Isolation and Characterization of the Androgen Receptor of Murine Preputial Gland

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The androgen receptor of murine preputial gland showed in binding experiments a biphasic saturation curve and a biphasic Scatchard plot. The receptor converted from an 8.5–9 S form to a 4.5–5 S form in high ionic strength buffer. The apparent dissociation constant was K_D 0.5 ± 0.2 nM for the 8.5–9 S receptor form. A 6.5–7 S receptor form could be detected in some experiments. The ligand specificity was evaluated by competition experiments: testosterone > androstenedione > dihydrotestosterone > androstanediol > estradiol > progesterone > dexamethasone. The receptor of murine preputial gland was less stable than the androgen receptor of skeletal muscle of the same mice.

Introduction

The preputial gland is an accessory sexual gland associated with the caudal end of the urogenital tract. The androgen-dependence of adult preputial gland is well documented in several species [1–5]. On the other hand, estrogens affect the preputial gland adversely by markedly reducing its size, weight and function [6, 7]. The preputial gland of male mice contains considerable amounts of androgen receptors. Furtheron this gland gives a typical androgenic answer under androgen influence. This androgen target tissue turned out to be a good source to isolate androgen receptor allowing us to compare it with the androgen receptor of skeletal muscle (a target tissue which gives a typical anabolic answer under androgen influence). The use of preputial gland for the experiments described here is of advantage, because it is easy to dissect and because it can be taken from the same mice as were the skeletal muscles taken from. The aim of the experiments was to isolate and to characterize the androgen receptor of murine preputial gland to compare these data with those of the androgen receptor of murine skeletal muscle and to see if there are significant differences between these two androgen receptors.

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Materials and Methods

(1,2,4,5,6,7)-^{3}H-Dihydrotestosterone (5.0 TBq/mmol); (17-α-methyl-^{3}H)-methyltrienolone (3.22 TBq/mmol); (1,2,6,7,16,17)-^{3}H-testosterone (5.78 TBq/mmol) and radioinert methyltrienolone were purchased from NEN (Dreieich); all other radioinert steroids were from Sigma (München). Charcoal Norit A, dextran 60, bovine serum albumin cryst. from Serva (Heidelberg). The other reagents used were of analytical grade and obtained from E. Merck (Darmstadt). The purity and identity of radioactive and radioinert steroids were ascertained by thin layer chromatography.

Buffers

KPP-buffer: 10 mM potassium phosphate, 2 mM EDTA adjusted to pH 7.4 at room temperature with KOH; KPPF-buffer: KPP-buffer + 0.1 mM PMSF; KPPFM-buffer: KPPF + 2 mM mercaptoethanol; MoKPPFM-buffer: KPPFM + 10 mM Na_2MoO_4. In some experiments DTT was used in varying concentrations instead of mercaptoethanol.

Dextran coated charcoal (DCC)

The respective buffer containing 0.1 g dextran 60, 4 g charcoal Norit A per 100 ml was stirred over night at 0–4 °C.

Scintillation cocktail

3.5 g PPO, 0.14 g POPOP, 300 ml triton-X-100 and 700 ml toluene were stirred under exclusion of light.

Preparation of cytosol

Sexually mature male C57BL6J mice were killed and the preputial gland dissected. The glands (average weight 50 mg) were washed with the respective ice cold homogenization buffer. All other steps were done at 0–4 °C in the cold room. Homogenization was performed with three strokes of a Polytron-homogenizer in a cooled vessel containing 3 ml of the respective homogenization buffer per gram tissue. The resulting homogenate was centrifuged for 10 min at 12,000 × *g*. The supernatant was again centrifuged in a Beckman SW 60 rotor for 1 h at 55,000 rpm. After removing the lipid layer the resulting supernatant was used as "cytosol" (protein content 7–12 mg/ml). This cytosol was used undiluted for density gradient centrifugation. For other experiments this cytosol was diluted with the respective homogenization buffer to a protein content of 0.5–1.7 mg/ml.

