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The Effect of Androgens and Antiandrogens on the Immunohistochemical Localization of the Androgen Receptor in Accessory Reproductive Organs of Male Rats

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The androgen receptor (AR) was localized immunohistochemically after different hormonal treatments in the ventral prostate, coagulating gland, seminal vesicle and epididymis of the adult rat. In the untreated controls AR-immunoreactivity was confined to the cell nuclei. One week after castration or treatment with the gonadotropin-releasing hormone antagonist Cetrorelix (150 μg/animal per day) a cytoplasmic staining occurred in the epithelial cells of the ventral prostate and in part of the coagulating gland and seminal vesicle. In contrast, the AR remained exclusively in the nuclei in the epididymal epithelium and the glandular smooth muscle layer even after 2 weeks of androgen depletion. Bolus injections of either dihydrotestosterone (1 mg/kg), the antiandrogen flutamide (40 mg/kg), or the novel non-steroidal antiandrogen casodex (40 mg/kg) to androgen-depleted animals eliminated cytoplasmic AR-immunoreactivity and restored the nuclear staining pattern in the ventral prostate. A sustained 2-week treatment with the antiandrogens resulted in a loss of weight in all organs but did not alter the distribution of AR-immunoreactivity. The data show an apparent cytoplasmic/nuclear ligand-dependent translocation of the AR in the ventral prostate, coagulating gland and seminal vesicle but not in the epididymis of the adult rat.

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INTRODUCTION

The action of androgens is mediated by the intracellular androgen receptor (AR). Similar to other steroid receptors, the binding of the hormone finally results in the regulation of the transcription rate of hormone-dependent genes. Initially the AR undergoes a transformation which enables the hormone–receptor complex to bind to specific DNA sequences designated as "hormone responsive elements" (HRE). Bound to the HRE, the complex interacts with other transcription factors that regulate the transcription of the target gene [1].

The rat, mice and human AR has been sequenced, specific antibodies have been produced and the AR was localized by immunohistochemistry in a variety of tissues [2–6]. Nevertheless there is only scant information about the effects of different hormonal treatments on the functional state of the AR and the corresponding AR-immunoreactivity. Androgen depletion renders the AR unoccupied and untransformed. The total cellular AR content is influenced by the presence or absence of androgens, a process designated as autoregulation [3]. Antiandrogens are known to bind to the AR without triggering a hormonal effect on target genes. Nevertheless, they may alter the functional state of the AR, the cellular AR content or the subcellular localization of the AR.

The subcellular localization of unoccupied steroid receptors has received much attention but there are still some open questions. According to the two-step-model established by Jensen and Gorski [7, 8] the unoccupied steroid receptors were supposed to reside in the cytoplasm and to translocate to the nucleus after hormone binding and transformation. In contrast to this, the unoccupied receptors for estrogens and progestins were localized immunmohistochemically in the nuclei of target cells [9, 10]. However, cytoplasmic staining after hormone depletion and hormone-induced nuclear

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translocation have been described for the glucocorticoid receptor (GR) [11, 12], the mineralocorticoid receptor [13] and the vitamin D receptor [14]. This was also shown for the AR in cells which were transfected with the AR-gene, thus overexpressing the AR *in vitro* [15, 16].

We intended to compare the effects of androgen depletion and the effects of the antiandrogen flutamide and the novel non-steroidal antiandrogen casodex on the subcellular distribution of AR in androgen target organs. The compound casodex is of special interest because it does not produce a compensatory rise—at least in the short term—of serum androgen levels as seen with flutamide [17]. Casodex and flutamide possibly have different effects on the AR itself. It is known that some antisteroids induce a transformation of the receptor into the DNA binding form (type II), while the others do not (type I) [18].

We therefore investigated whether different alterations of the functional state of the AR are reflected in the AR-immunoreactivity of natural target tissues. Untreated controls were compared with the AR-immunoreactivity after androgen depletion which renders the AR unoccupied and untransformed. A semi-quantitative estimation of changes in the cellular AR distribution was performed. Bolus injections of either dihydrotestosterone (DHT), flutamide or casodex to previously androgen-depleted animals were used to produce different AR–ligand complexes and to permit investigation of the subcellular localization and immunoreactivity of these complexes. In addition, the antiandrogens were administered chronically to investigate their effect on AR autoregulation.

EXPERIMENTAL

Animals and compounds

Adult male Sprague–Dawley rats, weighing 350–380 g, were obtained from Charles River Wiga (Sulzfeld, Germany) and kept in groups of 2–3 under a 12:12 light–dark cycle. The animals had free access to rat chow and tap water.

