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Androgen Action through Estrogen Receptor in a Human Breast Cancer Cell Line*

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ABSTRACT. Androgens stimulate the growth of the human breast cancer cell line MCF-7. Very high doses are required, however, and therefore, we inquired whether they might exert this effect through some mechanism other than the androgen receptor. Evidence is presented here that it is actually the estrogen receptor, activated by weak but specific binding of androgens, which mediates this effect.

Physiological 5α-dihydrotestosterone (DHT) at 10^{-8} M translocates androgen receptor to cell nuclei without affecting other steroid receptors or stimulating cell growth. High DHT (10^{-6} M) translocates estrogen as well as androgen receptor and stimulates cell growth. Receptors for progestins and glucocorticoids are not affected. Testosterone, 3β-androstanediol, and the antiandrogen, R2956, at 10^{-6} M also translocate the estrogen receptor, while progesterone, hydrocortisone, and the inactive isomer, 5β-DHT, have little or no effect.

5α-DHT at 10^{-6} M competes with estradiol for binding to cytosol estrogen receptor, while 10^{-8} M DHT does not.

Estrogen receptor translocated to the nucleus by 10^{-6} M DHT reaches a peak within an hour of DHT addition and is then depleted within 3 h to a much lower steady state level which is then maintained; these actions parallel those of 10^{-8} M estradiol.

Two responses considered specifically estrogenic follow treatment with 10^{-6} M DHT; neither occurs with physiological 10^{-8} M DHT. The first is stimulation of progesterone receptor synthesis. The second is rescue of cell growth from inhibition by the antiestrogen nafoxidine. The latter action is not prevented by the antiandrogen, R2956, or cyproterone acetate, as would be expected for an androgen receptor action. (*Endocrinology* **103**: 624, 1978)

THE MODEL human breast cancer cell line MCF-7 contains separate, specific receptors for estrogens, androgens, progestins, and glucocorticoids (1). It would be reasonable to expect each hormone to act through its respective receptor, and indeed the literature strongly documents such mechanisms (2–4).

It is known that both estrogens and androgens promote growth of MCF-7 cells (5, 6). Stimulation by estrogens occurs at hormone doses that are quite compatible with binding of estrogen to its receptor and transport of this hormone-receptor complex to nuclear sites for specific gene activation. Androgens, on the other hand, enhance growth only at doses about 1000-fold higher than is necessary to saturate the androgen receptor (6). Such results suggest that androgens might stimulate cell growth by some mechanism other than the androgen receptor system. Such an alternate mechanism is suggested by work performed with the rat uterus, where androgens at pharmacological concentrations bind not only the androgen receptor but also the estrogen receptor, resulting in transport of this receptor-ligand complex into the nucleus (7–10).

In this report we examine the mechanism of androgen stimulation of MCF-7 cells and show that androgens act by stimulating cell growth not through the androgen receptor, but rather through the estrogen receptor system.

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Materials and Methods

Hormones

The following radioinert ligands were used: 17β-estradiol (E_2), diethylstilbestrol (DES), 5α-dihydrotestosterone (5α-DHT), 5α,3β-androstanediol (Adiol), progesterone, dexamethasone (DEX), triamcinolone acetonide (TA; Sigma, St. Louis, MO), cyproterone (CP), cyproterone acetate (CPA), flutamide (Flu, SCH 13521; Schering Pharmaceuticals, Bloomingfield, NJ); methyltrienolone

(R1881), 17α,21-dimethyl-19-norpregna-4,9-diene (R5020), and 17β-hydroxy-2α,2β,17α-trimethylestra-4,9,11-trien-3-one (R2956; Roussel UCLAF, France); nafoxidine hydrochloride (Naf; Upjohn, Kalamazoo, MI); hydrocortisone acetate (F; Calbiochem, LaJolla, CA).

The radiolabeled isotopes [^{3}H]R1881 (58.2 Ci/mmol) and [^{3}H]R5020 (51.4 Ci/mmol) were gifts from Dr. J. P. Raynaud (Roussel UCLAF, France). [2,4,6,7-^{3}H]E_2 (100 Ci/mmol), and [1,2,4,5,6,7-^{3}H]-5α-DHT (133 Ci/mmol), and 1,2-[^{3}H]DEX (23 Ci/mmol) were purchased from Amersham Searle (Arlington Heights, IL).

