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ANDROGEN RECEPTOR IN GENITAL TUBERCLE OF RABBIT FETUSES AND NEWBORNS. ONTOGENY AND PROPERTIES

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Summary—In newborn rabbits of both sexes, an androgen receptor was characterized in the genital tubercle. Homogenates exhibited high affinity (K_d was about 0.4 nM) and saturable binding of [^{3}H]methyltrienolone. The half-life of the [^{3}H]5α-dihydrotestosterone–androgen receptor complex was 72 h at 4°C. The receptor was inactivated by heat and pronase and the binding was specific for potent androgens. Sucrose gradient analysis revealed a 8–9 S [^{3}H]methyltrienolone binding protein in cytosols from both sexes. Androgen binding, in the homogenate, was detected as soon as day 18 of gestation in both sexes and the number of binding sites increased until birth. During sexual organogenesis and at birth there were no major differences between males and females in the amount or affinity of androgen binding. Specific androgen binding was also detected in sexual ducts of male and female newborns.

INTRODUCTION

Embryos of both sexes develop in an identical fashion, irrespective of their genetic sex during the first period of gestation, and thereafter sexual differentiation is an androgen dependent process in the male fetus [1]. In fetal rabbits testicular synthesis and secretion of testosterone start between days 18 and 19 of gestation, just before the beginning of differentiation of the male genital tract [2–4]. Very few data are available concerning the molecular basis of androgen action in fetal target organs. As a result of studies in animals and humans with single-gene mutations that induce resistance to androgen, it has been suggested that androgens may act in fetuses by mechanisms similar to those described in adult life [5]. Recent studies have analysed the role of sex steroids on tissular interactions [6–9] and have characterized an androgen receptor in the urogenital tract [10]. The present study was undertaken in newborn and fetal rabbits (1) to characterize an androgen receptor in the genital tubercle (2) to assess whether qualitative and quantitative changes occur in androgen binding during sexual organogenesis, (3) to determine if the number of binding sites is related to the sex of the fetus.

EXPERIMENTAL

Animals

Female rabbits of the New Zealand strain were used. Gestation was considered as beginning at the time of copulation and parturition occurred at 32 days. The differentiation of the genital tubercle occurs between 23 and 25 days of gestation [11] and fetuses were removed on days 18, 22, 25 and 29. The sex of the fetuses was macroscopically determined with certainty from day 19 onwards. Newborns of both sexes and 10–15-day old males were also used. Organs (genital tubercle, sexual ducts) were immediately weighed, cut into pieces (genital tubercle) and frozen in liquid nitrogen. No loss of receptor binding activity could be detected after storage at −196°C for 3 months.

Abbreviations used: [^{3}H]DHT: 1,2,4,5,6,7 [^{3}H]5α-dihydrotestosterone; BSA: bovine serum albumin; [^{3}H]R-1881, methyltrienolone: 17β-Hydroxy-17α [^{3}H]methyl-4,9,11-estratrien-3-one; DTT: dithiothreitol; EDTA: Ethylenediaminetetraacetic acid; PMSF: Phenylmethylsulfonyl fluoride; HAP: Hydroxylapatite.

Materials

The following materials were purchased: [^{3}H]DHT (104 Ci mmol) and [^{14}C]BSA from the Radiochemical Centre (Amersham, Great Britain); [^{3}H]R-1881 (87 Ci mmol) as well as radioinert R-1881 from New England Nuclear Corp. (Boston, MA); unlabelled steroids from Steraloids Inc. (Wilton, NH) and cyproterone acetate from Schering (Berlin). The radiochemical purity of radioactive steroids was checked once every 2 months by thin layer chromatography. Radioactive steroids were conserved in a 9:1 benzene–ethanol solution at 6°C.

Buffers

The following buffers were used: A buffer, 50 mM Tris–maleate (pH 7.5) containing 1 mM DTT, 1 mM sodium azide and 0.25% (w/v) gelatin; A 40 buffer, A buffer containing 40 mM sodium molybdate and 3 mM EDTA (pH 7.5); TEM buffer, 10 mM Tris buffer, 1.5 mM EDTA, 1.5 mM mercaptoethanol (pH 7.4); TEMG buffer, TEM containing 10% (v/v) glycerol.