Steroid binding assay

Aliquots of 0.15 ml cytosol were incubated in duplicate or triplicate at 0–4 °C with labelled steroid (0.2–10 nM) alone (total binding) or in presence of a 100-fold excess of the corresponding unlabelled steroid (non-specific binding) for 20 h if not noted otherwise. Incubation was terminated by the addition of 0.1 ml of DCC to separate unbound from protein bound steroids. Treatment with DCC was performed for 2 min followed by centrifugation at 10,000 × *g* for 2 min. The supernatant was again centrifuged at 10,000 × *g* for 3 min. Radioactivity was determined from 0.2 ml portions of the supernatant diluted with water to 0.45 ml in a toluene-based scintillation cocktail. The counting efficiency was 45%. Corrections for sample quenching were made by the use of an external standard. Specific binding was defined as the difference in bound radioactivity between the tubes containing labelled steroid and the tubes containing both labelled and unlabelled steroid. The data obtained were submitted to further analysis using the Scatchard plot [8] with the modification proposed by Rosenthal [9].

Density gradient centrifugation

Aliquots of undiluted cytosol (7–12 mg protein/ml) 0.17 ml (Beckman SW 60 rotor), and 0.2 ml (Beckman VTi 80 rotor) respectively were layered onto the top of a 8–30% (w/w) linear glycerol gradient in the respective homogenization buffer (with or without 10^{-8} M [^{3}H]testosterone) with (high ionic strength) or without (low ionic strength) the addition of 0.3 M KCl. The tubes were centrifuged at 2 °C for 6 h at 60,000 rpm (SW 60) or 65 min at 80,000 rpm (VTi 80) in a Beckman Spinco L2-65B and in a Beckman L8-80 (with slow acceleration mode) respectively. Ovalbumin (3.6 S), bovine serum albumin (4.4 S), bovine gamma globulin (6.6 S) and β-amylase (9.4 S) were used as reference standards.

Protein determination

Protein content of solutions containing more than 2.5 µg protein per ml solution was measured according to Lowry *et al.* [10] and to Ross *et al.* [11] respectively.

Affinity chromatography

Affinity chromatography was done according to [12].

Results

To evaluate the metabolic behavior of DHT and testosterone during incubation with cytosol of murine preputial gland we performed thin layer chromatography with ether extracts of the incubates. Methyltrienolone was not examined because it has been proved by others that it is not metabolized [13]. As shown in Fig. 1a–c testosterone is not metabolized during incubation, whereas DHT is strongly metabolized under these conditions. That is why we used for most of the experiments testosterone as ligand. Since methyltrienolone is also stable to metabolism and doesn't bind to SHBG (sex hormone binding globulin), it was used in certain cases too.

Cytosol prepared of murine preputial glands in KPPFM-buffer showed in binding experiments with testosterone a biphasic saturation curve and a biphasic Scatchard plot (Fig. 2). To find out if the biphasicity of the saturation curve was due to metabolism, we examined this by using methyltrienolone (in MoKPP-buffer) instead of testosterone. In this case we obtained a biphasic curve too (Fig. 3). From the Scatchard plot of cytosol prepared in KPPFM-buffer (Fig. 2) 40 ± 10 fmol/mg protein binding sites were evaluated. The Scatchard plot of cytosol prepared in MoKPPF-buffer containing 10 mM DTT (Fig. 3) showed a significant increase of

