J. steroid Biochem. Vol. 17, pp. 421 to 426, 1982
Printed in Great Britain.

0022-4731/82/100427-06$03.00/0

RESOLUTION OF TWO FRACTIONS OF ANDROGEN RECEPTOR FROM MOUSE KIDNEY

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(*Received* 30 *June* 1981)

SUMMARY

Putative androgen receptors from mouse kidney were separated and re-chromatographed on DNA-cellulose columns. With either [^{3}H]-dihydrotestosterone or [^{3}H]-testosterone as ligands, two major fractions of androgen receptors were obtained in both elutions from DNA-cellulose columns; these eluted, respectively, at 140–150 mM NaCl (lower-salt) and 180–190 mM NaCl (higher-salt). Therefore, the two major peaks detectable with extracts of mouse kidney result from differences that are intrinsic to the receptor complexes rather than heterogeneity of the DNA-cellulose matrix. These findings are discussed in relation to two forms of androgen receptors that exist in a different proportion in extracts of brain and in relation to other steroid receptors.

INTRODUCTION

Androgen receptors from the kidney [1–6] and regions of the brain [1–4, 7–11] including hypothalamus of mice and of rats [6, 10–11] have been characterized qualitatively by DNA-cellulose chromatography. For hypothalamic extracts of wild-type mice a major lower-salt eluting receptor is detected, and there is a minor component of higher-salt eluting receptor; for the androgen-resistant mutant with testicular feminization (*Tfm*), brain extracts have exhibited only a fraction which elutes with higher salt concentrations [1, 3, 9]. In contrast, kidney extracts of wild-type mice have similar amounts of both lower-salt and higher-salt eluting androgen receptors [1, 3, 5]; again in *Tfm*, kidney has exhibited only a higher-salt eluting fraction [1, 3, 5]. These differences between wild-type and *Tfm* receptors are intrinsic to the binding complexes, since additive elution patterns are obtained when mixed extracts are chromatographed on DNA-cellulose columns [3]. It is undetermined whether the putative receptor from *Tfm* mice (10% of the wild-type level) represents a normal component that is not easily apparent in wild-type extracts or a mutant protein product that fortuitously resembles a component of the wild-type. An apparent isoelectric focusing variation for *Tfm* was also obtained when the separated higher-salt fraction from wild-type mice was compared [5]. This finding emphasizes that resolution of the nature of the *Tfm* mutation requires determination of which heterogeneous fractions of androgen receptors normally exist in wild-type extracts.

In this study, we demonstrate that the lower-salt and higher-salt eluting fractions of androgen receptors from mouse kidney can be separated from one another and that they retain distinguishable elution characteristics.

EXPERIMENTAL PROCEDURES

Chemicals

Extract buffer contained 10 mM Tris–HCl, pH 8.1 (21°C), 1 mM β-mercaptoethanol, 1 mM Na_2EDTA, 10% glycerol, and 50 mM NaCl; the molarity of NaCl during chromatography is indicated in the text and figures. Non-radioactive steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Labeled steroids [1,2,4,5,6,7-^{3}H(*N*)]-dihydrotestosterone, 123 Ci/mmol, [1,2,4,5,6,7,16,17-^{3}H(*N*)]-dihydrotestosterone, 190 Ci/mmol, [1,2,6,7-^{3}H(*N*)]-testosterone, 85 Ci/mmol, and [1,2,6,7,16,17-^{3}H(*N*)]-testosterone, 152 Ci/mmol, were obtained from New England Nuclear, Inc. (Boston, MA). Purity of these components was monitored by thin layer chromatography and high-performance liquid chromatography. The [1,2,4,5,6,7,16,17-^{3}H(*N*)]-dihydrotestosterone stocks were purified by high performance liquid chromatography prior to use [4, 6].

Extracts

Mice (C57BL/6J, or C57BL/6 × C3H F_1 hybrids) at 3–4 weeks of age were used. Neither genetic background nor sex affected the yield or qualitative chromatography of the resultant receptor preparations. Animals were killed by cervical dislocation, and were decapitated. Kidneys were removed, stripped of their capsules, then homogenized in

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extract buffer by hand in a glass–glass Duall homogenizer (Kontes Glass Co., Vineland, NJ) at 2°C at a concentration of 600 mg tissue/ml of buffer. Homogenates were then diluted to 300 mg tissue/ml of buffer, and centrifuged at 140,000 *g* for 45 min. The supernatants were decanted without inclusion of the floating lipid layer.