Preparation of homogenates and cytosols

Homogenates were used to determine total androgen binding (cytosolic + nuclear binding sites), as demonstrated by Hechter *et al.*[12], cytosols for sucrose gradient analysis and dissociation studies. All procedures were carried out at 0–4°C. The genital tubercles were pulverized after freezing in liquid nitrogen and the powder obtained was homogenized in 5 vol. A buffer in the presence of 0.5 mM PMSF by 25 strokes at 800 rpm of a motor driven Teflon-glass Potter homogenizer. Three aliquots were removed for DNA assay and the remaining homogenate was diluted (v/v) in A 40 buffer. The homogenate was centrifuged at 105000 *g* for 60 min at 2°C to obtain the cytosol fraction. The cytosol preparations used contained about 6 mg protein/ml for binding assays, 15 mg for sucrose gradients.

Steroid binding assays

Equilibrium constants of the [^{3}H]R-1881 to the homogenates were determined by application of the Scatchard method [13]. One-hundred and fifty or 200 μl of homogenate were incubated with increasing concentrations of [^{3}H]R-1881 (0.1–20 nM) in the presence and absence of a 100-fold excess of radioinert R-1881. The difference between [^{3}H]R-1881 binding in the absence and presence of a 100-fold excess of non radioactive R-1881 represents the specific ligand binding. The incubation was performed for 18 h at 4°C with constant shaking in the presence of 5 μM triamcinolone acetonide to inhibit binding of [^{3}H]R-1881 to glucocorticoid and progesterone receptors. After incubation, the homogenate was separated into two fractions: the first was used to determine the radioactivity of total homogenate by counting in 5 ml of scintillation fluid (Picofluor, TM_{15} Packard); the second was used to separate bound and free fractions using the HAP method previously described [12]. Briefly, 100 μl samples were diluted 1:1 with A buffer containing 20 mM sodium molybdate and 60% glycerol (v/v), then 500 μl of HAP suspension in 3 mM phosphate buffer containing 30% glycerol (B buffer pH 7.4) were added. To remove "free" and "loosely bound" radioligand, the HAP pellets were washed three times with B buffer containing 0.5% Triton X-100. The [^{3}H]R-1881 was extracted from the pellet by 2 ml ethanol and the radioactivity measured. Specific binding measured in this way was linearly correlated ($y = 0.734\ x + 0.223$; $r = 0.998$; $P < 0.001$) with concentrations of DNA between 80 and 800 μg/ml homogenate. Specific [^{3}H]R-1881 binding is expressed in units of picomoles [^{3}H]R-1881 bound per milligram DNA.

Sucrose density gradient sedimentation

Cytosols were incubated (12 h at 0–4°C) with 10 nM [^{3}H]R-1881 with or without a 100-fold excess of unlabelled R-1881 or cyproterone acetate. Labelled cytosol samples (300 μl) were treated 10 min at 0–4°C with dextran-charcoal (charcoal: 0.25%; dextran: 0.025% in TEM buffer) to remove "free" and "loosely bound" steroids. Two-hundred μl aliquots (containing 2–3 mg protein) plus 10 μl (2000 dpm) of [^{14}C]BSA were layered on linear 5–20% (w/v) sucrose gradients (5 ml) prepared in TEMG buffer in the presence of 0.5 mM PMSF. Gradients were centrifuged at 234000 *g* for 23 h in a Kontron TST 41–14 rotor at 2°C. Fractions (200 μl) were collected with a needle from the bottom and counted. BSA ($S_{20,w}$4.6) was used to calculate the sedimentation coefficient.

Protein and DNA assays

The quantity of protein in each sample was determined according to Lowry *et al.*[14] using BSA as a standard. DNA concentration was measured according to Burton[15].

RESULTS

Saturability and affinity

Figure 1 shows the Scatchard plot of the binding of [^{3}H]R-1881 by the homogenate of genital tubercles from newborn females. The specific binding of [^{3}H]R-1881 was saturable and Scatchard analysis was compatible with the presence of a single class of binding sites ($y = -3.31x + 2.0$; $r = -0.989$; $P < 0.001$). The K_d value was 0.3 nM and the total number of binding sites 2.28 pmol/mg DNA.

