Regulation of Cytoplasmic Dihydrotestosterone Binding in Dog Prostate by 17β-Estradiol

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ABSTRACT 17β-estradiol enhances androgen-induced prostate growth in the castrate dog to a degree comparable to that seen in spontaneous prostatic hypertrophy. To investigate the mechanism of this synergism, cytosol androgen binding was measured by a density gradient technique in prostates of control and 17β-estradiol-treated castrate dogs. [^{3}H]Dihydrotestosterone was bound principally to a moiety that averaged 8.6S in size. Approximately twofold enhancement of this binding by 17β-estradiol was demonstrable after 1 wk of treatment with 750 μg/wk and after 3 wk with 75 μg/wk. Under conditions in which binding in the 8S region was demonstrable with dihydrotestosterone and testosterone no binding of 3α-androstanediol or progesterone was detectable. Thus, enhancement by 17β-estradiol of a prostate cytosol androgen-binding protein occurs under circumstances in which 17β-estradiol enhances androgen-mediated prostatic growth.

INTRODUCTION

Benign prostatic hypertrophy—enlargement of the prostate to the degree that it produces an obstruction of the urethra and(or) rectum—is known to occur commonly in only two species, man and dog (1, 2). The disorder does not develop in the castrate, and an endocrine etiology has long been suspected. In studies designed to explore the pathogenesis of prostatic hypertrophy, we observed that 17β-estradiol alone is ineffective in inducing prostatic growth, but 17β-estradiol has a synergistic effect in promoting growth of the gland in the castrate dog when administered along with the androgen 3α-androstanediol[1] (3). Administration of the two hormones together uniformly results in development of prostatic hypertrophy within 12 wk comparable to that seen in the naturally occurring disorder (3, 4). The nature of the augmentation of androgen action by estrogen is unknown. We have shown that estrogen has no measurable effect on the metabolism of androgen within the gland (4). However, 17β-estradiol is known to enhance the production of the progesterone receptor protein in target tissues such as rabbit uterus (5), guinea pig uterus (6), rat breast (7), and human breast (8, 9). Therefore, experiments were designed to determine whether 17β-estradiol also regulates activity of the cytoplasmic androgen receptor protein that is believed to be essential for transporting androgen from the cytosol to the nuclear sites of action of the hormone within the prostatic cell.

Received for publication 30 November 1977 in revised form 9 October 1978.

[1] *Nomenclature used in this paper*: Dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 3α-androstanediol, 5α-androstane-3α,17β-diol; methyltrienolone (R1881), 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one; promegestone (R5020), 17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-acetonide.

METHODS

Male mongrel dogs of varying ages and weighing 16–27 kg were used in these experiments. At the commencement of each study the prostates were exposed through an abdominal incision, three dimensional measurements of the glands were made with calipers (length, width, and depth), and prostate weight was determined as previously described from a nomogram that allows estimation of actual weight from the apparent volume (3, 4). Only animals with normal prostates (5–14 g) were used. After the initial measurement of prostate size, the animals were castrated and divided into control and treatment groups. Pharmacological treatment was instituted on the day of castration. The standard dosage consisted of either 1 ml triolein (control) or 1 ml triolein containing 250 μg 17β-estradiol (treatment group) administered intramuscularly three times a week (weekly dose, 750 μg). The dogs were killed either on day 0 or after 1, 2, 3, or 6 wk, and the prostates were removed, cleaned, weighed, and cut into small fragments. All subsequent procedures were done at 0–4°C. 2 vol of 20 mM Tris chloride, pH 7.4, 1.5 mM EDTA, 0.25 mM dithiothreitol, and 10% (wt/vol) glycerol (TEDG buffer) were added, and the suspension was subjected to three 10-s treatments with a Polytron PT10 tissue homogenizer (Polytron Corp., Elkhart, Ind.) at a power setting of 90% of full intensity and with 60 s waiting periods between each treatment to maintain the homogenates at 0–4°C. The homogenates were centrifuged at 38,000 rpm (104,000 *g*) for 1 h, and

the supernates (cytosols) were removed for subsequent use. Cytosol preparations contained 11–18 mg protein/ml.