DNA-cellulose chromatography

DNA-cellulose chromatography was performed essentially as before [6] with some modifications. Samples were incubated with the appropriate concentration of radioactive hormone for 1 hour before application to the DNA-cellulose columns. Ionic strength of extracts and buffers was monitored by measuring conductivity. The ionic strength of the extract was reduced, when necessary, by addition of buffer containing no added NaCl, to be equivalent to extract buffer with 50 mM NaCl. Samples were loaded by gravity onto the DNA-cellulose columns, which had been equilibrated with buffer containing 50 mM NaCl.

Columns were washed for a minimum of 12 column-volumes with a peristaltic pump at 1 column-volume per hour. Radioactivity in the wash fractions was monitored until it reached a low, constant background. Samples were then eluted with a linear gradient of NaCl (50–300 mM). One ml or 0.5 ml fractions were collected and counted in a two-phase system using Omnifluor (New England Nuclear, Boston, MA)–toluene [7].

RESULTS

When extract of mouse kidney was chromatographed on DNA-cellulose, two major peaks of androgen receptors eluted with a gradient of NaCl (Fig. 1). Androgen ligands, dihydrotestosterone (Fig. 1) and testosterone (Fig. 2, solid line), both labeled DNA-binding material which eluted in these two major fractions. By diluting wild-type kidney cytosol 10-fold before application to a DNA-cellulose column, we tested the possibility that one of the two elution forms represented a reversible, concentration-dependent complex containing the other form. The salt-dependent elution profile of the diluted extract (Fig. 2, dotted line) is grossly similar to that of the more concentrated extract (Fig. 2, solid line); therefore, the concentration of extract does not appear to influence significantly the subsequent elution of the receptors. The recoveries obtained with the diluted sample are similar to values reported by others [12].

The heterogeneity of the androgen receptor elution profile might have resulted from a single receptor form binding to multiple sites on DNA-cellulose, rather than from intrinsic differences among receptor fractions. These alternatives were resolved by collecting the two separate fractions and re-chromatographing them on DNA-cellulose (Fig. 3). The fraction which eluted with a lower concentration of NaCl re-

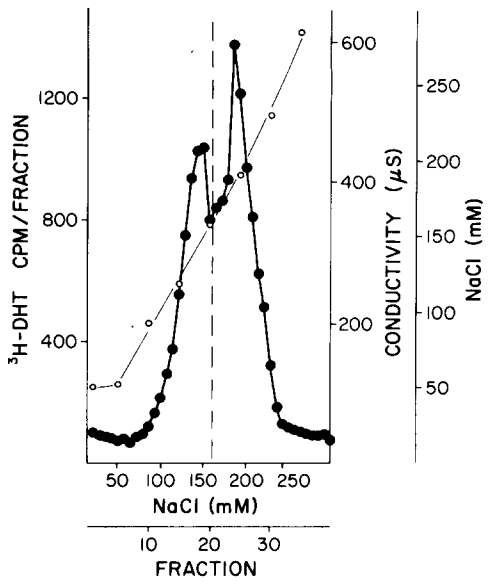

Fig. 1. DNA-cellulose chromatography of kidney androgen receptor. A cytosol extract of two kidneys was prepared and labeled with 10 nm [^{3}H]-dihydrotestosterone (DHT) (190 Ci/mmol). After application of the labeled cytosol to the DNA-cellulose column, the column was washed, and then eluted with a gradient of NaCl ranging from 50 to 400 mM NaCl. All radioactivity (●——●) eluted between 100 and 250 mM NaCl. Conductivity (○——○) was determined for every fifth sample. The conductivity curve can be read directly as NaCl concentration, as shown by the outer right-hand axis. The vertical dashed line denotes 160 mM NaCl.

chromatographed with a major species again eluting at the lower NaCl concentration, while the fraction which initially eluted with a higher concentration of NaCl re-chromatographed with a major species eluting at the higher NaCl concentrations. The separabi-

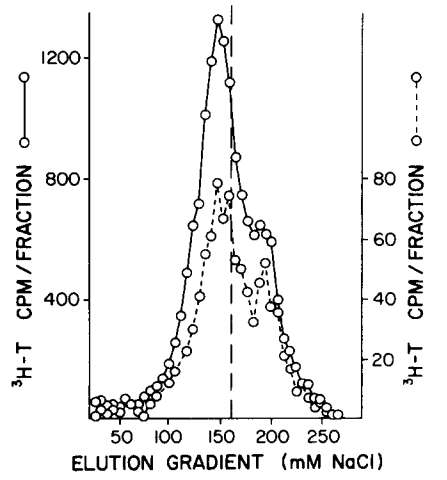

Fig. 2. Chromatography of diluted androgen receptor. A cytosol extract of 5 kidneys in 1.25 ml (○——○), and a diluted sample of the same cytosol, equivalent to 0.5 kidney in the same volume (○----○), were labeled with 10 nM [^{3}H]-testosterone (T) (85 Ci/mmol) and applied to parallel DNA cellulose columns. The columns were then washed and eluted as in Fig. 1. The vertical dashed line denotes 160 mM NaCl.