DHT (5α-androstane-17β-ol-3-on, Sigma Chemical, Deisenhofen, Germany) was dissolved in ethanol–sesame oil (1:3, v/v) and injected intraperitoneally. Casodex [ICI 176,334: (2RS)-4-cyano-3-(4-fluorophenylsulphonyl)-2-anilide, ICI Pharmaceuticals, Macclesfield, England] [19] and flutamide (4-nitro-3-trifluoromethyl-isobutyranilide, Schering, Bloomfield, NJ, U.S.A.) were dissolved in sesame oil or sesame oil ethanol (3:1, v/v), respectively, and were administered subcutaneously or intraperitoneally. The gonadotropin-releasing hormone (GnRH)-antagonist Cetrorelix (AcDNAL-D4C1PHE-DPAL-SER-TYR-DCIT-LEU-ARG-PRO-DALA) [20] (Asta Pharma, Frankfurt a.M., Germany) was dissolved in a 5% glucose aequous solution and injected subcutaneously. The studies were performed in accordance with the regulations of the German Federal Law on the Care and Use of Laboratory Animals.

Experimental protocols

Experiment 1. 3 groups of 10 animals each were treated for 1 week. The first group received daily subcutaneous injections of the GnRH-antagonist (150 μg/animal), the second group was treated with vehicle and the third group was castrated at the beginning of the experiment. After 1 week the animals were anesthetized with carbon dioxide and decapitated. Trunk blood was collected for testosterone measurements. The ventral prostate, seminal vesicles and the epididymides were removed for immunohistochemical investigation.

Experiment 2. 5 groups of 10 animals each were treated for a period of 2 weeks as follows: castration at the beginning of the experiment, daily subcutanous injections of GnRH-antagonist (150 μg/animal per day), casodex (10 mg/kg per day, subcutaneously), flutamide (10 mg/kg per day, subcutaneously). The controls received daily vehicle injections. The animals were decapitated at the end of the experiment. Trunk blood was collected for testosterone measurement as well as tissue for the immunohistochemical investigations.

Experiment 3. 16 rats were castrated and 1 week thereafter received intraperitoneal injections of either DHT (1 mg/kg, $n = 5$), casodex (40 mg/kg body wt, $n = 3$) or flutamide (40 mg/kg body wt, $n = 3$), the controls ($n = 5$) were given sesame oil injections. One hour after injection the animals were decapitated. Trunk blood was collected for testosterone measurement and tissue for immunohistochemistry.

Immunostaining of AR

The tissue samples were placed in embedding medium for cryosections (Reichert-Jung, Nußloch, Germany), immediately frozen in liquid nitrogen and stored at -70°C until sectioning. 7 μm cryosections were thaw-mounted on poly-L-lysine coated slides and air-dried for 10 min. The sections were fixed by immersion in 3% paraformaldehyde containing 100 mM sodium phosphate and 5% sucrose, pH 7.2 at room temperature for 5 min. After rinsing in PBS (phosphate buffered saline)-buffer (pH 7.6) for 10 min the sections were incubated for 30 min at room temperature with 2% normal rabbit serum (Vector Laboratories Inc., Burlingname, CA, U.S.A.) to block non-specific binding. Excess serum was removed and the incubation with the AR52-antiserum against the AR was performed overnight in a humid chamber at 4°C (polyclonal rabbit IgG-fraction, final dilution 5 μg IgG/ml in PBS with 1.5% normal rabbit serum, pH 7.5). The AR52 was raised against the synthetic peptide 875 corresponding to a 15 amino acids sequence positioned N-terminal to the DNA-binding domain of the human and rat AR as described previously [3].

After incubation with the AR52, the sections were washed in PBS and the biotinylated second antibody was incubated for 45 min at room temperature

