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ANDROGEN RECEPTORS IN RAT VENTRAL PROSTATE MICROSOMES

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SUMMARY

The androgens 17β-hydroxy-5α-androstane-3-one (androstanolone, dihydrotestosterone) and 5α-androstane-3β,17β-diol (3β-androstanediol) bind to a single specific component of the "microsomes" (105 000 $\times g$ sediment) of adult rat ventral prostate. The apparent association constant is $2.5 \pm 0.9 \cdot 10^{10}$ M^{-1} (mean $\pm$S.E.) for androstanolone and $6.5 \pm 4.4 \cdot 10^{10}$ M^{-1} for 3β-androstanediol. The number of binding sites approaches 10 fmoles/mg microsomal proteins. The specific binding is inhibited by proteolytic enzymes and–SH blocking agents. Competition studies show that testosterone and 5α-androstane-3α,17β-diol are weak competitors whereas estradiol, progesterone and cortisol are ineffective. No such high affinity binding was observed in rat liver microsomes. On the basis of apparent affinity and steroid specificity determinations, the microsomal receptor seems different from the androgen receptor previously described in rat ventral prostate cytosol.

INTRODUCTION

In addition to testosterone and androstanolone, several other androgens were shown to prevent the regression of normal rat ventral prostate explants kept in organ culture [1, 2]. All these compounds belong to the 5α-androstane series, and thus their activity might be explained by their possible conversion into androstanolone inside the prostate cell, as well documented for a variety of 3α-hydroxy-5α-androstane compounds [3, 4]. Indeed, androstanolone is the main steroid found in rat ventral prostate nuclei after the injection of testosterone in vivo [5] or incubation in vitro [6] and androstanolone has the highest affinity for the receptor of rat ventral prostate cytosol [7–9]. However, it was observed that the oxidation to a 3-ketone of the hydrox-

Abbreviations: testosterone: 17β-hydroxy-androst-4-ene-3-one; androstanolone: 17β-hydroxy-androstane-3-one (also known as dihydrotestosterone); 3β-androstanediol: androstane-3β, 17β-diol; 3α-androstanediol: androstane-3α, 17β-diol; epiandrosterone: 3β-hydroxy-androstane-17-one; dehydroepiandrosterone: 3β-hydroxy-androst-5-ene-3-one; estradiol: 1,3,5(9)-estratriene-3,17β-diol; progesterone: pregna-4-ene-3,20-dione; cortisol: 11β, 17α, 21-trihydroxy-pregna-4-ene-20-one.

yl group of the 3β-hydroxy steroids tested (3β-androstanediol, epiandrosterone, dehydroepiandrosterone) was exceedingly small [3]. Thus, the possibility was raised for molecules like 3β-androstanediol to be active as such. However, an obvious drawback to this assumption was the failure to demonstrate any high affinity, low capacity, specific binding (receptor) for 3β-androstanediol in rat ventral prostate cytosol [10, 11].

Evidence for a receptor which selectively binds androstanolone and 3β-androstanediol in rat ventral prostate microsomes is reported here. A preliminary report was previously published [10].

MATERIALS AND METHODS

Steroids

Testosterone, androstanolone, androstanediol, 3β-androstanediol, estradiol, progesterone and cortisol were given by Roussel-UCLAF and guaranteed 99.5 % pure. [1,2-$^3H_2$]testosterone was obtained from Mol (40 Ci/mmole) and [1,2-$^3H_2$]androstanolone (40 Ci/mmole) was made by M. Pichat (C.E.A.) according to a tech-nique by R. Emiliozzi using Δ_1-androstanolone (courtesy of Schering AG, Berlin). It was reduced by Tritium gas with a charcoal–palladium catalyst at room temperature for 30 min. Thin-layer chromatography on silica gel GF plates in the system benzene–ethyl acetate (3 : 2, v : v) gave a product, the purity of which was checked by isotopic dilution with the reference steroid, and crystallization to constant specific activity.