lity of these two forms is apparent with either [^{3}H]-dihydrotestosterone (Fig. 3a) or [^{3}H]-testosterone (Fig. 3b) as the labeling ligand. When we sampled the elution envelope with four sequential fractions and re-chromatographed these, the resulting elution patterns were similar to those in Fig. 3 and likewise revealed two major receptor species. Thus the elution characteristics of the two detected fractions of androgen receptor are stable and appear to be intrinsic to the cytosol components. Since these results were necessarily obtained with only portions of the eluates, they confirm the finding (Fig. 2) that diluted receptors retain intrinsic chromatographic characteristics.

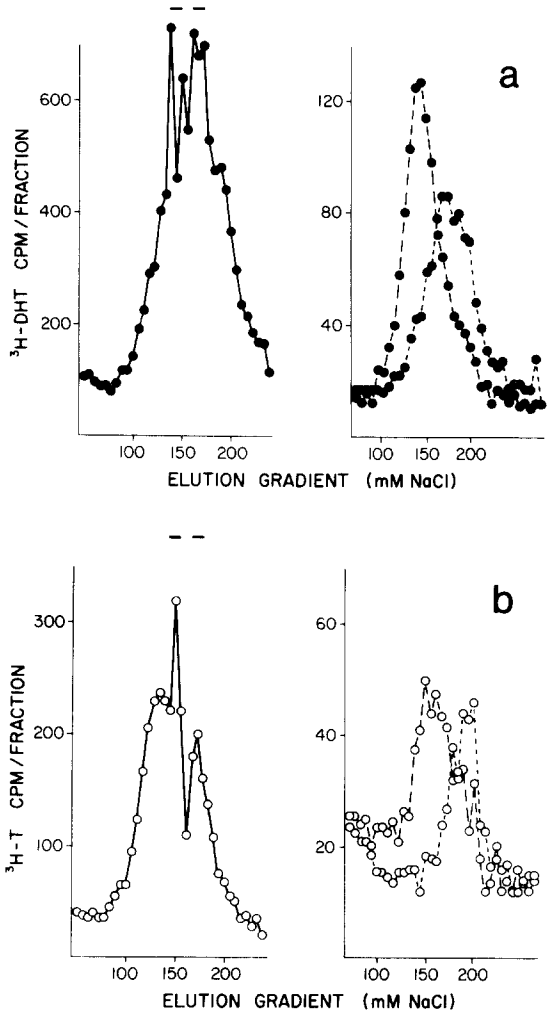

Fig. 3. Re-chromatography of two fractions of androgen receptors. (a) Kidney cytosol was incubated with [^{3}H]-dihydrotestosterone (DHT) and chromatographed on a DNA-cellulose column (left-hand panel). Fractions (represented by heavy bars) containing either lower-NaCl eluting or higher-NaCl eluting material were obtained after measuring sample aliquants. The samples were diluted to 50 mM NaCl and re-applied to other DNA-cellulose columns. These columns (right-hand panel) were washed and eluted with a salt gradient in the same manner as the original column. The re-chromatographed lower-salt peak is denoted by long dashes; the re-chromatographed higher-salt peak is denoted by short dashes. (b) The same protocol was followed as in (a) above, using [^{3}H]-testosterone (T) as the ligand. Original column is shown in left-hand panel; re-chromatographed samples are shown in right-hand panel. The lower salt peak is shown with long dashes, the higher salt peak with short dashes.

The experiments in this study establish that the heterogeneity of elution patterns for androgen receptors from mouse kidney extracts is intrinsic to the binding species and not attributable to the column matrix. Other data suggest that the two major species may be present in the tissue when the extract is prepared and not be a result of artifactual degradation in the extract. When wild-type and *Tfm* extracts, of either kidney or brain, were mixed and chromatographed on DNA-cellulose, additive patterns were obtained [3]. This indicates that the differences in elution patterns for wild-type and *Tfm* are not caused by soluble factors or enzymes that are active in the respective extracts. Furthermore, when radioactively labeled androgen receptor in brain extracts—which is predominantly the lower-salt form—was mixed with kidney extract of wild-type mice—which has both receptor forms—or with kidney extract of *Tfm* mice—which has only a higher-salt receptor—the elution profiles from DNA-cellulose were identical. Thus wild-type and *Tfm* kidney extracts, with higher-salt receptor, did not modify the brain receptor, which is the lower-salt form, or vice versa. Altogether, these mixing experiments suggest strongly that both major species of androgen receptors are stably present in mouse kidney cytosols.