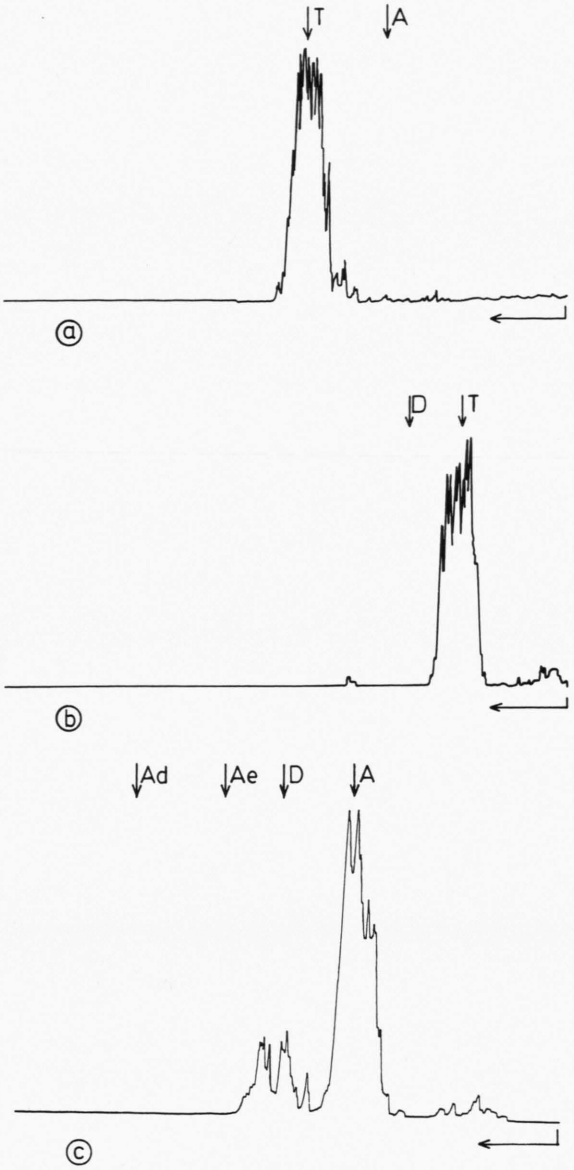

Fig. 1. Thin layer chromatography of 10 nM [^{3}H]testosterone (T) incubated for 20 h at 0–4 °C in cytosol of murine preputial gland prepared in MoKPP-buffer containing 2 mM DTT. The steroid was extracted with ether. As references were used: [^{3}H]5α-androstane-3α,17β-diol (A) and [^{3}H]5α-dihydrotestosterone (D). Arrow: direction of migration. The solvent system used was: acetone/chloroform/ehtyl acetate = 8:7.5:17 (v/v) (a). In this solvent system T and D are not separated from each other, therefore the solvent system chloroform/ether = 90:10 (v/v) was used (b). This solvent system allows the separation of T and A. The analogous procedure to (a) was done with 10 nM [^{3}H]5α-dihydrotestosterone (D). As references were used: [^{3}H]5α-androstane-3α,17β-diol (A), 4-androstene-3,17-dione (Ae) and 5α-androstane-3,17-dione (Ad) (c). As Ae and Ad have greater R_f-values than D, T and A this chromatogram shows that 5α-dihydrotestosterone is metabolized to androstanediol.

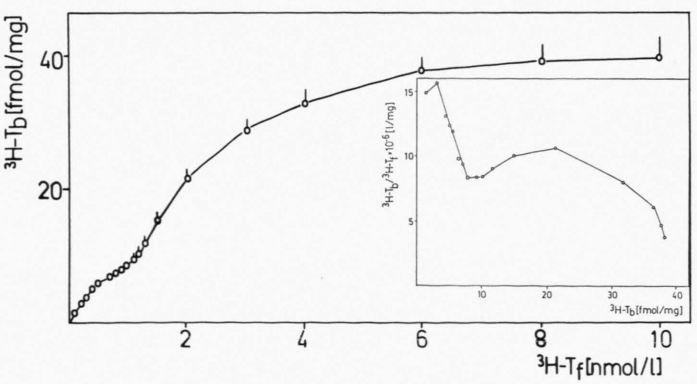

Fig. 2. Testosterone binding curve obtained with cytosol of murine preputial gland (prepared in KPPFM-buffer, protein content 0.45 mg/ml). ^{3}H-T_b: specifically bound ^{3}H-testosterone; ^{3}H-T_f: unbound ^{3}H-testosterone. The bars indicate S.E.M. (n = 3). *Inset:* Scatchard plot to this binding curve. (^{3}H-T_b is expressed in fmol/mg protein and ^{3}H-T_f is expressed in mol/l. Therefore ^{3}H-T_b/^{3}H-T_f has the dimension l/mg.)