Association and dissociation of the binding

Homogenates from genital tubercles of newborn males were incubated at 0–4°C with a saturating concentration (20 nM) of [^{3}H]R-1881 in the presence or absence of a 100-fold excess of unlabelled R-1881 for varying lengths of time from 30 min to 72 h (Fig. 2A). Specific binding activity measured by the HAP method described above increased rapidly (maximum at 4 h). The slight decrease observed after 24 h is probably due to some degradation of androgen receptors. For dissociation studies, genital tubercle cytosols from newborn males were incubated with 10 nM [^{3}H]DHT at 0–4°C, in the presence or absence of a 100-fold excess of unlabelled DHT (DHT is the predominant endogenous androgen of the genital tubercle) [16]. After incubation for 12 h, a 500-fold excess of unlabelled DHT was added to each tube and specific binding determined at various times up to 80 h (Fig. 2B). The apparent half-life of [^{3}H]DHT dissociation corrected for androgen receptor inactivation is 72 h at 4°C.

Sensitivity to heat and pronase

When labelled homogenates of genital tubercles from newborn males were heated at 50°C for 30 min, cooled at 0°C and centrifuged to remove the precipitated proteins, the specific binding of [^{3}H]R-1881 was destroyed by 75%. In aliquots of homogenates incubated with pronase for 30 min at 25°C, the specific

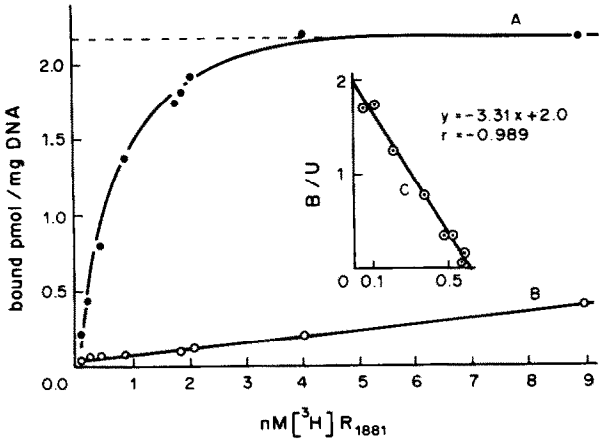

Fig. 1. Saturation analysis of [^{3}H]R-1881 binding in genital tubercle of newborn female rabbit. Homogenate was incubated with increasing concentrations of [^{3}H]R-1881 for 18h at 4°C. Bound and free fractions were separated by HAP. Non specific binding (B) was determined in the presence of 100-fold excess of non radioactive hormone and was subtracted from total binding to give specific (A) binding. A Scatchard plot of the same data is shown (C). $K_d = 0.30$ nM; $B_{max} = 2.28$ pmol/mg DNA; B/U = bound to unbound ratio.

binding of [^{3}H]R-1881 was abolished. These results showed the protein nature of the androgen binding component.