Aliquots of the supernates (250 μl) were added to tubes in which radioactive steroids in 25 μl methanol had been taken to dryness (final concentration was 3 nM except when indicated). The tubes were mixed and allowed to stand at 0°C for 3 h with repeated mixing at hourly intervals. [^{14}C]-Albumin (5 μg, 8,100 dpm) was added, and 0.2 ml of the mixture was layered over a 5.3-ml continuous 5–20% wt/wt sucrose gradient in TEDG buffer. After centrifugation at 50,000 rpm (234,000 *g*) in a Beckman SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 18.5 h, 10-drop fractions (5-drop fractions in the initial studies) were obtained using a Beckman fraction recovery system (Beckman Instruments, Inc.). 5 ml of counting fluid were added (2,000 ml toluene, 1,000 ml Triton X-100 [Rohm and Haas Co., Philadelphia, Pa.], 80 ml Permafluor [Packard Instrument Co., Downers Grove, Ill.], 210 ml water), and radioactivity was assessed in a Packard 2650 liquid scintillation counter (Packard Instrument Co.) under circumstances in which the counting efficiencies averaged 25% for ^{3}H and 65% for ^{14}C. Sedimentation values were estimated as described by Martin and Ames (10). In routine assays in which 10-drop fractions were collected, binding in the 8–9S region was estimated as the amount of radioactivity in the peak tube plus four tubes on each side of the peak. In a preliminary series of experiments [^{3}H]dihydrotestosterone binding in the 8–9S region was shown to be stable to freezing in liquid nitrogen for as long as 2 mo, but all the physiological studies reported here were done with fresh cytosol. To assess the extent of metabolism of radioactive ligand during the procedure aliquots of the cytosol-hormone mixture were extracted with chloroform-methanol, and thin-layer chromatography was performed on the extracts as described (11). Uniformly, >95% of the radioactive dihydrotestosterone was recovered unchanged. Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard.

Sources. [1,2,4,5,6,7,16,17-^{3}H]Dihydrotestosterone, (190 Ci/mmol), [1,2-^{3}H]progesterone (55 Ci/mmol); [1,2-^{3}H]3α-androstanediol (44 Ci/mmol), and [1,2,6,7-^{3}H]testosterone (85 Ci/mmol) were from New England Nuclear, Boston, Mass. [6,7-^{3}H]Methyltrienolone (58 Ci/mmol) was from Roussel-Uclaf, Romainville, France as a gift from Patrick C. Walsh, Johns Hopkins Hospital, Baltimore, Md. [^{14}C]-Acetyl human serum albumin was prepared by acetylation with [^{14}C]acetic anhydride (New England Nuclear) (13). All radioinert steroids were from Steraloids, Inc., Pawling, N. Y. except for promegestone which was from New England Nuclear.

RESULTS

In the first study the binding of [^{3}H]dihydrotestosterone was assessed in cytosol from a castrated dog (Fig. 1). A distinct peak of bound [^{3}H]dihydrotestosterone was resolved from [^{14}C]albumin and was characterized by a sedimentation coefficient of ≅8.6S. In similar animals, no binding in the 8S region was demonstrable in serum (results not shown), and all demonstrable serum binding from both 17β-estradiol-treated and castrate controls sedimented in the same area as albumin (≅4.4S). In occasional experiments with cytosol, some binding of [^{3}H]dihydrotestosterone was also demonstrable in the 4S as well as the 8S region,

