 3).

5. *Gel-filtration on Sephadex G* 200. 1 M NaCl nuclear extracts were filtered through a column of Sephadex G 200. As shown in Fig. 4, proteins were eluted into two fractions: the first was excluded from gel, the second was slightly retained. DNA was eluted together with the first peak of proteins. Only one peak of radioactivity occurred, at the same time as DNA and the first fraction of proteins. Taking into account that the sedimentation constant of the nuclear receptor was 4–5 *S*, it could be concluded that gel filtration involved the formation of aggregates.

DISCUSSION

Our results are in agreement with and corroborate previous data obtained by Clark *et al.* [10] concerning the specific binding of estradiol in nuclei from immature male rat pituitary.

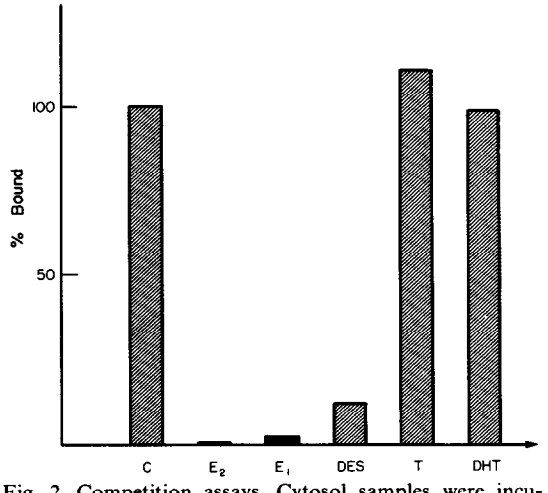

Fig. 2. Competition assays. Cytosol samples were incubated for 1 h at 0°C, in the presence of 2×10^{-9} M tritiated estradiol and of 2×10^{-7} M steroid competitors. Purified nuclei were then incubated in these samples for 1 h at 25°C. They were extracted with 1 M NaCl and bound radioactivity was isolated by gel filtration. Results are expressed as a percentage in relation with controls considered as 100. C: Control, E_2: 17β-estradiol, E_1: estrone, DES: Diethylstilboestrol, T: testosterone, DHT: 5α-dihydrotestosterone.

Table 2. Action of enzymes on the nuclear binding of estradiol.

Enzymes	d.p.m./mg proteins	% of controls
None (controls)	7 382	100
Pronase	0	0
Trypsine	4 579	62
RNAse A	5 781	78
DNAse	4 107	55

1 M NaCl nuclear extracts were incubated for 30 min at 37°C in the presence of various enzymes as described in Materials and Methods. Bound radioactivity was isolated by gel filtration. Results are expressed as bound radioactivity either in d.p.m./mg proteins, or in % of controls. (Each assay was made in duplicate).

We demonstrated the presence of a receptor in purified nuclei from immature male rat pituitary; its sedimentation constant is 4–5 *S*. It originates from cytoplasm since no estradiol binding was found following direct incubation of nuclear extracts in the presence of labelled hormone. It is thus obvious that the presence of a cytosol receptor is required for such a nuclear binding and that this receptor is translocated to nucleus by a "two-step", temperature-dependent mechanism. Translocation is a quantitative phenomenon and the fraction of bound radioactivity lost in cytosol is recovered in nuclei.

The nuclear receptor is very specific for estrogens since androgens are not competitive towards their binding sites. It is at least in part a protein, and pronase completely abolished the binding of estradiol. However trypsin is less efficient than pronase. This fact may be due both to the primary structure of the receptor and also to the specificity of action of this enzyme.

However, the biological significance of our results is not at all obvious, inasmuch as the precise role of gonadal steroids in pituitary regulation remains to be clarified. It is now demonstrated that androgens are efficient in the regulation of LH and FSH secretion. The most active of them is 5α-dihydrotestosterone. 5α-Androstane-3α,17β-diol was also considered as an efficient hormone. But the lack of pituitary receptors for it and its intensive conversion into dihydrotestosterone suggest that it is only efficient via DHT [9, 18].

However estradiol-17β is more potent than androgens in inducing the decrease of gonadotropins secretion in male rats [19, 20, 22–26]. It is also able to reduce plasma concentrations of LH and FSH following LH-RH administration [27–29]. On another hand, it is known that growth hormone secretion [30, 31] as well as adrenocorticotropin or prolactin secretions [32] are increased by estrogens administration to male rats. Moreover thymidine kinase activity in the pituitary is increased by estrogen injections to prepubertal male rats [33].