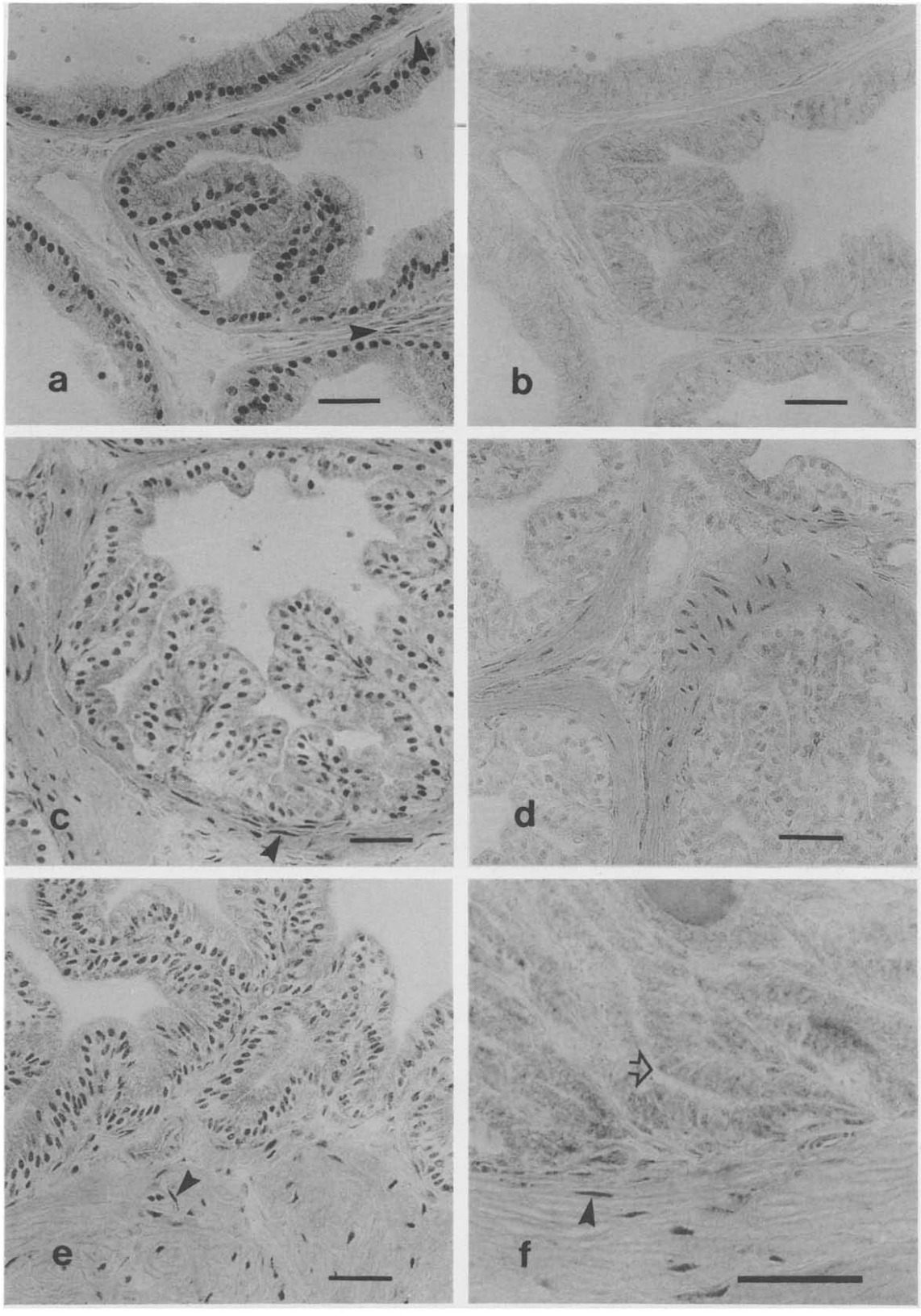

Fig. 1. Cryosections stained with AR52. (a) Ventral prostate, (c) coagulating gland and (e) seminal vesicle of vehicle-treated intact rats. Note AR52-positive epithelial nuclei and nuclei in the smooth muscle layer (arrowheads). (b) Adjacent section to (a) stained with AR52 after preadsorption with antigen. (d) Coagulating gland after 2 weeks of treatment with the antiandrogen casodex (10 mg/kg/day). (f) Seminal vesicle 1 week after castration, epithelial cells show some cytoplasmic label (arrow), the nuclear stain in the smooth muscle layer remains. Bars correspond to 40 μm.

followed by washing in PBS and incubation with the avidin–biotin solution for 45 min at room temperature (the second antibody and the avidin–biotin solution from the Vectastain Elite kit were prepared according to the manufacturer's protocol, Vector Labs). After washing in PBS followed by Tris–HCl-buffer (0.05 M, pH 7.6) the staining reaction was performed in 0.05% diaminobenzidin tetrahydrochloride and 0.0075% hydrogen peroxide (in Tris–HCl, pH 7.6) for 5 min. The reaction was terminated by washing in Tris–HCl. The sections were dehydrated and covered without counterstaining.

The specificity of the immunostaining was controlled by incubating the sections either with the preimmunserum or by preadsorbtion of the AR52 with the antigen: the diluted AR52 (5 μg IgG/ml) was incubated overnight at 4°C with the peptide 875 (5 μg/ml). After centrifugation the supernatant served as primary antiserum.

An evaluation of the staining pattern was performed on coded sections and attempted to provide semi-quantitative data on the distribution of the AR-immunoreactivity. Each section (3 per organ and animal) was examined independently by three different people who evaluated the difference between the cytoplasmic and nuclear immunostaining of the AR assigning rank values of 0 to 3 as follows: 3 denotes exclusive or maximal preponderance of nuclear staining and 0 indicates a lack of excess of nuclear over cytoplasmic staining.

Testosterone measurements

Serum testosterone was measured in unchromatographed serum using a double-antibody radioimmunoassay as described by Chandolia *et al.* [21]. The assay detection limit was 0.67 nmol/l and the intra-assay coefficient of variation was 4.3%.

Statistical evaluation

Data for testosterone and organ weights were analyzed using one-way analysis of variance followed by Tukey's test to determine significant differences. Rank data were subjected to non-parametric analysis of variance (Kruskal-Wallis' test) followed by multiple comparisons. Data are expressed as mean ± SEM or as median ± range (rank values). The probability level was set at 5%.