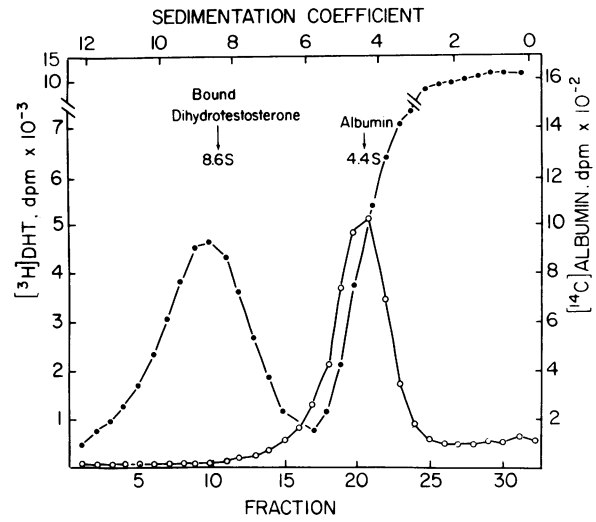

FIGURE 1 Density gradient sedimentation analysis of [^{3}H]-dihydrotestosterone binding of prostate cytosol and [^{14}C]-albumin. Prostate cytosol (15.7 mg protein/ml), prepared from a 3-wk castrate control, was incubated 3 h at 0°C with 3 nM [^{3}H]dihydrotestosterone in a total volume of 0.25 ml. 10 μl of [^{14}C]albumin was added, and 200 μl of the mixture was layered on a 5.3-ml 5–20% sucrose gradient in TEDG buffer. After centrifugation for 18.5 h at 49,500 rpm in the SW 50.1 rotor, the gradient was fractionated and analyzed for ^{3}H (●) and ^{14}C (○) as described in the text.

possibly the result of varying degrees of contamination of prostate (and hence of cytosol) with plasma.

[^{3}H]Dihydrotestosterone binding by cytosol was ≅80% saturable with 3 nM dihydrotestosterone, was essentially complete within 3 h incubation time, and was linear between 1 and 4 mg cytosol protein, both for preparations from castrate and 17β-estradiol-treated animals (Fig. 2). As a result of these studies, a standard assay was derived for the comparison of control and 17β-estradiol treatment that used incubation of cytosol with 3 nM [^{3}H]dihydrotestosterone at 0° for 3 h at protein concentrations that varied from 2 to 3.5 mg in a total volume of 0.2 ml.

Binding of [^{3}H]dihydrotestosterone in the 8S region was displaced by radioinert dihydrotestosterone—22% by a 0.5-fold excess, 36% by a 1-fold excess, and 83% by a 10-fold excess (Fig. 3A). Binding of [^{3}H]dihydrotestosterone was also displaced by a 10-fold excess of testosterone, partially by a similar amount of progesterone and not at all by 3α-androstanediol (Fig. 3B). When the abilities of several steroids to form an 8S complex were compared, definite 8S binding could be demonstrated with [^{3}H]dihydrotestosterone and [^{3}H]testosterone but not with [^{3}H]progesterone or [^{3}H]3α-androstanediol (Fig. 3C). [^{3}H]Methyltrienolone, a steroid that binds to the androgen receptor in many tissues (14–17), had a pattern of binding that was