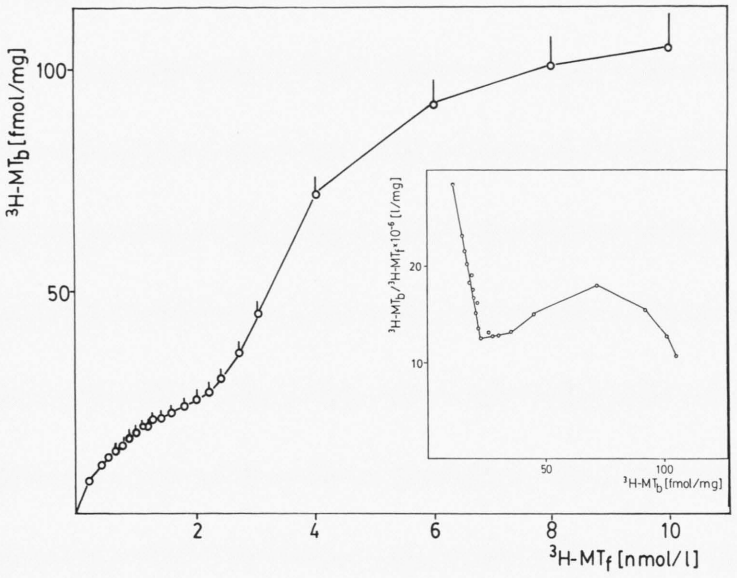

Fig. 3. Methyltrienolone binding curve obtained with cytosol of murine preputial gland (prepared in MoKPPF-buffer containing 10 mM DTT, protein content 1.7 mg/ml). ^{3}H-MT$_b$: specifically bound ^{3}H-methyltrienolone; ^{3}H-MT$_f$: unbound ^{3}H-methyltrienolone. The bars indicate S.E.M. (n = 3). *Inset:* Scatchard plot to this binding curve. (Dimension of ^{3}H-T$_b$/^{3}H-T$_f$ see legend to the inset Fig. 2.)

binding sites up to 120 ± 10 fmol/mg protein (Fig. 3). As shown by Gaubert *et al.* [15] molybdate is able to protect the receptor, but the high yield of receptor is not only due to molybdate but also to DTT which is a better reductant than mercaptoethanol. The apparent dissociation constant evaluated from the Scatchard plot was K_D = 0.5 ± 0.2 nM for the first bend of the saturation curve in both cytosols. Remarkable is the marked plateau of the first bend in the saturation curve of molybdate containing cytosol compared with that of molybdate free cytosol. The phenomenological positive and negative cooperativity, which can be seen in the Scatchard plots (Fig. 2, 3) is also detectable in the Hill plot (Fig. 4). The Hill plot also allows the presumption that the androgen receptor of murine preputial gland is a protein consisting of probably at least four identical binding sites (n_H = 3.6).

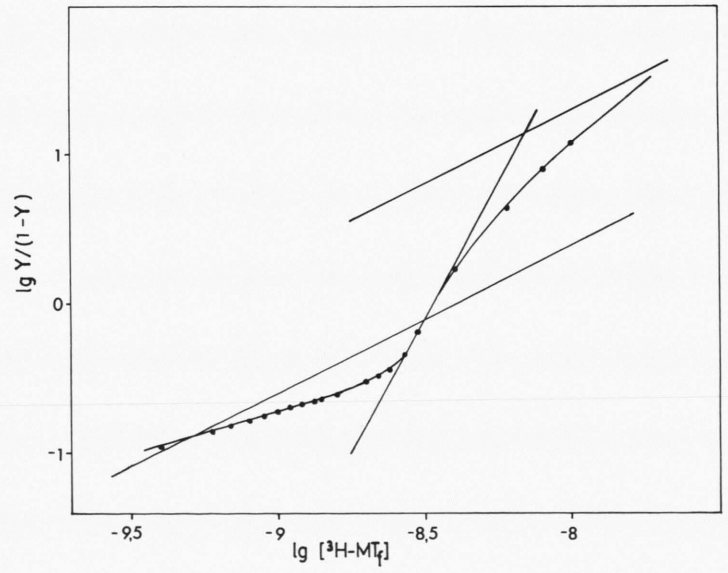

Fig. 4. Hill plot to Fig. 3. Maximum slope: n_H = 3.6. ^{3}H-MT$_f$: unbound methyltrienolone.