RESULTS

Localization of AR

The control stains were performed on sections of different tissues from animals of all experimental groups. No staining was observed in sections incubated with either preimmune serum or preadsorbed antiserum with the exception of a low background staining and occasional dark spots in the connective tissue [Fig. 1(b)]. Staining for AR52 in all organs of untreated control animals was characterized by intensively labeled nuclei of the epithelial cells (Figs 1 and 2). Most of the cells in the stromal compartment exhibited only slightly stained nuclei. In contrast, the nuclei of cells in the peri-epithelial smooth muscle layer were as intensively stained as the epithelial cell nuclei (Fig. 1). No cytoplasmic staining was observed in the vehicle-treated animals.

The effect of androgen depletion

Castration and GnRH-antagonist treatment led to a similar suppression of testosterone concentrations (Table 1). The effects of both treatments on the distribution and intensity of AR-immunoreactivity were indistinguishable. After 1 week of androgen depletion the epithelial cells in the ventral prostate were stained throughout the entire cell area. There was no difference in the intensity of nuclear and cytoplasmic label [Fig. 2(c)]. The epithelia in the coagulating gland and in the seminal vesicle did not stain uniformly after androgen depletion. In some cells the nuclear immunoreactivity was decreased whilst others additionally contained a cytoplasmic label [Fig. 1(f)]. In contrast to the epithelia of the accessory glands, the epididymal epithelium showed exclusively nuclear immunoreactivity [Fig. 2(b)]. Likewise, among the stromal cells of the smooth muscle layer only the nuclei exhibited the AR-signal (Figs 1 and 2) with the exception of the ventral prostate, in which the stroma showed several staining patterns as described above.

Two weeks after castration or GnRH-antagonist treatment the pattern of immunoreactivity was similar to that of the 1-week experiment. The nuclear localization of AR in the epididymal epithelial cells and in the smooth muscle layers of the seminal vesicle and coagulating gland persisted during the 2-week period. The intensity of nuclear staining in the epididymal epithelium and the glandular smooth muscle layer appeared to decrease.

Table 1. Serum testosterone levels after 1 and 2 week treatment with GnRH-antagonist, anti-androgens or following castration in adult rats

Group	*N*	Testosterone (nmol/l)
Experiment 1 (1 week)		
Control	10	21 1 ± 2.8
Castration	10	1 2 ± 0.1*
GnRH-antagonist	10	1.1 ± 0 1*
Experiment 2 (2 weeks)		
Control	10	26.1 ± 4 9
Castration	10	0 7 ± 0 03*
GnRH-antagonist	10	0.67 ± 0*
Casodex	10	32 6 ± 7 0
Flutamide	10	57 4 ± 7.7*

Values are mean ± SEM. Asterisks denote values significantly different from the control group ($P < 0.05$) For testosterone concentrations below the assay detection limit (castration and GnRH-antagonist) the lowest detectable concentration of 0 67 nmol/l was used for statistical analysis GnRH-antagonist (150 μg/kg per day), casodex and flutamide (10 mg/kg per day)

Treatment with antiandrogens for 2 weeks led to a weight reduction of the reproductive organs compared to the vehicle control, but the values remained higher than the castrated or GnRH-antagonist treated animals (Fig. 3). Casodex was more effective than flutamide which failed to induce a significant reduction of ventral prostate weight. The AR-immunoreactivity showed no alterations after treatment with casodex or flutamide (Fig. 4) except for a distinct decrease of the nuclear staining intensity for AR in parts of the coagulating gland [Fig. 1(d)].

The semi-quantitative analysis of the distribution of the AR revealed significant effects of castration and GnRH-antagonist treatment in ventral prostate, seminal vesicles and coagulating glands when compared to vehicle treatment ($P < 0.05$, Fig. 4). In contrast, administration of antiandrogens did not exert a significant influence on the cytoplasmic/nuclear localization of the AR ($P > 0.05$). Similarly, the localization of the AR was not significantly influenced by any treatment in the smooth muscle cells of the seminal vesicles and coagulating glands ($P > 0.05$, Fig. 4).

Castration and androgen replacement

In this experiment the influence of androgen replacement or antiandrogens on the immunoreactivity in the ventral prostate 1 week after castration was investigated. Animals of the control group were castrated and received sesame oil 1 h before decapitation. Again, this group showed staining in the cytoplasm of epithelial cells [Fig. 2(c)]. In contrast the DHT-injected animals exhibited an unequivocal nuclear staining pattern without cytoplasmic label [Fig. 2(d)]. In the casodex- and flutamide-injected castrated animals nuclear staining was also dominant, although a higher amount of cytoplasmic label was present. Quantification of the effects of DHT, casodex and flutamide on subcellular AR distribution revealed effects of all three compounds (Fig. 5) which, however, attained statistical significance for DHT only ($P < 0.05$).