Cell culture conditions

MCF-7 cells, a gift from Dr. H. Soule, were grown as monolayer cultures in either Falcon plastic flasks (75 cm^2), plastic roller bottles (490 cm^2), or glass roller bottles (692 cm^2) in an atmosphere of 95% air-5% CO_2 at 37 C. Cells were inoculated into appropriate growth chambers in growth media containing Earle's minimal essential medium (MEM; Gibco) supplemented with nonessential amino acids (Gibco), 2 mM L-glutamine (Gibco), 0.006 μg/ml insulin (Sigma), 5% calf serum (Gibco), and 50 μg/ml gentamicin (Schering). Routinely, after 2 days, the growth medium was changed to the same medium as above with the following exceptions: 5% calf serum was stripped of endogenous steroid hormones with charcoal (11) and 10^{-8} M hydrocortisone and 1 μg/ml PRL were supplemented; this control growth medium, used throughout these experiments, is referred to as "5% stripped calf serum."

Cell harvest and preparation of cell extracts

At appropriate time intervals, cells were removed by a 15-30-min incubation (37 C) with 1 mM EDTA in Ca^{++}/Mg^{++}-free Hank's balanced salt solution and washed once with Hank's without EDTA and once with phosphate buffer [5 mM sodium phosphate, pH 7.4 (4 C), 10 mM thioglycerol, and 10% glycerol].

Cells were resuspended in phosphate buffer (4 × 10^7 cells/ml buffer). Preparation of cytosol and nuclear extracts with 0.6 M KCl-Tris buffer (0.01 M Tris-HCl, pH 8.5, at 4 C, 1.5 mM EDTA, 10% glycerol) was performed as previously described (12). Cytosol preparations were diluted to protein concentrations ranging from 0.5–3.0 mg/ml (usually about 2 ml phosphate buffer/2 × 10^7 cells); nuclear KCl-Tris extracts were diluted 10-fold with phosphate buffer (1 ml nuclear extract plus 9 ml phosphate buffer/2 × 10^7 cells) to reduce the salt concentration for the protamine assay (13).

Receptor assays: protamine sulfate

After appropriate dilutions with phosphate buffer, cytosol (200 μl) and nuclear extracts (500 μl) were precipitated in 12 × 75-mm glass tubes (14) with 250 μl 2 mg/ml protamine sulfate (USP injection) without phenol preservative (Eli Lilly Co.). The precipitate was sedimented at 600 × *g*, and the supernatant was decanted. Receptor binding was then measured in the protamine pellet.

Unoccupied cytosol receptor sites were measured by incubating protamine receptor pellets at 0–2 C (18 h) with 5 × 10^{-8} M [^{3}H]estradiol (estrogen receptor), 5 × 10^{-9} M [^{3}H]DHT or [^{3}H]R1881 (androgen receptor), 1 × 10^{-8} M [^{3}H]R5020 (progesterone receptor), and 5 × 10^{-8} M [^{3}H]DEX (glucocorticoid receptor). To assess nonspecific binding in each assay, a parallel series of tubes contained a 100-fold excess of DES, DHT, R1881, R5020, or DEX, respectively. We have previously shown (12) that cytosol receptors occupied with estrogen are insignificant and therefore, were not monitored in these studies.

Unoccupied nuclear estrogen receptor sites were measured by incubating protamine pellets at 0–2 C (18 h) with 1 × 10^{-8} M [^{3}H]estradiol with or without a 100-fold excess of DES. Nuclear receptor occupied with radioinert estradiol is completely exchanged with excess [^{3}H]estradiol by incubating at 30 C (5 h); unfilled sites are also filled at 30 C so that total binding sites are measured. The difference in 30 and 0–2 C binding is used to determine estrogen-occupied nuclear receptor. After incubation, tubes were washed twice with 1 ml phosphate buffer at 4 C. Protamine pellets were then twice extracted with 2.5 ml toluene scintillation fluor (4.0 g PPO, 0.05 g POPOP, 1 liter toluene), and the extract was counted in a Beckman LS233 counter with a counting efficiency of 50%.