[1,2-$^3H_2$]Androstanedio land 3β-[1,2-$^3H_2$]androstanediol were made from [1,2-$^3H_2$]androstanolone. 5 nmoles of the latter were dissolved in 0.8 ml of methanol. 30 μl of a 20-mg/ml solution of KBH_4 in water were added, and stirred 2 min at room temperature. The reaction was stopped by the addition of 3 drops of glacial acetic acid. 2 ml of water were added and the steroids extracted with dichloromethane (10 ml). The extract was washed with water, dried over Na_2SO_4 and chromatographed on Whatman No. 1 paper in the solvent system hexane–benzene (1 : 1)–propyleneglycol at 25 °C for 18 h. This procedure resulted in the separation of a small amount of unreacted androstanolone (<3 %) in the outflow of the chromatogram and of the two isomers 3α-androstanediol (29 %) and 3β-androstanediol (68 %). The purity of the androstanediols, checked by isotopic dilution with reference steroid, and crystallisation to constant specific activity, was ≥ 98 %. The radioactive steroids were kept at − 20 °C in a benzene –(10 %)ethanol solution and their radiochemical homogeneity was checked every two months by thin-layer chromatography and isotopic dilution.

Tissue fractionation

12-week-old Wistar rats (body weight 250–300 g) were castrated via the scrotal route. Ventral prostates were removed 24 h later, dissected free of fat and connective tissue, and kept in ice-cold homogenization buffer. All subsequent operations were performed at 0–4 °C and the experiments were completed in the same working day unless otherwise stated. Approx. 2.5 g of ventral prostate were minced with scissors, suspended in 4 vol. of 0.25 M sucrose, 1.5 mM EDTA, 2 mM mercaptoethanol, 10 mM Tris–HCl(pH 7.4)buffer (homogenization buffer), and homogenization was performed in a Teflon-glass Potter homogenizer, with 15 strokes of a motor driven pestle (Heidolph, setting 30). Filtration through two layers of cheese cloth was fol-

lowed by centrifugation at $800 \times g$ for 15 min, and the resulting supernatant was centrifuged at $105\,000 \times g$ for 90 min. The final pellet was resuspended in 10 ml of the homogenization buffer and homogenized in a Teflon-glass Potter apparatus (10 strokes at low speed), and diluted to 50 ml with the same buffer.

The standard binding assay was performed as follows: an appropriate amount of radioactive androgen was taken to dryness in a 20-ml stoppered glass vial. A 3-ml aliquot of the microsome suspension containing approx. 1.5 mg of proteins was added and incubated at 0 °C for 2 h with occasional stirring. This time length allowed complete dissolution of the radioactive steroid and binding equilibrium. Thereafter, the incubates were transferred into 3 ml cellulose nitrate tubes fitted to a Beckman Ti 50 rotor by means of adaptators, and spun at $105\,000 \times g$ for 60 min. The supernatants were carefully decanted. The translucent yellowish pellets were rinsed with a few drops of chilled homogenization buffer then the bottom of the tube corresponding to the microsomal pellet was cut and deposited in a liquid scintillation vial. To elute the radioactive steroid, 3 ml of absolute ethanol were added and after vigorous shaking for 1 min, the vials were kept overnight. 10 ml of scintillator solution were then added (4 g Omnifluor in 1 l of toluene). Similarly, 0.2 ml of supernatant were taken up in 3 ml of ethanol–10 ml of Omnifluor. Counting was performed in a Packard Tricarb or an Intertechnique SL 30 spectrometer. Quenching was corrected with the use of external standardization. The radioactivity of the pellet was considered as bound, whereas the radioactivity in the supernatant was considered as free.

Control experiments were made to account for the possible adsorption of [^{3}H]androgen onto the wall of the cellulose nitrate tube. Surfaces of the bottom of the tubes equivalent to the surface of the pellet were cut in several experiments and counted. This represented less than 10 % of the radioactivity in the pellet. In other experiments, the 3-ml of microsomal incubate were layered upon 2 ml of homogenization buffer made 0.88 M in sucrose, and the pellet was collected after centrifugation at 45 000 rev./min for 60 min in a Beckman SW 50 rotor. The microsomal pellet was cut as usual. With that procedure, very small amounts of radioactivity are adsorbed at the bottom of the cellulose nitrate tube. The results obtained were not significantly different of those given by the simplest procedure. In some instances, the incubated microsomes were passed through small Sephadex G-25 columns prepared in homogenization buffer, to separate the bound and free radioactive steroids, with comparable although less reproducible results when compared to ultracentrifugal sedimentation. The concentrations of radioactive androgens used were in the 0.005–5 nM range. Scatchard-type plots were drawn in order to determine the binding affinity constants and the number of sites. Correction was made for the concentration of low affinity binding as described by Rosenthal [12].