Stromal AR-immunoreactivity appeared increased after ligand injections in the ventral prostate and the stain appeared to be mainly nuclear. In the seminal vesicle and in the coagulating gland where cytoplasmic

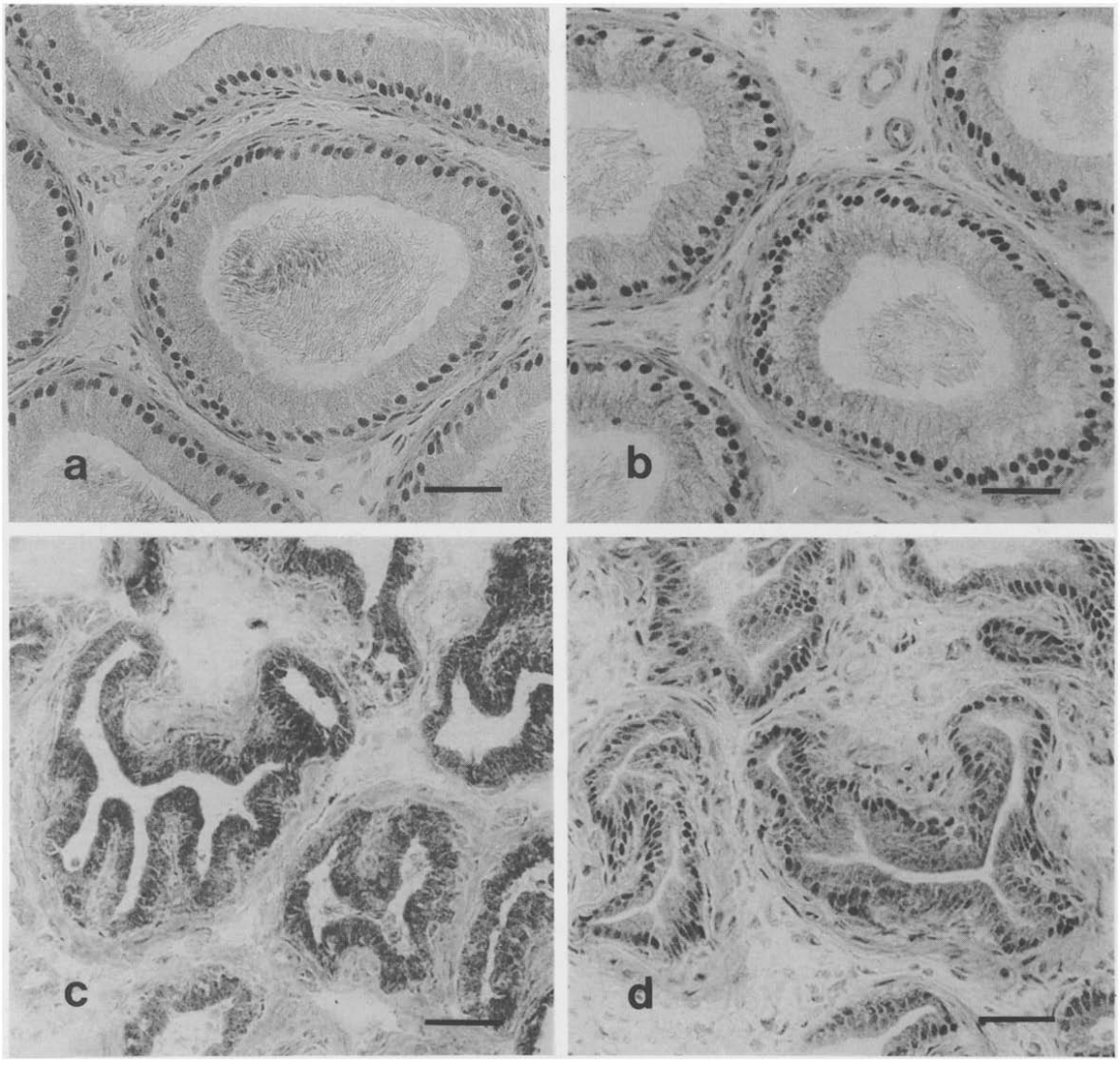

Fig. 2. Cryosections stained with AR52. (a) Epididymis from intact rats and animals treated for 1 week (b) with GnRH-antagonist (150 μg/animal/day). (c, d) Ventral prostate of rats 1 week after orchidectomy and injection of (c) sesame oil or (d) DHT (1 mg/kg). Bars correspond to 40 μm.