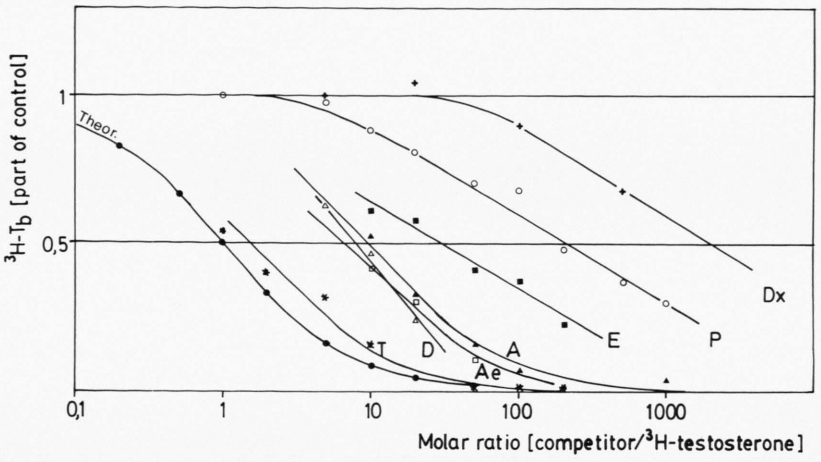

Fig. 5. Ligand specificity of testosterone binding in cytosol of murine preputial gland (prepared in MoKPPF-buffer containing 10 mM DTT). 8 nM [^{3}H]testosterone were incubated together with ascending concentrations of various steroids for 20 h at 0–4 °C. Theor.: theoretically expected competition curve with the identic competitor without other influences (●——●), T: real testosterone competition curve (*——*), D: 5α-dihydrotestosterone (△——△), Ae: 4-androstene-3,17-dione (□——□), A: 5α-androstane-3α,17β-diol (▲——▲), E: 17β-estradiol (■——■), P: progesterone (○——○), Dx: dexamethasone (x——x).

Ligand specificity was determined by competition experiments. The ligands chosen for these examinations were along with the identic competitor (testosterone) the most important *in vivo* metabolites of testosterone namely androstenedione, androstanediol and DHT. Furtheron other steroid hormones like 17β-estradiol, progesterone and dexamethasone were used as competitors. Fig. 5 and Table I show the following relative binding affinities: testosterone > 4-androstene-3,17-dione > DHT > androstanediol > estradiol > progesterone > dexamethasone. From these results it can be seen that dexamethasone and progesterone respectively don't have any affinity to the androgen receptor of murine preputial gland. Estradiol has just a little affinity to this receptor. The other hormones used in this experiment were highly competitive. Remarkable is the relative high affinity of androstenedione and the low affinity of DHT. This might be due to metabolism [15–20].

Table I. Relative binding affinity (RBA) of the steroids used in the competition assay. The labelled ligand was [^{3}H]testosterone.

Steroid	RBA in %
Testosterone	100
Androstenedione (4-androstene-3,17-dione)	16
5α-Dihydrotestosterone	13
Androstanediol (5α-androstane-3α,17β-diol)	10
17β-Estradiol	3.2
Progesterone	0.5
Dexamethasone	0.05

Density gradient centrifugation performed in linear 8–30% (w/w) glycerol gradients (glycerol + KPP-buffer) spun for 65 min at 80,000 rpm in a VTi 80 rotor yielded, when 0.2 ml of receptor containing cytosol (receptor loaded with [^{3}H]testosterone) were layered onto the top of the gradient, a peak at 8.5–9 S with a shoulder at 6.5–7 S and a peak at 4.5–5 S. A loss of [^{3}H]testosterone from the receptor during centrifugation can be observed (tailing in the peak profile in Fig. 6a). The procedure in MoKPPFM containing gradients and with cytosol prepared in MoKPPFM-buffer is shown in Fig. 6b. Here only a peak at 8.5–9 S appears.

Besides the density gradient centrifugation in the VTi 80 rotor other centrifugation experiments were performed using other rotors and varying amounts of reductant in the different buffers. Results of these experiments are shown in Fig. 6c and 7. As already observed in Fig. 6a a more or less marked peak at 6.5–7 S appears in the peak profiles of these density gradient centrifugation experiments. The most distinct appearance of this receptor form occurs, when cytosol prepared in MoKPP-buffer containing 15 mM DTT is layered onto the top of an 8–30% glycerol gradient (containing MoKPP-buffer, 15 mM DTT and 10^{-8} M [^{3}H]testosterone) and spun for 65 min at 80,000 rpm in a VTi 80 rotor (Fig. 7). This receptor form (6.5–7 S) was not detected in density gradient centrifugation experiments