Appropriate aliquots of the microsomal suspensions were kept for protein measurement by the Lowry procedure.

RESULTS

Binding parameters of androstanolone, testosterone, 3β-androstanediol and 3α-androstanediol

Representative Scatchard plots are given in Fig. 1. It can be seen that androstanolone and 3β-androstanediol are bound to two kinds of binding components.

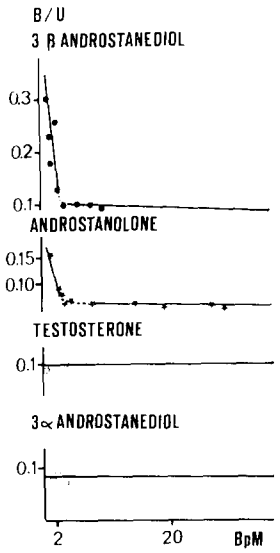

Fig. 1. Scatchard-type plot of the binding of 3β-androstanediol, androstanolone, testosterone and 3α-androstanediol to rat ventral prostate microsomes. B, radioactivity in the resedimented microsomal pellet. U, radioactivity in the final supernatant. Increasing amounts of radioactive androgens (0.005–5 nM) were added to 3 ml of microsomal suspensions (0.5 mg/ml). The number of specific binding sites was deduced from the intercept of the "specific" and "non-specific" lines according to Rosenthal [12].

TABLE I

APPARENT BINDING CONSTANTS OF ANDROSTANOLONE AND 3β-ANDROSTANEDIOL TO RAT VENTRAL PROSTATE MICROSOMAL RECEPTOR

Three separate determinations were performed with both steroids.

	Association constant ($\cdot 10^{10}$ M^{-1})	Number of binding sites × fmoles/mg microsomal protein
Androstanolone	2.5 ± 0.9*	9.5 ± 3.0
3β-Androstanediol	6.5 ± 4.4	8.1 ± 6.0

* Mean ± standard error of the mean.

The first binding was observed for concentrations below 0.1 nM. The apparent association constants and numbers of binding sites are given in Table I. The small difference in the number of binding sites falls within experimental errors. The second binding was "non-specific" (characterized by large capacity and low affinity); its binding parameters could not be determined since it was not saturated within the limit of solubility of the steroids. Testosterone and 3α-androstanediol binding was different because no high affinity component similar to those observed with androstanolone and 3β-androstanediol could be detected.

Although the influence of castration was not fully investigated, the binding parameters of 3β-androstanediol to intact rat ventral prostate microsomes were

determined in one experiment. The affinity constant ($3.6 \cdot 10^{10}$ M^{-1}) and the number of specific binding sites (8.6 fmoles/mg protein) were identical to those observed 1 day after castration (Table I).

Competition experiments

Usually the Scatchard plots were performed with increasing concentrations of radioactive androgen for practical reasons, in order to reduce the counting time required for an accurate measurement of low counts. However, in control experiments, a constant amount of radioactive steroid was used with increasing concentrations of the non-radioactive corresponding molecule. In such conditions, a 50 % reduction of bound radioactivity in the microsomal pellet will occur when 10 pM of [^{3}H]androstanolone competes with 100 pM of the same unlabelled molecule, the further decrease in bound radioactivity being small with greater concentrations of androstanolone, due to the presence of a great amount of non-specific binding. Therefore, for the competition studies it was decided to incubate a fresh microsomal preparation with 10 pM of[^{3}H]androstanolone or 3β-[^{3}H]androstanediol, with or without a 10-fold concentration of competitor. The results of such competition experiments are given on Table II. They suggest that androstanolone and 3β-androstanediol are bound to the same set of binding sites with similar affinities. Testosterone and 3α-androstanediol are much weaker competitors, which might explain why no specific binding could be demonstrated with these compounds. Estradiol, progesterone and cortisol do not seem to compete for the binding of androstanolone and 3β-androstanediol to microsomes.