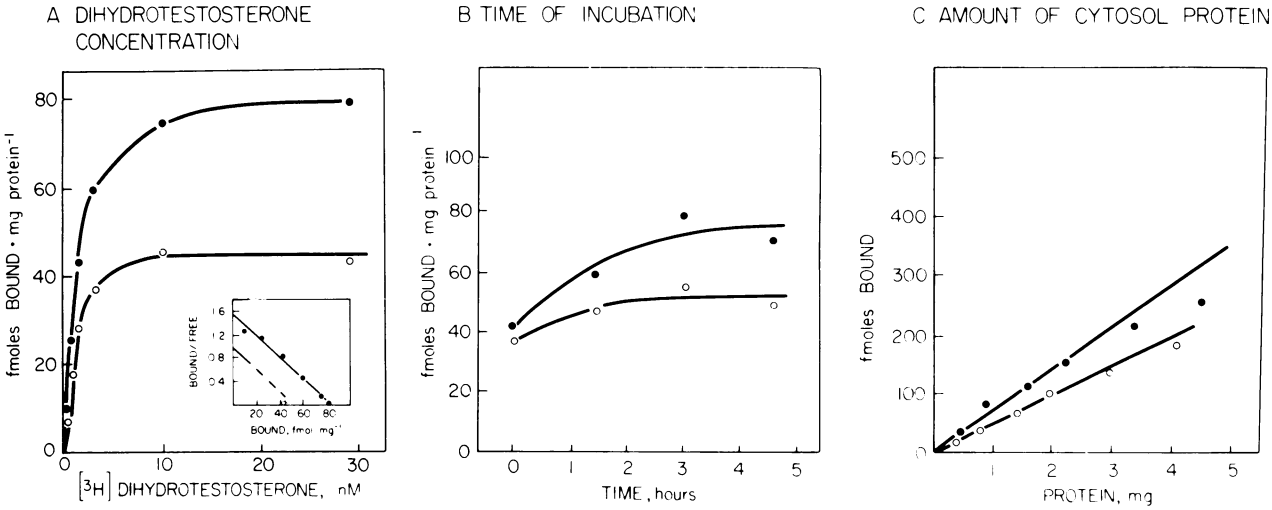

FIGURE 2 Influence of dihydrotestosterone concentration, time of incubation and amount of cytosol protein on 8S binding of [^{3}H]dihydrotestosterone by cytosol from castrate controls (○) and 17β-estradiol-treated (●) castrate dogs. (A) Binding as a function of increasing concentrations of dihydrotestosterone. Cytosol from control and 17β-estradiol-treated animals containing 17.6 and 16.7 mg protein/ml was incubated 3 h at 0°C with 0.3, 0.75, 1.5, 3.0, 10.0, or 30 nM [^{3}H]dihydrotestosterone and analyzed by density gradient centrifugation. Binding was calculated from the radioactivity in the 8S region as described in the text. Inset. Scatchard analysis of binding. The linear regression of bound:free vs. femtomole dihydrotestosterone bound per milligram cytosol protein yielded intercepts on the abcissa (binding site concentration) of 49 and 85 fmol/mg protein, respectively, for control and 17β-estradiol-treated animals. The association constants, estimated from the slopes of the regression equation, were 1.13 and 1.07 nM^{-1}, respectively, for control and estradiol-treated animals. (B) Binding as a function of time of incubation. Cytosol from control and 17β-estradiol-treated animals containing 14.9 and 14.6 mg protein/ml was incubated with 3 nM [^{3}H]dihydrotestosterone for 0, 1.5, 3.0, or 4.5 h and analyzed by density gradient centrifugation. Binding was calculated from the radioactivity in the 8S region as described in the text. (C) Binding as a function of amount of cytosol protein. Varying amounts of cytosol from control and 17β-estradiol-treated animals (containing 10.1 and 11.5 mg protein/ml) was incubated with 3 nM [^{3}H]dihydrotestosterone and analyzed by density gradient centrifugation. Binding was calculated from the radioactivity in the 8S region as described in the text.

almost identical to that of [^{3}H]dihydrotestosterone (results not shown).

Because the binding of [^{3}H]dihydrotestosterone in the 8–9S region was partially displaced by progesterone, the specificity of the binding was investigated further. Progesterone was only about a tenth as effective in displacing [^{3}H]dihydrotestosterone binding in the 8–9S region as was dihydrotestosterone itself, and the synthetic progestin promegestone (R5020) which binds avidly to the progesterone receptor (18) was even less effective in this regard (Fig. 4). In addition, the 8–9S binding of 3nM [^{3}H]dihydrotestosterone and 3nM [^{3}H]methyltrieneolone was not influenced by the presence of 0.3 μM triamcinolone acetonide, which blocks binding to the progesterone receptor (19) (results not shown). We concluded from these studies that the 8–9S binding of [^{3}H]dihydrotestosterone has the characteristics to be expected of an androgen receptor.

With the standard assay, binding of dihydrotestosterone in cytosol from control and 17β-estradiol-treated animals was compared in five paired experiments (Fig. 5). The difference between the two groups using a paired t test was significant ($P < 0.01$). The results of all the studies of castrate and 17β-estradiol-treated animals (paired and nonpaired) and five measurements in intact controls are summarized in Fig. 6. Again the difference between control and 17β-estradiol-treated groups was significant ($P < 0.001$). Similar differences between the two groups were detected when [^{3}H]dihydrotestosterone was employed at a concentration of 30 nM, eliminating the possibility that differences reflected differing average affinities between the two types of preparation. There was no difference between the levels of the intact controls and that of the castrate controls, recognizing that direct comparison of binding between castrate and intact animals may be invalid because of occupancy of the receptor by endogenous hormone.