Sucrose gradient

To 250 μl cytosol (~5 mg protein/ml phosphate buffer) or to concentrated KCl-Tris nuclear extracts (~3 mg/ml), [^{3}H]estradiol (5 μl) was added to give a final concentration of 1 × 10^{-8} M. Nonspecific binding was determined with parallel samples containing 100-fold excess DES. Incubations were carried out for 4 h at 0–2 C. After incubation, unbound radioactivity was removed with dextran-coated charcoal (11). The [^{3}H]estradiol-occupied receptors were layered on 5–20% sucrose-phosphate buffer (cytosol) or 5–20% sucrose-0.4 M KCl Tris buffer (nuclear) gradients and sedimented for 16.3 h at 297,000 × *g* in a Beckman SW60 rotor. ^{14}C-Labeled bovine serum albumin [BSA (15)] was used as an

internal marker. Fractions (200 μl) were collected and counted in 5 ml modified Bray's solution (16) with a counting efficiency of 42%.

Results

Androgens and growth

As seen in Fig. 1, the growth of MCF-7 cells can be significantly enhanced by pharmacological androgens (10^{-6} M) as well as physiological estrogens (10^{-8} M). Physiological androgens (10^{-8} M), however, have little or no effect despite the fact that at this dose androgen receptor is translocated to the nucleus (see below). In light of other experiments (7–9) showing that androgens at high doses are capable of interacting with the estrogen receptor in the rat, we thought that perhaps in a similar way pharmacological androgens could be promoting MCF-7 cell growth. Verification of this hypothesis required proof that high dose androgens do bind only to the estrogen receptor but not to the progesterone or glucocorticoid receptors also found in this cell line.

Androgen effects on different receptors

To determine the androgen effect on each receptor, intact cells were exposed to low and high dose DHT, and after 1 h, the four known cytosol receptors in this cell line were examined (Table 1). At 10^{-8} M DHT, only cytoplasmic androgen receptor was depleted, presumably by translocation to the nucleus. By contrast, 10^{-6} M DHT, in addition to its effect on the androgen receptor, depleted the cytoplasmic estrogen receptor with no change in progesterone or glucocorticoid receptors.

Thus, it seems that high dose androgens may indeed act through the estrogen receptor system. Therefore, experiments were designed to determine if androgens can mimic the actions of estrogens on the estrogen receptor system and ultimately yield products of estrogen action.

Direct competition by DHT

The interactions of DHT with the estrogen receptor were studied to determine if DHT competes with estradiol for the estrogen-binding site on the receptor molecule. Two types of assays were employed. In the first, cytoplasmic estrogen receptor was incubated in solution with [^{3}H]estradiol and increasing concentrations of DHT and then assayed by sucrose gradient centrifugation (Fig. 2). In the second, estrogen receptor was first immobilized by precipitating with protamine, then incubated with [^{3}H]estradiol and increasing

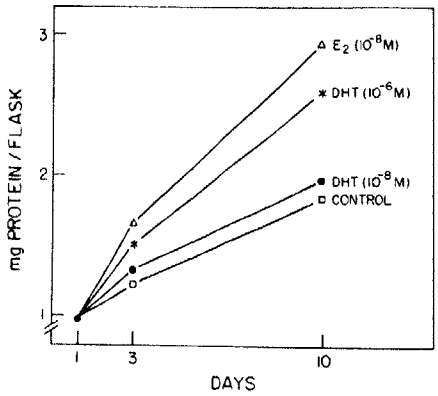

FIG. 1. Effect of estrogen and androgen on MCF-7 cell growth. MCF-7 cells were grown in T-75 flasks containing 5% stripped calf serum (control) or serum containing 10^{-8} M E_2 or 10^{-8} and 10^{-6} M DHT. At 1, 3, and 10 days, total soluble cytosol protein pooled from triplicate flasks was measured.