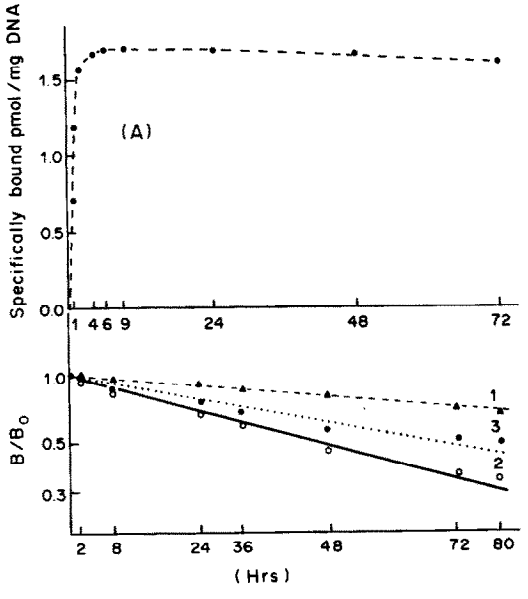

Fig. 2. (A) Time course of [^{3}H]R-1881 specific binding to genital tubercle of newborn male rabbit. Homogenate was prepared in the presence of sodium molybdate and incubated at 0–4°C with 20 nM [^{3}H]R-1881 in the absence or presence of a 100-fold excess of unlabelled R-1881. Each point is the mean of two determinations. (B) Dissociation at 0–4°C of the specific binding of [^{3}H]DHT to genital tubercle cytosol from newborn male rabbit. Cytosol (6 mg protein/ml) was incubated 12 h at 0–4°C with 10 nM [^{3}H]DHT with or without a 100-fold excess of non radioactive DHT. At this time (0 h), buffer alone (1) or 5 μM of non radioactive DHT in buffer (2) were added. Bound and free steroids were separated with HAP at the indicated times and non specific binding was subtracted. The dissociation rate was corrected (3) for androgen receptor inactivation estimated by the decrease in [^{3}H]DHT binding observed in 1. B and Bo = specific binding at time to and t respectively.

Sucrose gradient centrifugation

Sucrose density gradient analysis of the genital tubercle cytosols from newborns, in the presence of sodium molybdate (20 mM), showed the presence of a distinct [^{3}H]R-1881 binding component with a sedimentation coefficient of 8–9 S (Fig. 3). There were no differences between coefficients of sedimentation measured in newborn males (8.73 ± 0.69 mean $\pm$ SEM; $N = 6$) and females (8.62 ± 0.01; $N = 6$). Under the same conditions, the value measured from rat ventral prostate was 10.11 ($N = 3$). In the rabbit, this protein has a limited binding capacity since in the presence of a 100-fold excess of unlabelled R-1881 or cyproterone acetate, [^{3}H]R-1881 binding was completely or partially abolished (Fig. 3B). The 8–9 S binding of R-1881 was not affected in genital tubercles stored in liquid nitrogen for 1 month. When genital tubercles of newborns were layered on gradients containing 0.4 M KCl no 4–5 S binding was observed either in the presence or absence of sodium molybdate.

Steroid specificity

Table 1 shows the competition by various unlabelled steroids for binding of [^{3}H]R-1881 in genital tubercle homogenates from newborn males. Only R-1881 (76%), DHT (66%) and testosterone (57%) markedly inhibited [^{3}H]R-1881 binding; estradiol (36%) and progesterone (18%) competed to a lesser extent whereas cortisol produced negligible inhibition.

Ontogenesis of the amount and affinity of binding sites

Tissue specificity, assessed in organs from newborns of both sexes, showed that specific androgen binding in brain (0.16 pmol/mg DNA) was lower than that determined in sex target organs (Table 2). Scatchard analysis of [^{3}H]R-1881 binding was made