Evidence in support of the concept that the differences in androgen binding between 17β-estradiol-treated and castrate controls reflect the in vivo situa-

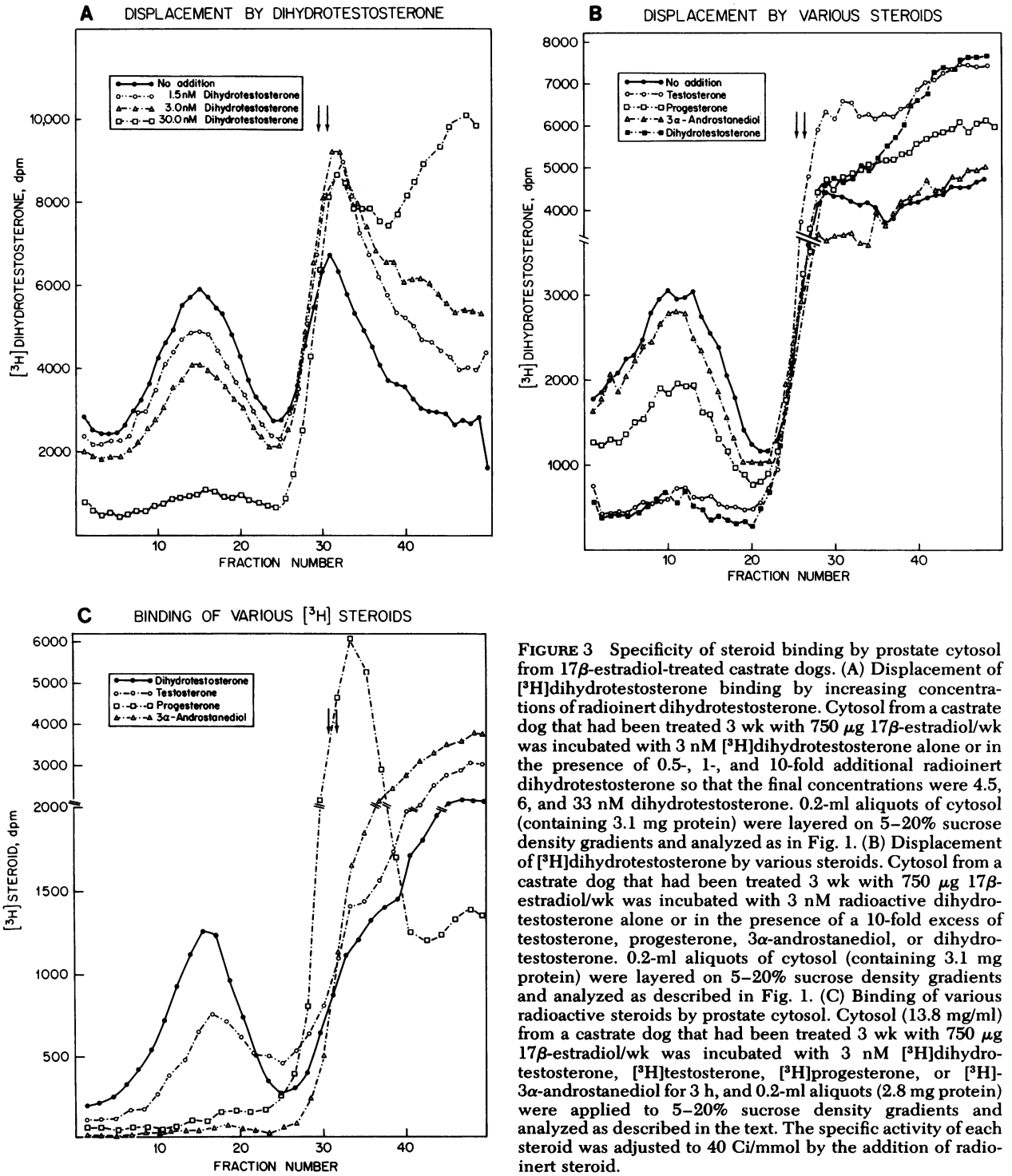

FIGURE 3 Specificity of steroid binding by prostate cytosol from 17β-estradiol-treated castrate dogs. (A) Displacement of [^{3}H]dihydrotestosterone binding by increasing concentrations of radioinert dihydrotestosterone. Cytosol from a castrate dog that had been treated 3 wk with 750 μg 17β-estradiol/wk was incubated with 3 nM [^{3}H]dihydrotestosterone alone or in the presence of 0.5-, 1-, and 10-fold additional radioinert dihydrotestosterone so that the final concentrations were 4.5, 6, and 33 nM dihydrotestosterone. 0.2-ml aliquots of cytosol (containing 3.1 mg protein) were layered on 5–20% sucrose density gradients and analyzed as in Fig. 1. (B) Displacement of [^{3}H]dihydrotestosterone by various steroids. Cytosol from a castrate dog that had been treated 3 wk with 750 μg 17β-estradiol/wk was incubated with 3 nM radioactive dihydrotestosterone alone or in the presence of a 10-fold excess of testosterone, progesterone, 3α-androstanediol, or dihydrotestosterone. 0.2-ml aliquots of cytosol (containing 3.1 mg protein) were layered on 5–20% sucrose density gradients and analyzed as described in Fig. 1. (C) Binding of various radioactive steroids by prostate cytosol. Cytosol (13.8 mg/ml) from a castrate dog that had been treated 3 wk with 750 μg 17β-estradiol/wk was incubated with 3 nM [^{3}H]dihydrotestosterone, [^{3}H]testosterone, [^{3}H]progesterone, or [^{3}H]-3α-androstanediol for 3 h, and 0.2-ml aliquots (2.8 mg protein) were applied to 5–20% sucrose density gradients and analyzed as described in the text. The specific activity of each steroid was adjusted to 40 Ci/mmol by the addition of radioinert steroid.