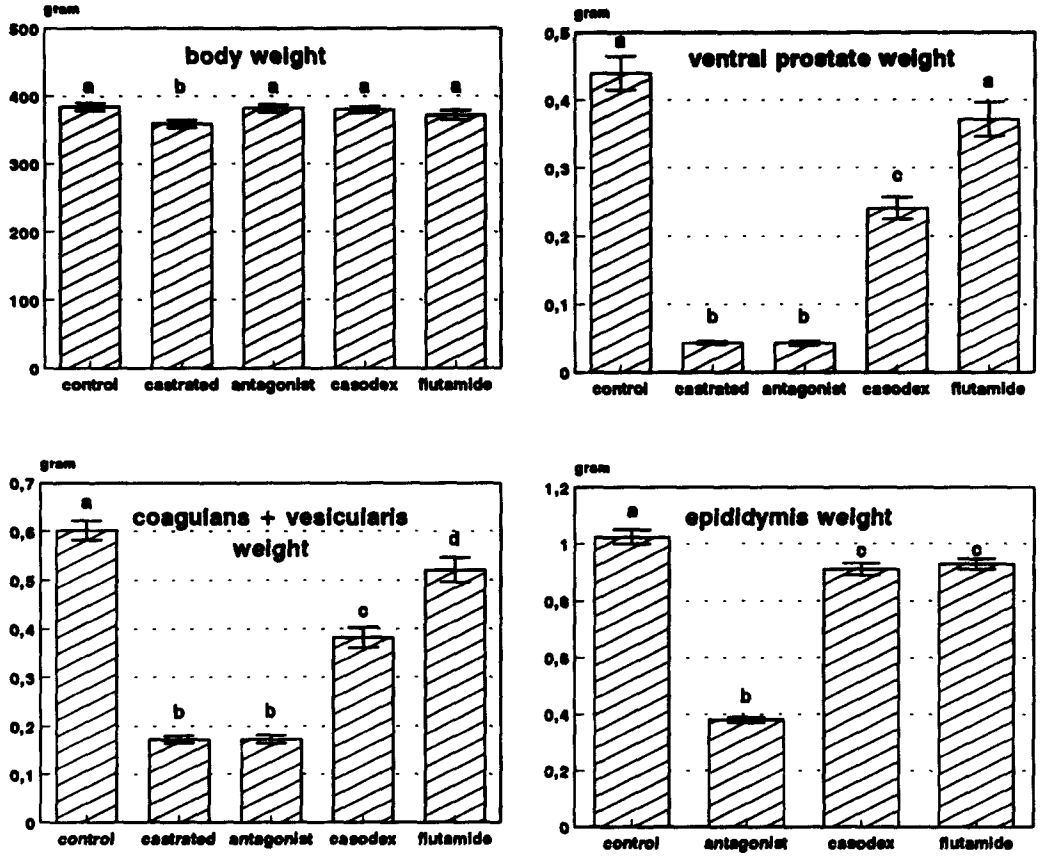

Fig. 3. Weights of reproductive organs after 2 weeks of treatment with GnRH-antagonist vehicle (control), after castration, treatment with GnRH-antagonist (150 μg/animal/day), casodex (10 mg/kg/day) or flutamide (10 mg/kg/day). Different superscripts indicate significant differences ($P < 0.05$). Values are means ± SEM, $n = 10$.

immunoreactivity after castration was less compared to the prostate, the effect of ligand injections appeared also weaker (data not shown).

DISCUSSION

In the present study we observed ligand-dependent changes of the AR-immunostaining in the ventral prostate, seminal vesicle and coagulating gland but not in the epididymis of the adult rat. In the glandular epithelia the nuclear staining predominated vs cytoplasmic staining in the presence of ligands compared to androgen-depleted animals. Restoration of the AR localization occurred in the presence of ligands compared to gondotropin/androgen-depleted animals. While most of the steroid receptors are downregulated by their ligands [22], the AR protein levels were reported to be upregulated by androgens [23, 24]. In contrast, some authors reported a suppression of receptor mRNA expression by androgens [3, 25]. Takeda *et al.* [26], however, found a decrease of AR mRNA in mice ventral prostates after castration using *in-situ* hybridization.

Interestingly, no decrease of nuclear AR-immunoreactivity occurred after treatment with antiandrogens, except for a tendency in the coagulating gland. The antiandrogens apparently maintained the AR level, although their antagonistic effect was evident by the loss in organ weights. This suggests that the maintenance of normal AR-levels does not depend on androgen-induced expression of the AR-gene. The AR-protein may rather be protected from degrading enzymes in its occupied form. Thus antiandrogens suppress hormone responses that depend on transcription, but protect the cellular receptor content.

Our data demonstrate that a certain type of target cell in the rat reproductive tract exhibits a cytoplasmic AR-immunoreactivity after androgen depletion, while in other cells only the nucleus remained labeled. To date we are not aware of other data on cytoplasmic AR-immunoreactivity in animal or human tissues after androgen depletion [26–31], except for a few of human prostate tumor specimens [32]. On the other hand, in AR-transfected COS-7 cells, overexpressing the receptor *in vitro*, the AR was localized in the cytoplasm in the absence of androgens [15, 16]. Jenster *et al.* [33], however, reported AR-transfected COS-1 cells to mainly display a nuclear localization of the AR following androgen depletion.