TABLE 1. Effect of DHT on depletion of receptors for estrogen, androgen, progesterone, and glucocorticoids

	% of Control	
	10^{-8} M DHT	10^{-6} M DHT
Estrogen receptor	87	38
Androgen receptor	30	18
Progesterone receptor	100	105
Glucocorticoid receptor	100	115

Cells were grown to confluence in 5% charcoal-stripped calf serum (see text for details). To intact cells was added vehicle (0.1% ethanol), 10^{-8} or 10^{-6} M DHT. After 10 days of exposure, cells were collected and cytosols were prepared as described in the text. Receptors for estrogen, androgren, progesterone, and glucocorticoids were each assayed by both the protamine (see text) and dextran charcoal (1) methods. No significant quantitative variations were noted between the two assays. Control levels were 1.9 (estrogen), 0.2 (androgen), and 3.3 (glucocorticoid) pmol receptor/mg DNA. For progesterone receptor cells were first primed with estradiol for 4 days, then exposed to DHT for 1 h. In these cells, control progesterone receptor is 1.3 pmol/mg DNA. Values represent the mean of triplicate determinations for the protamine assay from two pooled flasks.

doses of DHT or other competitors (Fig. 2, insert). Both techniques yield essentially the same results: DHT effectively inhibits estradiol binding to the estrogen receptor but only at high doses. The androgen analogues, CPA and Flu, are not effective competitors, whereas the estrogen analogue, Naf effectively competes with estradiol.

We conclude that DHT does interact directly with the estrogen receptor but with an affinity approximately 1000-fold less than estradiol. This would explain why exceedingly high doses of androgens are required to elicit growth effects comparable to physiological doses of estrogens.

Translocation of estrogen receptor with DHT

The DHT-induced depletion of cytoplasmic estrogen receptor seen in Table 1 is paralleled by the entry of a near equal complement of estrogen receptor sites into the nucleus, seen as a 4S component by sucrose gradient centrifugation (data not shown). This is identical to the action of estrogen on the estrogen receptor previously reported for MCF-7 cells (12).

Figure 3 represents an extended time course of estradiol and DHT action on estrogen receptors. Estradiol induces a rapid translocation of receptors into the nucleus within 1 h. This is then followed by nuclear receptor depletion to a steady state level within 3 h which, although not shown here, is maintained at these levels as long as hormone is present. At 10^{-8} M DHT, there is no effect on the estrogen receptor level, but at 10^{-6} M DHT, the entire sequence of estradiol effects on the estrogen receptor, from translocation to nuclear depletion, is mimicked.

Translocation of estrogen receptor with other androgens

Conversion of DHT to Adiol by the enzyme, 3-keto reductase, occurs rapidly in the MCF-7 cell line (6) and many other mammary tumor systems as well. In addition, one might expect the formation of other DHT metabolites which could potentially exert varying degrees of effects on the estrogen receptor system.

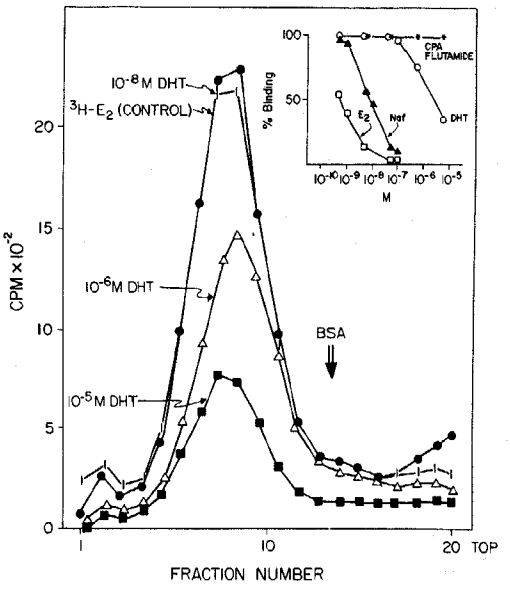

FIG. 2. Competition of [^{3}H]estradiol binding to cytosol estrogen receptor. Cytosol was prepared from cells grown to confluence in 5% charcoal-stripped calf serum. Competition of [^{3}H]estradiol binding to estrogen receptor was measured by sucrose gradient and protamine sulfate (insert) methods. Sucrose gradient: Cytosol (250 μg 5 mg/ml) was preincubated 15 min with 10^{-8}, 10^{-6}, and 10^{-5} M DHT (added in 5 μl ethanol) or ethanol alone before addition to each tube of [^{3}H]estradiol (added in 5 μl ethanol to give a final concentration of 2×10^{-9} M). Incubations were carried out 4 h at 4 C before layering on 5–20% sucrose-phosphate gradients. Protamine assay: Cytosol (200 μl ~ 2 mg/ml) was precipitated with protamine sulfate by methods described in the text. To triplicate series of tubes containing the protamine receptor pellet was added in 500 μl phosphate buffer 5×10^{-10} M [^{3}H]$_A$ estradiol containing vehicle only (ethanol) or increasing concentrations of [^{3}H]estradiol plus unlabeled estradiol, Naf, CPA, or Flu (5×10^{-10}–10^{-6} M). Incubations were carried out 18 h at 4 C. Data is presented as the percentage binding relative to tubes containing [^{3}H]estradiol only.