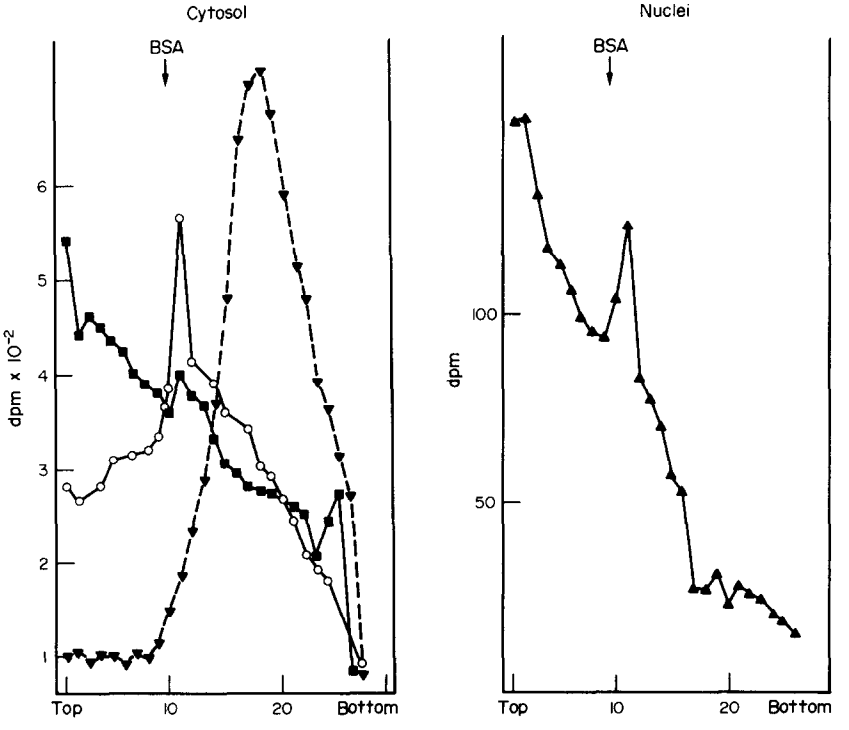

Fig. 3. Sucrose gradient analysis. Cytosol samples and nuclear extracts were layered on 5–20% sucrose gradients, and ultracentrifuged at 130,000 *g* for 16 h at 4°C. Left panel: ▼--▼ 8–9 *S* peak from cytosol incubated alone at 0°C in the presence of 2×10^{-9} M tritiated estradiol. ■——■ 4–5 *S* peak from cytosol incubated alone for 1 h at 0°C and then for 1 h at 25°C. O——O 4–5 *S* peak from cytosol incubated successively for 1 h alone at 0°C, and then for 1 h at 25°C in the presence of nuclei. Right panel: 4–5 *S* receptor from nuclei incubated for 1 h at 25°C in prelabelled cytosol.

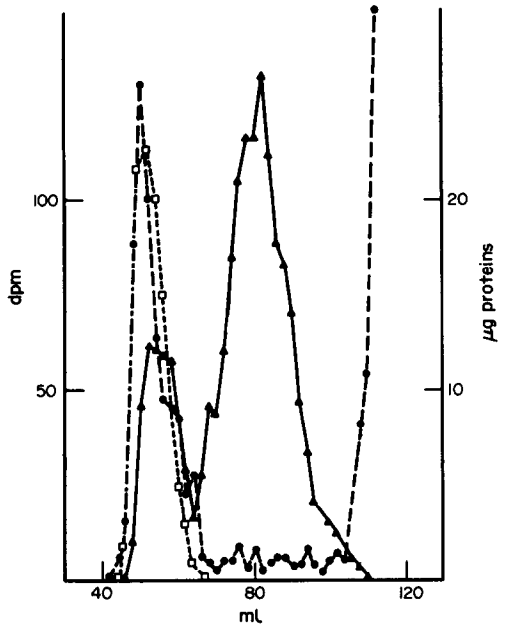

Fig. 4. Gel-filtration through Sephadex G 200 of 1 M NaCl nuclear extracts. ●——● Elution of radioactivity.□····□ Elution of DNA. ▲——▲ Elution of proteins.

From all these data, it seems that estrogen actions at pituitary level in the male are mediated by its receptors and that anterior pituitary could perhaps be considered as a target organ for estrogens.

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