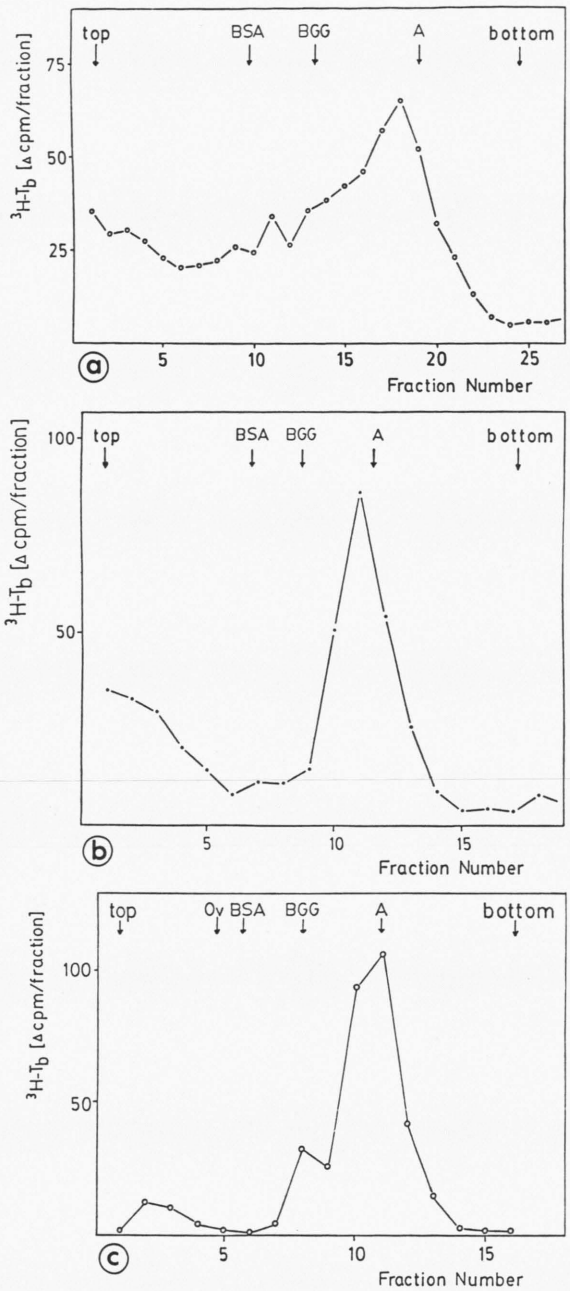

Fig. 6. Density gradient centrifugation of cytosol of murine preputial gland prepared in KPPFM-buffer (protein content 6.6 mg/ml). Preincubation for 2 h at 0–4 °C with 10 nM [^{3}H]testosterone and 1 μM radioinert testosterone respectively. At the end of incubation free steroid was removed by DCC. Gradient: 8–30% glycerol, free of steroid. Rotor: VTi 80. Specific binding found after centrifugation 9%. A: β-amylase (9.4 S), BGG: bovine gamma globulin (6.6 S), BSA: bovine serum albumin (4.4 S) (a). Same experiment with cytosol prepared in MoKPPF-buffer (protein content 6.8 mg/ml) (b). Specific binding found after centrifugation 18%. (c): this experiment is analogous to (b), but the gradient contained 10^{-8} M [^{3}H]testosterone. Specific binding found after centrifugation and DCC treatment was 15%. Ov: ovalbumin (3.6 S).

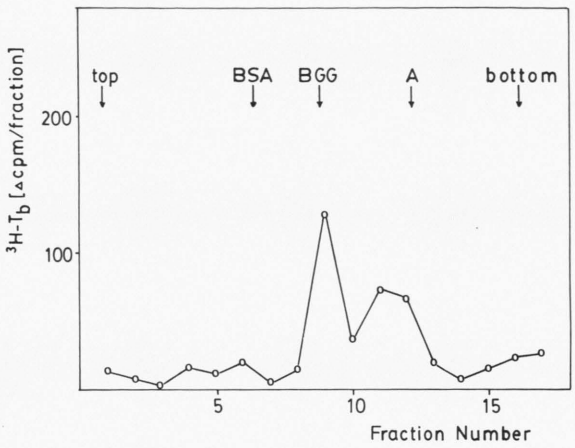

Fig. 7. Density gradient centrifugation of cytosol of murine preputial gland prepared in MoKPP-buffer containing 15 mM DTT (protein content 9 mg/ml). Preincubation for 2 h at 0–4 °C with 10 nM [^{3}H]testosterone and 1 μM radioinert testosterone respectively. Gradient: 8–30% glycerol and free of steroid. Rotor: VTi 80. Specific binding found after centrifugation and DCC treatment was 15%.

done with cytosol of mouse skeletal muscle (data not shown).