To test if one receptor form or the other is generated when the tissue homogenates are made, we homogenized mouse kidneys in the presence of several different agents which inhibit proteases (T. O. Fox, M. S. Kindy, L. I. Siegel, unpublished data). PMSF (phenylmethylsulfonyl fluoride, 1–4 mM), leupeptin (synthetic acetyl-L-leucyl-L-leucyl-L-argininal, 1 mM), Bacitracin (1 mM), and EGTA (ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*′-tetraacetic acid, 1–5 mM) did not reduce the quantity of either peak. In some cases one peak was increased, but not with concomitant decrease of the other receptor form. Thus we conclude to date that either both receptor forms exist in the kidney, or the formation of one occurs very early in the procedure and is not sensitive to several types of inhibitors and concentrations which we have examined so far.

While both major receptor fractions were labeled by either dihydrotestosterone or testosterone (Fig. 3), these two ligands were associated with distinguishable elution profiles (Figs 1 and 2). This difference in detected forms was quantified by designating material eluting with less than 160 mM NaCl in the gradient as the lower-salt eluting fraction and material eluting with more than 160 mM NaCl as the higher-salt eluting fraction. The average values of the lower-salt peak : higher-salt peak ratios obtained from several experiments were 1.47 (SEM = 0.085, n = 11) for testosterone and 0.83 (SEM = 0.057, n = 14) for dihydrotestosterone.

One possibility for the difference in chromatographic profiles of testosterone- and dihydrotestosterone-labeled kidney cytosols was that these two ligands bound preferentially to the different receptor species. Therefore, we compared the hormone-binding properties of the androgen receptors for these two ligands. This was done by incubating for one hour in the presence of alternate competing androgen ligands prior to DNA-cellulose chromatography. Since dissociation rates are relatively low [12, 13] little dissociation occurs in one hour. Thus, a competition effect in this experiment primarily should reflect the relative association of those ligands with receptors in these extracts. For example, if a given form of androgen receptor (lower- or higher-salt eluting) bound testosterone more readily than it did dihydrotestosterone, these two ligands would compete unequally. This would result in differential elution patterns, and the ratios of the peaks would be altered.

We compared the relative binding to the two forms of receptor detected on DNA-cellulose by cross competition with each androgen at a concentration of 5 nM, after determining that saturation was achieved for both ligands at the same concentration. Quantitatively, both non-radioactive ligands inhibited 50% of the radioactive binding; the elution patterns of the remaining 50% are shown in Fig. 4. The choice of the competing ligand did not significantly alter the elution profile associated with each radioactive ligand, and the respective ratios (low:high) of the eluted peaks were unchanged: for [^{3}H]-testosterone, 1.27 and 1.32; for [^{3}H]-dihydrotestosterone, 0.81 and 0.86. Thus the characteristic elution differences of testosterone compared with dihydrotestosterone appear to be determined by events occurring after ligand binding.

In ideal testing conditions, association rates should be diffusion limited; the association rates of most hormones is approximately $10^8\ M^{-1}$/min at 37°C [14], which is in the estimated range for purely diffusion-controlled processes [15]. The rates will differ significantly if alternate ligands differ significantly in size, in solubility, or in interactions with other components of the solution, and if proper orientations or conformations are required for binding. The results in Fig. 4 indicate that both androgen ligands tested associated similarly with the two androgen receptor fractions. Therefore, the differential elution patterns require another explanation. The differences obtained may result from differential stability of the hormone-receptor complexes, of the protein-DNA complexes, or of the proteins themselves.

Prior studies [4] suggested that a small proportion of 3-ketosteroid reductase in mouse kidney might complex with androgen receptors under the conditions of these experiments. If this enzyme or another factor were preferentially sequestered with the lower-salt receptor prior to elution, a lower ratio of dihydrotestosterone to testosterone in the lower-salt peak might result and thus cause the observed ligand-dependent peak ratios. This could be physiologically relevant or simply be an additional indicator that mouse kidney extracts contain distinguishable receptor forms.