tion was obtained by two types of mixing experiments. In one, aliquots of castrate control and 17β-estradiol-treated prostates were mixed and homogenized together; in the other, aliquots of the cytosols from the two animals were mixed and incubated together (results not shown). In both instances, binding was intermediate between that observed in the castrate and 17β-estradiol-treated preparations. In addition, loss of

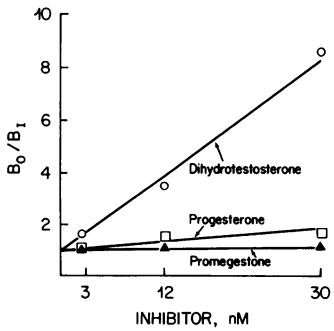

FIGURE 4 Displacement of [^{3}H]dihydrotestosterone binding by dihydrotestosterone, progesterone, and promegestone. Cytosol from a castrate control dog (14.8 mg protein/ml) was incubated with 3 nM [^{3}H]dihydrotestosterone alone or in the presence of 3, 12, or 30 nM radioinert dihydrotestosterone, progesterone, or promegestone. The samples were subjected to density gradient centrifugation, and binding was calculated from the radioactivity in the 8S region of the gradient as described in the text. Binding in the absence of added radioinert steroids (39 fmol/mg protein), B_0; and binding in the presence of added radioinert steroids, B_1. Identical results were found with cytosol from a 17β-estradiol-treated dog (results not shown).

binding after brief exposure to elevated temperature (15 or 30°C for 1 h) was identical for cytosol preparations from control and 17β-estradiol-treated animals, suggesting that differential stability or differential inactivation by protease did not influence the observed differences between the two treatment groups.

Finally, a time sequence study and a dose-response study were performed. When the standard dose (750 μg 17β-estradiol/wk) was administered, similar enhancement of dihydrotestosterone binding was observed at 1, 2, 3, and 6 wk (results not shown). At the standard 3-wk time, 75 μg of 17β-estradiol/wk appeared to be as effective as larger doses in enhancing dihydrotestosterone binding (Fig. 7).