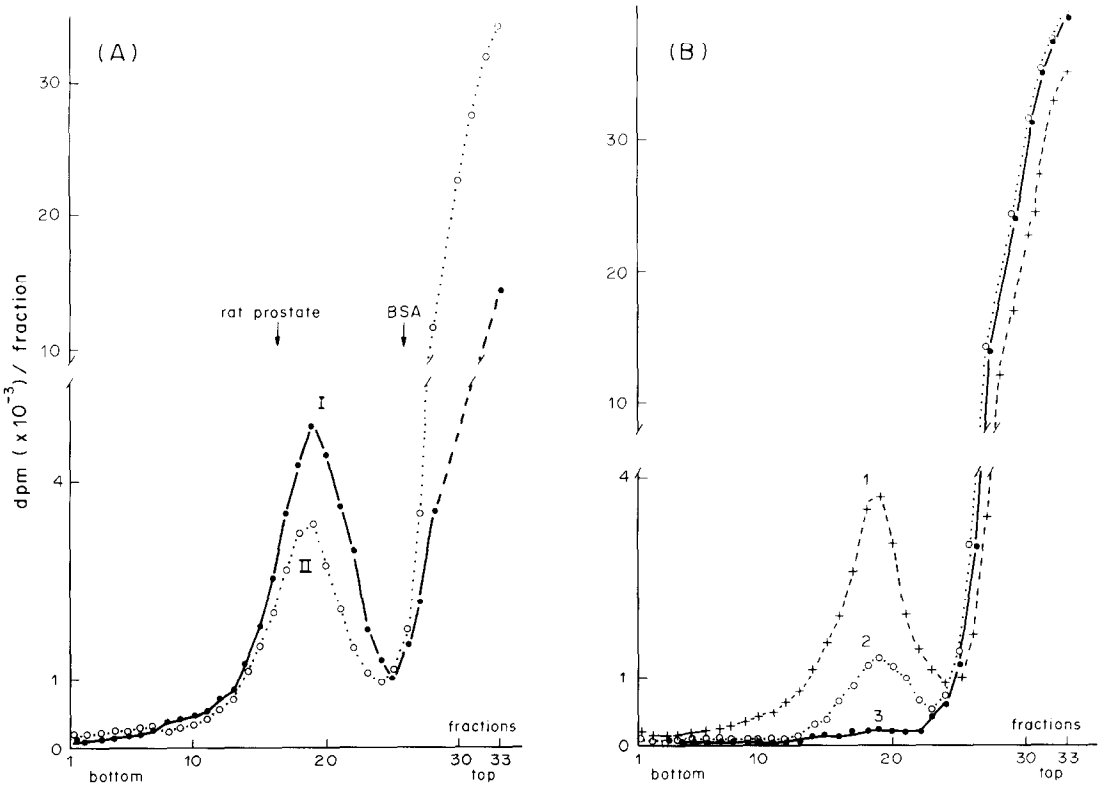

Fig. 3. Sucrose gradient centrifugation of genital tubercle androgen receptor from newborn rabbits. (A) Cytosols from male (I) and female (II) genital tubercles were incubated with 10 nM [^{3}H]R-1881 for 12 h at 0–4°C. After 10 min of charcoal treatment, samples were applied to 5–20% sucrose gradient in TEMG buffer. Bovine serum albumin (BSA: $S_{20,w}$ 4.6) was used as internal marker. The gradients were centrifuged at 234,000 *g* for 23 h and fractionated. Bound and free hormone were separated with HAP. The arrow "rat prostate" indicates the position of the peak obtained with cytosol from adult rat prostate. (B) Cytosols from newborn females were incubated with 10 nM [^{3}H]R-1881 in the absence (1) or presence of a 100-fold excess of unlabelled cyproterone acetate (2) or R-1881 (3).

in genital tubercles of both sexes from day 18 of gestation until birth (Table 2). The affinity constants varied little during the period studied. Androgen binding was detected as soon as 18 days of gestation in pooled males and females and regardless of the mode of expression used, the total number of binding sites increased from the 18th day till birth (Table 2). At each stage studied there were no major differences between males and females in the amount or affinity of androgen binding.

Table 1. Specificity of [^{3}H]R-1881 binding to homogenate from genital tubercle of newborn male rabbits

Competitor	Percentage inhibition of total [^{3}H]R-1881 binding
Methyltrienolone	76
Dihydrotestosterone	66
Testosterone	57
Cyproterone acetate	50
Estradiol	36
Progesterone	18
Cortisol	0.4

Hormonal specificity was determined by measuring binding of 15 nM [^{3}H]R-1881 in the presence of a 100-fold excess (500-fold for cortisol) of various non radioactive competitors. Bound and free hormones were separated by HAP. Each value is the mean of two determinations. [^{3}H]R-1881 binding = 2.33 pmol/mg DNA.

Measurements of the amount and affinity of androgen binding in epididymis, vas deferens, uterine horn of newborns and phallus of 10–15 day old males are summarized in Table 2. The number of binding sites was similar in epididymis and sexual ducts as well as in the phallus after birth but lower than in genital tubercles of both sexes (Table 2).