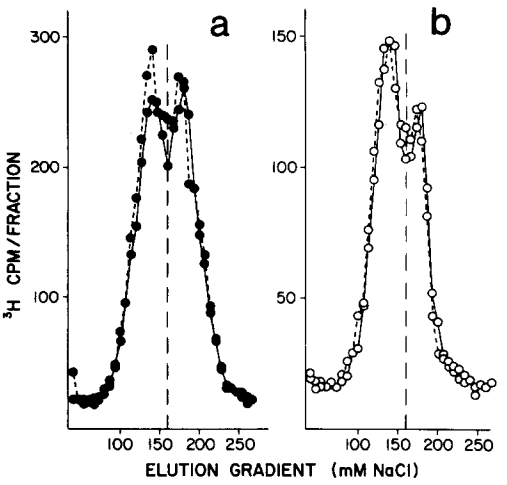

Fig. 4. Equimolar competition by testosterone and dihydrotestosterone. Aliquots of cytosol equivalent to two kidneys each were simultaneously labeled with 5 nM [^{3}H]-dihydrotestosterone (a) or 5 nM [^{3}H]-testosterone (b). Each radio-ligand was pre-mixed with either 5 nM non-radioactive dihydrotestosterone (dashed lines in panels a and b) or 5 nM non-radioactive testosterone (solid lines in panels a and b). Samples were then chromatographed on DNA-cellulose.

DISCUSSION

Two fractions of androgen receptors from mouse kidney were distinguished. This is in keeping with the suggestion of their existence from comparisons of hypothalamus and kidney [1, 3] and of wild-type mice with the androgen-resistant mutant, *Tfm* [1, 3, 9]. With the recognition of distinguishable forms for estrogen [16, 17], progesterone [18, 19], and glucocorticoid receptors [20], it is possible that each class of steroid receptors exhibits dichotomous properties [21]. Our findings for androgen binding complexes of mouse kidney are consistent with this possibility. The androgen receptors from mouse kidney differed in their elution from DNA-cellulose columns and in their apparent relative abundance with testosterone versus dihydrotestosterone as the radioligand.

The detection of multiple forms of androgen receptors has been reported in other tissues. Norris and Kohler [22] reported that androgen receptors from a hamster ductus deferens tumor cell line chromatographed as two peaks on DEAE-cellulose. Multiple forms seen [23] in rat prostate cytosols were distinguishable by sedimentation and by gel chromatography. The origin of some of these forms was traced to proteolytic activity in the prostate cytosols [24]. These smaller forms of prostate androgen receptors were similar to the smaller forms of hepatic glucocorticoid receptors produced [25] through con-

trolled proteolysis. In addition, smaller "mero-receptors" from chick oviduct progesterone receptors have been produced [26, 27] by the action of calcium-activated protease. In contrast to these examples, differences in sedimentation have not been detected [5] between the two forms of androgen receptors from mouse kidney investigated here, and the differences in DNA-binding were subtle. This is consistent with a greater degree of physical similarity, although effects of some type of modification have not been ruled out. It remains to be determined whether one form is derived from, or is a multimeric form of, the other and whether multiple genes are required for the production of these receptors.

Regarding estrogen receptors [16, 17], the sedimentation rate for those derived primarily from the cytosol (8S in low ionic strength, 4S in high ionic strength) differs from that of estrogen receptors obtained primarily from nuclear preparations (5S). Two forms of estrogen receptors have also been resolved by their differential binding to DNA-cellulose [28], ability to complex with a serum factor [29] and differences in estrogen affinity [30]. In addition, two classes of estrogen receptors have been suggested on the basis of thermal stability [31]. For progesterone receptors, two forms from chick oviduct were detected by agarose gel chromatography [18]. These forms were resolved and characterized on the basis of differences in ion-exchange chromatography and DNA-binding [19, 27]. For glucocorticoid receptors, also, two forms can be distinguished by nuclear and DNA binding, and by DEAE-cellulose chromatography [20, 32–34]. Thus many examples of multiple forms exist for steroid receptors, though it is not known how the different hormone receptor systems relate to one another or how significant these dichotomies will prove to be physiologically. The demonstration in this report of two fractions of androgen receptors for mouse kidney, as well as their relative distributions among tissues [1, 2, 3, 9] and in androgen-resistant mutants [3, 6], may help to explain how and why multiple receptor forms are detected.

Acknowledgements—We thank Mark S. Kindy for assistance with these experiments and W. Frost White for constructive discussions. Portions of this work were supported by a Basic Research grant from the March of Dimes Birth Defects Foundation (T. O. F.), a predoctoral National Research Service Award (to S. J. W.) in Cell and Developmental Biology (Grant 5T32GM07226), Grant HD10818 and Research Career Development Award (National Institute of Child Health and Human Development, to T. O. F.), and the Mental Retardation Research Center.

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