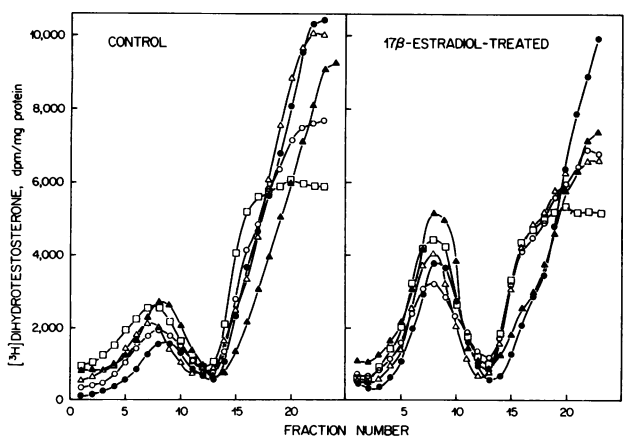

FIGURE 5 Composite density gradient analyses of [^{3}H]-dihydrotestosterone binding by prostate cytosol from control and 17β-estradiol-treated animals. Five paired analyses were performed in which cytosol was prepared from control and 17β-estradiol-treated animals at the same time and subjected to density gradient analysis. Cytosol preparations from controls contained 10.1–17.6 mg protein/ml and 10.9–16.7 mg protein/ml for 17β-estradiol-treated animals. The results have been expressed as disintegrations per minute ^{3}H per milligram protein in the original cytosol.

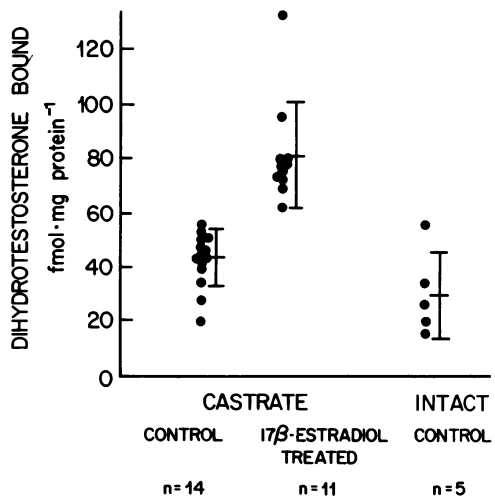

FIGURE 6 8S binding of [^{3}H]dihydrotestosterone by prostate cytosol from castrate control, 17β-estradiol-treated castrate, and intact control dogs. Cytosol preparations from each treatment group were incubated with 3 nM [^{3}H]dihydrotestosterone and subjected to density gradient sedimentation. Binding was calculated from the radioactivity in the 8S region as described in the text. Values (±SD) for each treatment mean, respectively, were 43.7±10.4, 81.9±19.5, and 29.1 ±15.6 fmol dihydrotestosterone bound per milligram cytosol protein.

DISCUSSION

The administration of 17β-estradiol to the castrate dog results in the enhancement of [^{3}H]dihydrotestosterone binding in prostate cytosol. This enhanced binding cannot be explained by contamination with plasma, is demonstrable within a week after commencing therapy, and occurs after the administration of as little as 75 μg 17β-estradiol/wk. Furthermore, the specificity of the binding of radioactive steroids in the 8S region indicates that the binding is not a result of the presence of a progesterone receptor. We conclude that the 8S binding is probably because of an androgen receptor and that the activity of this receptor in dog prostate is regulated by estrogen.

If one assumes that the amount of cytoplasmic androgen receptor is rate limiting for androgen action, as in certain other androgen-target tissues (20), these findings provide a potential explanation for the development of prostatic hypertrophy with aging because estrogen formation is known to increase with age in men (21). It is attractive to hypothesize that enhancement of