As to the 4.5–5 S receptor form it can be seen that under certain conditions when cytosol prepared in MoKPPFM-buffer is layered onto the top of an 8–30% glycerol gradient (containing MoKPPFM-buffer and 10^{-8} M [^{3}H]testosterone) is spun for 65 min at 80,000 rpm in a VTi 80 rotor this receptor is not detectable (Fig. 6c). When the determination of sedimentation coefficients was performed in high ionic strength glycerol gradients (containing KPP-buffer + 0.3 M KCl; 5 nM [^{3}H]testosterone) in a SW 60 rotor spun for 6 h at 60,000 rpm the sedimentation profile shown in Fig. 8 was obtained.

Comparing the amount of receptor found after density gradient centrifugation with the amount of receptor contained in the cytosol layered onto the top of the respective gradient, it can easily be seen that in all centrifugation experiments a significant portion of the receptor (about 80%) is lost.

The attempt to purify the androgen receptor of preputial gland by affinity chromatography was un-

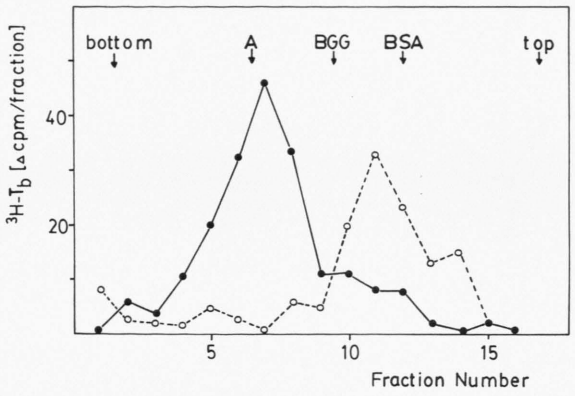

Fig. 8. Density gradient centrifugation of cytosol of murine preputial gland prepared in KPPFM-buffer with (– – –) or without (——) addition of 0.3 M KCl (protein content 9.5 mg/ml). Preincubation for 2 h at 0–4 °C with 5 nM [^{3}H]testosterone and 1 μM radioinert testosterone respectively. Gradient: 8–30% glycerol with (– – –) and without (——) addition of 0.3 M KCl and in both cases labelled with 10^{-8} M [^{3}H]testosterone. Rotor: SW 60. Specific binding found after centrifugation and DCC treatment was 5% and 8% respectively.

successful. No receptor could be detected in the affinity eluate. Isoelectric focusing of this receptor wasn't successful too. This failure in both experiments was probably due to the same effect that caused the loss of receptor during density gradient centrifugation.

Discussion

The androgen receptor of murine preputial gland showed in binding experiments (using 10 to 14 measuring points in the critical hormone concentration range where we find sigmoidity of the binding curve which is in contrast to other authors who use one to three points in this area) a biphasic saturation curve and a biphasic Scatchard plot independently of buffer and androgen used. This phenomenon was already observed with the androgen receptor of murine skeletal muscle [21]. When we examined partially purified androgen receptor of murine skeletal muscle, we obtained also biphasic saturation curves with testosterone and methyltrienolone respectively (data not shown). Dahlberg *et al.* [22] also obtained a biphasic saturation curve with the androgen receptor of rat skeletal muscle with methyltrienolone but they proved that this biphasicity was due to glucocorticoid receptor also present in this experiment (this receptor binds to methyltrienolone too [23]). This could not be the reason in our experiments, because biphasicity was also achieved with testosterone which does not bind to the glucocorticoid receptor as outlined by [24]. A biphasic binding curve found by Thampan [25] is not comparable with our results, because he found the critical area to appear at concentrations 10-fold higher than ours and is most probably due to androgen binding protein (ABP). As to sex hormone binding protein (SHBG) it is also unlikely to be responsible for biphasicity, because we got the same curve with testosterone and methyltrienolone, and methyltrienolone is not able to bind to SHBG [13]. We assume that the biphasicity derives from transformation processes, which occur during the binding experiments or reflect the dissociation equilibrium of the 8.5–9 S receptor form and the 4.5–5 S receptor form.