Therefore, we examined a variety of androgens along with other steroids of less similar structure for their efficacy in depleting cytoplasmic estrogen receptor. At 10^{-6} M, all androgens, with the exception of the inactive isomer 5β-DHT, deplete cytoplasmic estrogen receptor (Table 2). Ligands such as progesterone and hydrocortisone have absolutely no effect, suggesting that specific regions of the androgen structure must be critical for their action as a weak estrogen.

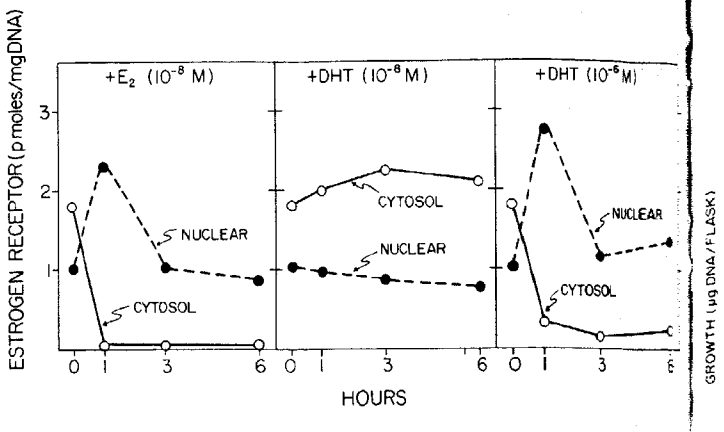

FIG. 3. Translocation and nuclear depletion of estrogen receptor as a function of time and hormone. T-75 flasks were exposed to 10^{-8} M E_2, 10^{-8} or 10^{-6} M DHT, or vehicle control only. Cytosol and nuclear estrogen receptors were then measured at 1-, 3-, and 6-h time points by procedures described in the text. Values represent the mean of triplicate protamine determinations from three pooled T-75 flasks.

TABLE 2. Cytoplasmic depletion of estrogen receptor with various steroids

	Cytoplasmic[a] estrogen receptor
Control	1.06
Estradiol (10^{-8} M)	0.01
DHT (10^{-8} M)	1.12
DHT (10^{-6} M)	0.26
Adiol (10^{-6} M)	0.16
Testosterone (10^{-6} M)	0.19
R2956 (10^{-6} M)	0.25
5β-DHT (10^{-6} M)	0.75
Progesterone (10^{-6} M)	1.15
Hydrocortisone (10^{-6} M)	1.04

Cells in T-75 flasks were grown to confluence in 5% charcoal-stripped calf serum. One hour before cell harvest, steroids were added to the final concentrations: vehicle control (0.1% ethanol), E_2 (10^{-8} M), 5β-DHT (10^{-8} and 10^{-6} M), Adiol, testosterone, progesterone, and hydrocortisone (all at 10^{-6} M). Cytosol estrogen receptor was measured by the protamine assay described in the test. Values represent the mean of duplicate determinations from three pooled T-75 flasks.

[a] Picomoles per mg DNA.

Induction of progesterone receptor

If the DHT-translocated estrogen receptor is functional, then stimulation of products specific for the action of estrogen should result. As progesterone receptor is such a product of estrogen action, we examined its synthesis after DHT compared to estradiol (Table 3). Intact cells exposed to 10^{-8} M DHT failed to increase progesterone receptor, but cells exposed to 10^{-6} M DHT showed almost as much stimulation as those exposed to 10^{-8} M estradiol. These results clearly show that like the estrogen-translocated receptor, the DHT-translocated estrogen receptor can activate the synthesis of specific cell products.