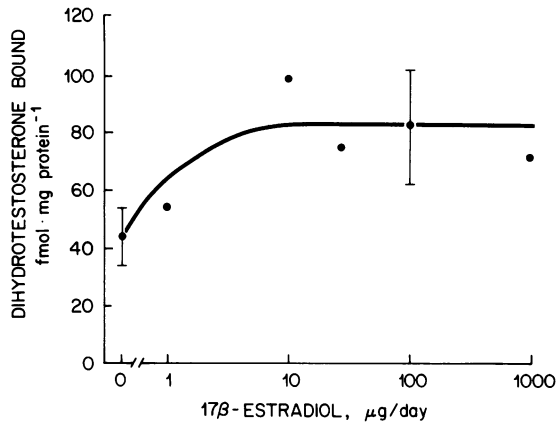

FIGURE 7 Effect of varying doses of 17β-estradiol on binding of [^{3}H]dihydrotestosterone. Castrate dogs were treated 3 wk with triolein (control) or 17β-estradiol in amounts of 7.5, 75, 225, 750, or 7,500 μg/wk administered in three doses. Data are plotted as microgram 17β-estradiol administered per day. Prostates were homogenized in TEDG buffer, and cytosol was prepared and incubated with 3 nM [^{3}H]dihydrotestosterone and analyzed by density gradient centrifugation. Binding was calculated from the radioactivity in the 8S region as described in the text. Data points for the controls (zero dose of 17β-estradiol) and the standard dosage of 17β-estradiol (750 μg/wk; 100 μg/d) represent the mean±SD of 10 animals in each group. Data points corresponding to 1 and 10 μg 17β-estradiol/d represent the average of three and two preparations, respectively, and those for 35 and 1,000 μg 17β-estradiol/d represent determinations based on single preparations.

the cytoplasmic androgen receptor by 17β-estradiol in older men results in an augmentation of the androgen-mediated growth of the gland, and this in turn results in pathological manifestations. If this were the case, inhibition of estrogen synthesis or action in such individuals might prevent its development.

The enhancement of androgen receptor activity by 17β-estradiol has not been described, but estrogen is known to regulate the activity of progesterone receptor in several target tissues (5–9). Furthermore, because androgen and estrogens have synergistic actions on physiological processes other than prostatic growth, such as the initiation of normal male sexual behavior in the rat (22–24) and the development of the magnum of the oviduct in the immature chicken (25), it is attractive to speculate that these synergistic effects may also be mediated by effects of estrogen on the androgen receptor. Of course, in most physiological situations, androgens and estrogens have independent or even opposite effects (26); it is presumed that in these instances either estrogen does not regulate the androgen receptor protein or a subsequent step in androgen action, not determined by the amount of androgen receptor, is rate limiting in the action of the hormone.

It is of considerable interest that 8S binding was not demonstrable with 3α-androstanediol, whereas in our previous studies 3α-androstanediol had been shown to be a more potent effector of prostatic growth than dihydrotestosterone (3, 4). It is known, of course, that the interconversion of 3α-androstanediol and dihydrotestosterone takes place in prostate (27, 28) and that when 3α-androstanediol is administered to the rat, dihydrotestosterone is the principal androgen recovered from the prostate nuclei (29). Considered together with the report that the content of 3α-androstanediol is decreased (30) under conditions in which dihydrotestosterone content is increased in the hypertrophic prostate of man (30, 31), these findings suggest that dihydrotestosterone may be the effective intracellular mediator of prostate growth in the dog.

Is it safe to assume that the 8S binding of dihydrotestosterone demonstrable in cytosol, in these studies, is the androgen receptor? Additional work will have to be done to establish the validity of this assumption, but in all other instances in which the androgen receptor has been characterized, such as the mouse kidney and submandibular gland (11), the rat prostate (32), and cultured human fibroblasts (20), high affinity dihydrotestosterone binding in the 8–10S region is characteristic of the cytosol receptor. Furthermore, although human prostate is known to contain a progesterone receptor in cytosol (33, 34), the fact that progesterone and promegestone bind weakly if at all to the 8S moiety characterized here is compatible with our conclusion that the 8S binding is a result of a cytosol receptor that is involved in androgen action. Moreover, progestins are known to compete weakly for binding sites on androgen receptors (35).

Even if these various assumptions are correct, it does not necessarily follow that the cytosol androgen receptor of human prostate is regulated by estrogen or that the predominant effect of estrogen when given in large quantities is to enhance prostate growth. Indeed, the administration of estrogens in large quantities to intact dogs inhibits prostatic growth, probably as the result of inhibition of gonadotropin release and the secondary induction of a chemical castration, and when administered in large amounts to castrated dogs estrogens cause cystic dilatation but not growth of the prostate (36). At best, the current results would explain those situations in which elevated estrogen levels occur in the presence of adequate androgen so that the synergistic effects of the two hormones can be demonstrated.

ACKNOWLEDGMENT

This work was aided by grant AG00306 from the National Institutes of Health.

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