One reason for this discrepancy may be that the nuclear transportation or anchoring mechanisms of steroid receptors are different depending on the target cell. Specific nuclear importer proteins [34], the presence of heat shock proteins (HSP-70) [35, 36] and

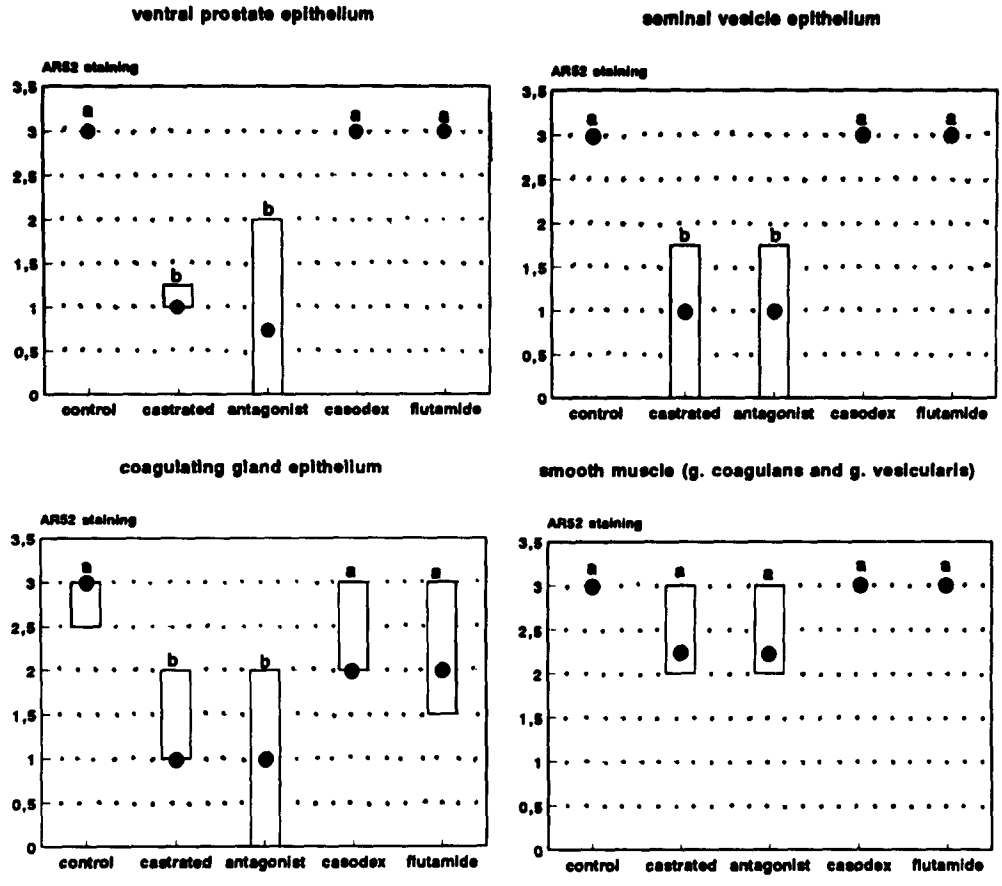

Fig. 4. Score values for the nuclear/cytoplasmic distribution of AR-immunoreactivity. The difference between the cytoplasmic and nuclear immunostaining of the AR assigning rank values of 0 to 3 as follows: 3 denotes exclusive or maximal preponderance of nuclear staining and 0 indicates a lack of excess of nuclear over cytoplasmic staining. Tissue was analyzed 2 weeks after administration of GnRH-antagonist vehicle (control), castration, GnRH-antagonist (150 μg/animal/day), casodex (10 mg/kg/day) or flutamide (10 mg/kg/day). Different superscripts indicate significant differences ($P < 0.05$). Values are medians (●) ± range (□) ($n = 5$). 3 sections per organ and animal were evaluated independently by three observers on coded sections.

receptor phosphorylation [37] have been suggested to be responsible for a nuclear versus cytoplasmic localization of unoccupied steroid receptors. A high degree of overexpression in receptor transfected cells may be an explanation for the cytoplasmic label [35, 38].

In case of the rat ventral prostate methodological differences seem to play an important role. The effect of castration and androgen replacement on the AR-immunoreactivity in the rat ventral prostate has been previously investigated by Sar *et al.* [30] and Husmann *et al.* [29]. Both authors observed a decrease in the nuclear staining intensity after castration and a restoration of the nuclear immunoreactivity by DHT injection, but no cytoplasmic staining was reported. Husmann *et al.* utilized an antibody which possibly does not recognize the unoccupied AR. This might explain why the unoccupied AR was not observed in the cytoplasm. However, as in the present study, Sar *et al.* [30] used the AR52 AR-antibody. They suggest that the unoccupied AR disappears during the immunostaining procedure or that the AR52 is unable to detect the unoccupied AR. The latter suggestion is not confirmed by our data. Moreover, the AR52 recognized the unoccupied AR in AR-transfected cells [15, 16]. This rather suggests that even minimal deviations in the processing of the tissue may lead to notable differences in the immunoreactivity, especially for the unbound AR.