TABLE II

COMPETITION EXPERIMENTS

Rat ventral prostate microsomes were resuspended in the homogenization buffer (1.5 mg protein/3 ml), incubated with 10 pM radioactive precursor alone or in presence of 100 pM competitor, the microsomes were resedimented and the pellets counted as described in Materials and Methods.

Radioactive compound (10 pM)	[^{3}H]Androstanolone (dpm bound)	3β-[^{3}H]Androstanediol (dpm bound)
Competitor (100 pM)		
None	918	874
Androstanolone	465	441
Testosterone	731	699
3β-Androstanediol	444	502
3α-Androstanediol	700	736
Estradiol	883	846
Progesterone	917	728
Cortisol	827	819

Action of –SH blocking agents and of proteolytic enzymes

The nature of the binding component was further investigated with the use of proteolytic enzymes and –SH blocking agents. Samples of microsomal suspensions in homogenization buffer were treated with 1 mg of pronase /ml for 2 h at 0 °C, the

TABLE III

ACTION OF INHIBITORS

[^{3}H]Androstanolone with or without an excess of unlabelled androstanolone was added to 3 ml of microsomal suspensions for 2 h at 0 °C, then resedimented and counted. Pronase 1 and *p*-hydroxymercuribenzoate, inhibitors were added immediately prior to incubation. Pronase 2, inhibitor was added 2 h before [^{3}H]androstanolone. Concentration of microsomal proteins: 1 mg/ml (experiment with pronase) or 0.5 mg/ml (experiment with *p*-hydroxymercuribenzoate).

	Radioactivity in pellet (dpm)	
	[^{3}H]Androstanolone (10 pM)	[^{3}H]Androstanolone (10 pM) + androstanolone (1 nM)
Control	815	396
Pronase (1 mg/ml) 1	518	392
Pronase (1 mg/ml) 2	432	426
Control 1	352	182
Control 2	344	200
p-Hydroxymercuribenzoate (1 mM) 1	237	226
p-Hydroxymercuribenzoate (1 mM) 2	266	224

addition of 10 pM [^{3}H]androstanolone, with or without a 100-fold excess of androstanolone, being made either at the beginning or at the end of the pronase treatment. Thereafter, the microsomes were resedimented and the radioactivity in the pellet was measured. To investigate the action of a –SH blocking agent, 1 mM *p*-hydroxymercuribenzoate was added to one half of the microsomal suspensions before incubation with [^{3}H]androstanolone, in the same conditions as above. After resedimentation, the radioactivity in the pellets was measured (Table III). Whereas in control incubations the radioactivity in the pellet is almost halved by the addition of 100-fold unlabelled androstanolone, no significant difference is observed after the addition of pronase or *p*-hydroxymercuribenzoate, which is probably due to the enzymatic destruction of the specific component of the microsomes. Similar observations were made with the cytosol androgen receptor [13].

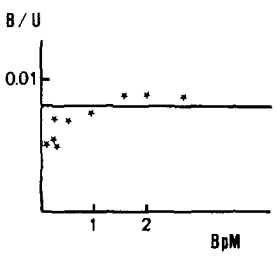

Fig. 2. Scatchard-type plot of the binding of [^{3}H]androstanolone to liver microsomes. B, radioactivity in the resedimented microsomal pellet. U, radioactivity in the final supernatant. Increasing amounts of radioactive androgens (0.005–5 nM) were added to 3 ml of microsomal suspensions (0.5 mg/ml).

Organ specificity

In addition to its hormone specificity, the microsomal receptor also shows an organ specificity. The binding of androstanolone to rat liver microsomes was investigated: only a very small amount of low-affinity, great-capacity binding was observed (Fig. 2).