The apparent dissociation constant evaluated from the Scatchard plot is 0.5 nM for the 8.5–9 S form. The amount of binding sites is 40 fmol/mg protein in cytosol prepared in KPPF-buffer and 120 fmol/mg protein in cytosol prepared in MoKPPF-buffer. This points to the fact that molybdate is able to stabilize the receptor during preparation and incubation.

The experiments with DHT were strongly influenced by metabolism of this hormone in cytosol. It could be shown that DHT is converted to androstanediol (Fig. 1c).

From determination of ligand specificity by competition assay an atypical behaviour of DHT could be observed (Fig. 5 + Table I). This behaviour of DHT is explanable by metabolism of DHT as shown by thin layer chromatography (Fig. 1c). The results obtained with androstenedione are due to the activity of 17β-hydroxy steroid dehydrogenase (EC: 1.1.1.63 or 64), which converts this steroid to testosterone, which is a more affine ligand than androstenedione as proved by Brind *et al.* [26] in murine preputial gland, whilst DHT is converted to compounds, which are less affine than DHT itself.

The determination of the sedimentation coefficients brought about some interesting results. Density gradient centrifugation of cytosol prepared in KPPFM-buffer on a 8–30% glycerol gradient containing KPPFM-buffer spun for 65 min in a VTi 80 rotor at 80,000 rpm yielded a peak profile as shown in Fig. 6a. A main peak seen at 8.5–9 S has a shoulder at 6.5–7 S and a small peak at 4.5–5 S. This is in contrast to Bullock *et al.* [27], who found only a 7.5 S androgen receptor in cytosol of murine preputial

gland. Since addition of molybdate is generally believed to stabilize the receptors of steroid hormones, the experiment was repeated with MoKPPFM-buffer. Here we found only a sharp peak at 8.5–9 S and a slightly greater amount of receptor (Fig. 6b). This means that the receptor is stabilized but only in the 8.5–9 S form. The use of high ionic strength buffer (KPPFM-buffer containing 0.3 M KCl) yielded after density gradient centrifugation a peak at 4.5–5 S (Fig. 8.). This points to the fact that the androgen receptor of murine preputial gland is also transformable from a high molecular weight form to a low molecular weight form in high ionic strength buffer as are other steroid receptors too [28]. The 3.5 S receptor form detected in some centrifugation experiments (Fig. 6a and 7) seems to be a degradation product which is also often observed by other authors [16, 28–33]. The density gradient centrifugation experiments yield not only the sedimentation coefficients of the different receptor forms but also other effects, which can be observed dependent on the different preparation and centrifugation conditions. Comparing the peak profile of Fig. 6b with that of Fig. 6a it can be seen that molybdate is able to inhibit the receptor dissociation (from the 8.5–9 S form into smaller receptor forms). But Fig. 6c shows that under the same conditions which were used in the experiment, which yielded the peak profile of Fig. 6b, a slight receptor dissociation (from the 8.5–9 S form to the 6.5–7 S form) can be observed, when hormone is in the gradient and so always available to the receptor. This receptor dissociation is markedly elevated, when high concentrations of SH-reductants are added (Fig. 7). Longer centrifugation times seem to increase this dissociation effect.

A receptor loss of about 80% was registered during density gradient centrifugation. What happened to the receptor? Our experiments concerning this question (data not shown) suggest an important role of the oxydation/reduction status of the receptor in influencing its binding capability: oxydation of the receptor molecule leads to a decrease of its ability to bind testosterone.

A support for this oxydation hypothesis is presented by Grippo *et al.* [34] who observed a similar oxydation phenomenon of the glucocorticoid receptor. These authors showed that the thioredoxin system (*i.e.* thioredoxin + thioredoxin reductase + $NADPH+H^+$) is involved in protecting the glucocorticoid receptor from oxydation [35]. The oxydation hypothesis regarding the androgen receptor of murine preputial gland is presently investigated in our laboratory.

The negative results achieved by trying to purify the receptor by affinity chromatography as well as the unsuccessful efforts with isoelectric focusing seem to be at least partially due to oxydation. This and the loss of receptor during density gradient centrifugation show that the androgen receptor of murine preputial gland is less stable than the androgen receptor of murine skeletal muscle of the same mice.

Acknowledgement

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