Unoccupied steroid receptors are known to be easily extractable. This is the reason for their presence in the cytosol after cell fractioning, even if they are nuclear proteins *in vivo* [39, 40]. This artifact has been the basis for the "two-step-model" of a hormone induced nuclear translocation. This extractability may also be the reason for the disappearance of unoccupied AR as suggested by Sar *et al.* [30]. The procedure used in the present study might have yielded a favorable preservation of the unoccupied AR for unknown reasons. However, this also raises the question whether the cytoplasmic immunoreactivity observed in the present study reflects the true *in vivo* localization of the unoccupied AR. Interestingly, there is growing evidence that in some instances the cytoplasmic localization of the unoccupied GR is due to an artificial redistribution which may be related to improper fixation methods [41–43].

From the present data it is not possible to decide whether the cytoplasmic staining and the nuclear translocation upon ligand injection reflect a translocation of the AR or an artificial redistribution of the

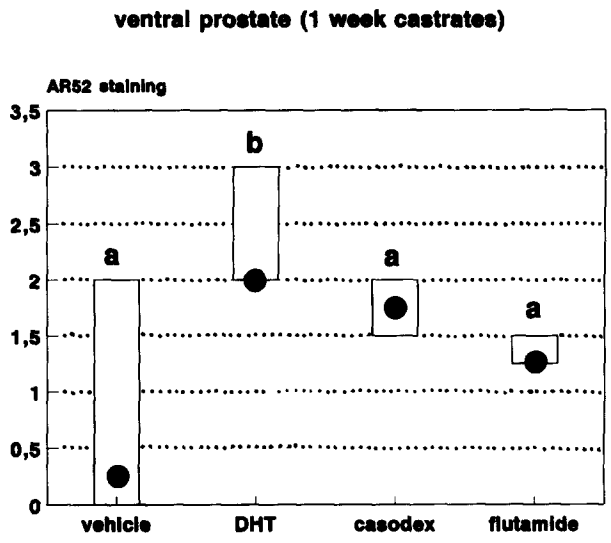

Fig. 5. Score values for the cytoplasmic/nuclear AR-immunoreactivity. The difference between the cytoplasmic and nuclear immunostaining of the AR assigning rank values of 0 to 3 as follows: 3 denotes exclusive or maximal preponderance of nuclear staining and 0 indicates a lack of excess of nuclear over cytoplasmic staining. All animals were orchidectomized and 1 week later received an intraperitoneal injection of DHT (1 mg/kg, $n = 5$), casodex (40 mg/kg, $n = 3$), flutamide (40 mg/kg, $n = 3$), or sesame oil (control, $n = 5$). One hour after the injection animals were decapitated. Different superscripts indicate significant differences ($P < 0.05$). Values are medians (●) ± range (□). 3 sections per organ and animal were evaluated independently by three observers on coded sections.

unoccupied AR which is prevented if the injection of a ligand induces a transformation of the AR into the tightly bound nuclear form. It should be noticed, that the interpretation of specific amino-acid sequences of steroid receptors as "nuclear targeting signals" which are activated by the hormone [15, 16, 33, 44] depends on the validity of the subcellular localization. Thus, a hormone-dependent "nuclear anchoring sequence" or a mediator of transformation may be misinterpreted as a hormone-dependent "nuclear targeting sequence".

However, the apparent translocation of the AR in the ventral prostate allowed an insight into the mechanism of antiandrogen action. After flutamide treatment and cell fractioning the AR has always been measured predominantly in the cytosol fraction which is similar to the results for the unoccupied AR [29, 45 ,46]. Based on these data flutamide would be classified as a type I antisteroid that binds to the receptor without triggering its transformation. But flutamide as well as casodex induced the nuclear "translocation" of the AR-immunoreactivity in our experiments and it has previously been demonstrated that flutamide translocates the AR-immunoreactivity from cytoplasm to the nucleus of AR-transfected COS-7 cells [15]. With a mutated form of the AR from the lymph node carcinoma cell line LNCaP Veldscholte *et al.* [36] report different results. In that system flutamide exerts agonistic activity whereas casodex stabilized the association of heat shock proteins with the untransformed AR. However, our data confirm the hypothesis that, on the wildtype AR, flutamide as well as casodex are type II antisteroids that transform the AR and build transcriptionally inactive complexes which are located in the nucleus.

In conclusion the data presented here show heterogeneity in the cellular localization of AR-immunoreactivity after androgen depletion. This indicates differences in the mechanism of AR transformation or translocation in different target cells and organs. The antiandrogen flutamide and the novel compound casodex can alter the functional state of the AR and build complexes which reside in the nucleus. The antiandrogens do not induce a detectable AR downregulation at the immunohistochemical level.

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