Rescue of antiestrogen-induced growth inhibition with DHT and estradiol

Antiestrogens, such as tamoxifen and Naf, inhibit MCF-7 cell growth (5, 17). Inhibition is maintained even when Naf is removed from the growth medium but can be reversed by subsequent treatment with estradiol (Fig. 4). As DHT at high doses interacts with the estrogen receptor, we wondered if perhaps, like estradiol, it could reverse the growth inhibition caused by Naf. Cell growth, therefore, was inhibited with Naf for 5 days. Media were then changed to MEM with 5% stripped calf serum alone or estradiol, or DHT with or without the antiandrogens, R2956 or CPA. Cells were maintained on these hormones for 6 days before the growth-enhancing effects of each were assessed (Fig. 5). As expected, estradiol rescued cell growth from Naf inhibition. DHT also rescued growth at 10^{-6} M but

TABLE 3. Induction of progesterone receptor with estrogen and androgen

	Progesterone receptor
Control	0.23[a]
Estradiol (10^{-8} M)	1.38
DHT (10^{-8} M)	0.16
DHT (10^{-6} M)	0.72

Cells were grown 10 days in 5% charcoal-stripped calf serum containing vehicle control (0.1% ethanol), 10^{-8} M E_2, or 10^{-8} and 10^{-6} M DHT. Progesterone receptor was measured using [^{3}H]R5020 by both protamine sulfate and dextran charcoal methods, with no significant variations in the two procedures used. Values represent the mean of duplicate protamine determinations from two plastic roller bottles pooled.

[a] Picomoles per mg DNA.

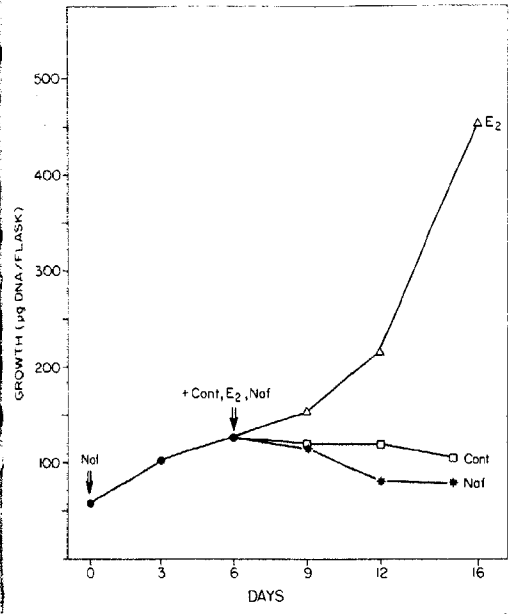

FIG. 4. Antiestrogenic effect on cell growth; rescue with estradiol. Three days after plating, cells were maintained on 5×10^{-7} M Naf in 5% stripped calf serum for 6 days. Growth media was then changed to 5% stripped calf serum containing vehicle control (ethanol), 5×10^{-7} M Naf, or 10^{-8} M estradiol. Growth (micrograms of DNA per flask) was then monitored 3, 6, and 10 days later. Values represent the mean of triplicate determinations from two T-75 flasks.

not at the lower doses. If DHT were stimulating Naf-inhibited cells through the androgen receptor, then R2956 or CPA should block this effect. Instead, when either of the antiandrogens were combined with DHT at 10^{-6} M, there was no inhibition but rather an increased stimulation; such an effect is in agreement with the data of Table 2 in which it is seen that R2956 also translocates estrogen receptor. Therefore, it seems that, like estradiol, androgens at high concentrations can overcome antiestrogenic inhibition of cell growth.

Discussion

In this report we have shown that high levels of androgens in MCF-7 human breast cancer cells can act as estrogens, binding and translocating the estrogen receptor and provoking both growth and induction of specific products (Fig. 3, Table 3). The androgen receptor is bound and translocated at much lower concentrations without causing these responses, while progestin and glucocorticoid receptors are not translocated by androgens at all (Table 1).

The rates of binding, translocation, and nuclear processing of estrogen receptor in response to androgens closely parallel the rates of these same steps after estrogen administration (Fig. 3). The induction of progesterone receptor by levels of androgen which translocate estrogen receptor proves that the specificity of the response lies in the receptor and not in the ligand (Table 3). Indeed, we cannot be sure that the androgen even remains with the receptor after translocation, as its affinity is too low to inhibit exchange with [^{3}H]estradiol in receptor assays even at 0 C (7-9).