DISCUSSION

Receptors for different steroid hormones are present in the fetuses of various species and two models have been well documented: the fetal lung, a target tissue for glucocorticoids, and the fetal uterus, a target organ for estrogens [review in 17]. In this study we have demonstrated that the binding site for [^{3}H]R-1881 in fetal rabbit genital tubercle represents the androgen receptor. Possible binding of steroid to other androgen binding proteins present in the homogenate was circumvented by the use of methyltrienolone, a synthetic steroid with high affinity for androgen receptors which does not bind to serum or prostatic proteins [18]. Binding of methyltrienolone to an eventual progesterone receptor (which binds R-1881 with high affinity) was probably not

Table 2. Affinity constants and number of total binding sites in sex target organs homogenate from rabbit fetuses and newborns

Tissue	Sex	Binding sites			
		fmol/ organ	pmol/g tissue	pmol/mg DNA	K_d nM
Fetal (day 18)					
GT	M + F (1)	2.6	1.70	0.34	0.39
Fetal (day 22)					
GT	M (1)	8	2.04	0.56	0.90
GT	F (1)	15	3.90	0.65	0.44
Fetal (day 25)					
GT	M (3)	23	1.87	0.93	0.35
GT	F (3)	25	2.38	1.09	0.28
Fetal (day 29)					
GT	M (3)	135	4.15	2.72	0.56
GT	F (3)	107	4.22	3.60	0.45
Birth					
GT	M (6)	319	6.19	2.02	0.39
GT	F (4)	275	6.03	2.64	0.45
Epididymis	M (3)	33	4.77	0.92	1.74
Vas deferens	M (2)	6	1.45	0.46	0.96
Uterine horn	F (2)	21	2.19	0.43	0.52
Cerebral cortex	M (2)	—	0.35	0.16	—
10–15 Days phallus	M (3)	353	1.96	0.47	0.52

Values are means of n assays. GT = genital tubercle. M = male. F = female.
Numbers in parentheses indicate number of assays. Genital tubercles were pooled as follows: day 18: 127, day 22: 30, day 25: 16, day 29: 8, birth: 6.

significant since an excess of triamcinolone acetonide, a blocker of progesterone receptor, did not affect the number of binding sites ($N = 1.84$ pmol/mg DNA in the presence of triamcinolone acetonide; $N = 1.62$ without this compound in genital tubercle of newborns). Additional evidence for the receptor nature of the binding site is provided by its sedimentation behavior (8–9 S in sucrose gradient) and by Scatchard plots. The localization of binding in sex target organs but not in cerebral cortex and competition by various compounds revealed the specific binding characteristic of a typical androgen receptor [19]. The apparent K_d for R-1881 is of the same order of magnitude as that reported for androgen receptors in the seminal vesicle of ram [20] and in the rat ventral prostate cytosol [12] but lower than that measured in the homogenate of rat prostate [12]. The results reported here, in keeping with others [10], show that the genital tubercle androgen receptor has many properties similar to those of androgen receptors in adult male target tissues.

The developmental processes responsible for the initiation of receptor synthesis are still unknown. In the genital tubercle, androgen receptor is not induced by circulating androgens since it is already present at day 18 of gestation while testosterone is first detected at day 19 in plasma and genital tubercle [4, 16]. Because hormonal action requires preexisting receptors it is logical that the hormone itself cannot induce its own receptor. There is an increase in androgen binding from the 18th day of gestation to birth. Specific testosterone binding macromolecules in the whole urogenital tract of fetal rat [21] and androgen receptors in the mammary gland of fetal mouse [7] also increased in late gestation. The increase reported here may be due, at least in part, to increasing endogenous levels of DHT in genital tubercle [16] since androgens may regulate the number of their own receptors [19, 22]. Present results also show that the rapid loss of the genital tubercle androgen responsiveness, which occurs after day 22 [1], is not due to modifications of the amount or affinity of androgen receptors. Similarly, the loss of the mammary gland androgen responsiveness in mouse fetuses is not accompanied by a decline of androgen receptors [7].

The sexual dimorphism in the embryonic development of genital tubercle is induced by testicular androgens [1]. However, the androgen environment in which male phenotype develops is not drastically different from that of females and considerable overlap in individual values are described in rabbits [23] and rats [24]. Present results clearly show that there are no changes related to sex in the affinity and number of specific sites during sexual organogenesis. Then it is possible that sexual differences may occur beyond the receptor level since the mechanism of steroid hormone action requires binding of the receptor hormone complex to DNA where it activates transcription of new RNA and protein synthesis [25].

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