DISCUSSION

Androstanolone and 3β-androstanediol bind with high affinity to the 105 000 $\times$ g pellet of rat ventral prostate. This binding is due at least partly to a protein, since it is sensitive to proteolytic enzymes and –SH blocking agents. It has the main characteristics of steroid hormone receptors: it is only present in target organs, it is steroid specific, and has a high affinity and limited capacity. The binding constants of the rat ventral prostate cytosol receptors have been determined by a technique using the specific precipitation of the receptors by protamine sulfate (Blondeau, J. P. and Robel, P., unpublished). In the 1-day castrated rat, the Scatchard plots obtained correspond to a complex binding pattern; the highest affinity component has a K_A of $2.5 \cdot 10^9$ M^{-1} for androstanolone and a K_A of $1 \cdot 10^9$ M^{-1} for testosterone at 0 °C. The 3β-androstanediol is not bound [10]. Thus the microsomal receptor and the cytosol receptors are obviously different in terms of affinity and ligand specificity. However, measurements made on receptors which are not in a soluble form can only give apparent estimates of affinity and specificity, and definitive conclusions should await solubilization of the microsomal receptor. Attempts to elute the binding protein from microsomes with high salt buffers were unsuccessful. Samples were incubated with 10 pM [^{3}H]androstanolone, in homogenization buffer with or without 1 M NaCl. After resedimentation, the specific activity of binding was determined (dpm [^{3}H]androstanolone/mg of protein) in the pellet and no significant difference was found. Although indirect, the above cited arguments do not favor the possibility that the specific macromolecule described here is either part of the cytosoluble receptor adsorbed onto the microsomes, or is an incomplete polysomal precursor of the cytosoluble receptor, as was suggested for the microsomal estrogen receptor molecules described in pig uterus [14]. If the observed microsomal binding system is in fact part of the cytosol receptor with a modified affinity and specificity because of its interaction with membrane, it is interesting to ask what is the significance of the cellular distribution of such a population of receptor molecules. In any case the microsomal receptor is also completely different from the sex steroid binding macromolecules detected in rat liver endoplasmic membranes which are observed with very high hormone concentrations [15]. Finally, the microsomal binding component is not an enzyme of testosterone metabolism, like the 5α-reductase [16, 17] or the 3α-hydroxysteroid dehydrogenase [18] described in rat ventral prostate, since the K_m of these enzymes are in the micromolar range and thus their affinity for substrate or product cannot be of four orders of magnitude greater. In addition, these enzymes are also present in rat liver microsomes, where no high affinity binding was observed.

The concentration of microsomal binding sites has been calculated from the data given Table I, and found to be 250 pmoles/g ventral prostate (wet weight). This is less than the amount of the cytosol receptor (1 000 pmoles/g ventral prostate) (Blondeau, J. P. and Robel, P., unpublished), but still is too much (25 %) to be

interpreted as contamination (see also washing experiments). Unexpectedly, the number of microsomal binding sites found in the intact rat was identical to that in the 1-day castrated animal: this cannot be due to the presence of endogenous androgens since the half-life of circulating testosterone is very short, and the testosterone and dihydrotestosterone measured by radioimmunoassay in rat ventral prostate fell to undetectable levels 24 h after castration (Corpéchot, C. and Robel, P., unpublished).

Therefore, one has to postulate that only a small part of the microsomal receptor sites are occupied by endogenous steroids, or that an exchange occurred during the 2-h incubation at 0 °C with radioactive 3β-androstanediol and subsequent ultracentrifugation. In the normal rat ventral prostate, on the contrary, almost all cytosol receptor sites are filled with endogenous androstanolone and do not exchange during a 2-h incubation at 0 °C.

The occurrence of an insoluble (in a fraction of homogenate different from "cytosol") steroid hormone receptor is not an unique observation. Besides the pig uterus microsomal estradiol receptor [14], evidence has been reported for the specific binding of aldosterone to rat kidney plasma membranes [19] and for estradiol in the "residual" or "insoluble" nuclear proteins of chick liver [20]. Finally the microsomal receptor of rat ventral prostate here described may be compared with the binding of androstanolone and 3α-androstanediol observed in rat mouse kidney microsomes (Kan, J., Dofuku, R. and Ohno, S., personal communication).

Although the regulation of transcription is almost certainly a decisive component of steroid hormone action, some additional effects at the translational level cannot be excluded. However, no experimental evidence has related such control to the direct interaction of androgens with the translational machinery. The 3β-androstanediol was shown to be active in rat ventral prostate organ culture [1], and the microsomal specific binding here described is so far the only known interaction of the steroid with a cellular component which can be related to such an activity. However, the active concentrations of 3β-androstanediol in culture are in the 100 nM range, well above those predictable from the K_D of the microsomal binding. At the present time, the physiological significance of the specific binding of testosterone metabolites to rat ventral prostate microsomes is not established.

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