Androgens and estrogens share close structural similarities (19), which may explain why androgens can function as weak estrogens. Presumably, as the structure of the androgen more closely approximates that of estrogens, their estrogenic potential would be increased. Thus, we have shown that several androgens, including Adiol, are able to translocate the estrogen receptor (Table 2). Adiol is a rela-

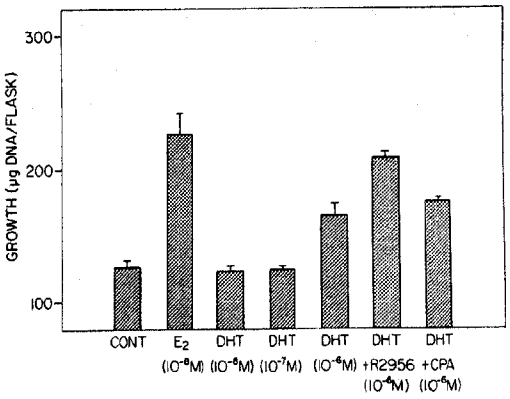

FIG. 5. Rescue of Naf-inhibited cell growth with estradiol and DHT. Two days after cell plating, growth media was changed to 5% stripped calf serum containing 2×10^{-7} M Naf. Cells were maintained on this media for 5 days then changed to 5% stripped calf serum containing vehicle control (ethanol), 10^{-8} M estradiol; 10^{-8}, 10^{-7}, or 10^{-6} M DHT; 10^{-6} M DHT plus 10^{-6} M R2956; or 10^{-6} M CPA. After 6 days, cell growth was monitored. Values represent the mean ± SEM of triplicate determinations from each of four T-75 flasks.

tively weak androgen but, in line with its resemblance to estradiol, it appears equally as active as DHT on the estrogen receptor, as reported by others (9). Likewise, 5α-androstene-3α,17β-diol, an androgen metabolite found in the plasma of fertile women, competes even more effectively than DHT for estrogen binding to estrogen receptor (20).

Our work with the antiestrogen Naf further supports the contention that pharmacological androgens mediate their trophic effects through the estrogen and not the androgen receptor system. That pharmacological androgens reinitiate antiestrogen-blocked cell growth and could not be inhibited by the antiandrogens R2956 or CPA further demonstrates the estrogenic action of high dose androgens (Figs. 4 and 5). Similar studies in the rat uterus have shown that pharmacological androgen induction of a specific protein (IP) can be blocked with antiestrogens but not antiandrogens (21).

The role of androgens in the control of estrogen-sensitive tissues, including breast tumors, is unclear. Physiological concentrations of DHT do not affect growth (4) (Fig. 1) or any other known function in MCF-7 cells, in spite of the translocation of androgen receptor to nuclei. In rat uteri also, translocation of androgen receptor by physiological androgen levels does not immediately cause an observable response (10, 22), though there is considerable reduction in uterine weight over a prolonged period of time (23). Perhaps this action follows the same mechanism as the regression of DMBA-induced rat mammary tumors (23–25) and human breast cancers (26) after low pharmacological doses of androgen.

Very high pharmacological androgens, on the other hand, actively stimulate growth in all of these systems, including MCF-7 cells (5), rat uterus (7, 8, 27), and DMBA tumors (23–25). Even for human breast cancer patients there is a report suggesting that high androgen doses are less effective than somewhat lower doses in causing tumor regression (28).

Therefore, it seems that the effect of androgen on estrogen-dependent tissues and tumors may be biphasic. The first phase, occurring at lower androgen concentrations, leads to inhibition of growth; this phase is present in rat uterus and in some rat and human breast cancers but apparently not in the MCF-7 human breast cancer cell line. This first phase may be mediated directly by androgen receptor in the tissues, or it may operate indirectly through an effect on the pituitary-hypothalamic axis or through some other mechanism. The second phase, leading to enhancement of growth, requires higher androgen concentrations and probably has a somewhat different androgen specificity pattern. This phase, at least in rat uterus and MCF-7 cells, is mediated by androgen binding and translocation of the estrogen receptor. This biphasic response to androgens must be considered both in interpreting studies on possible actions of androgens and in planning androgen therapy for human breast cancer patients.

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