Intracellular inactivation, reactivation and dynamic status of prostate androgen receptors

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The dynamic status of the androgen receptor in prostate cells was studied by incubation of rat ventral prostate with radioactive 17β -hydroxy- 5α -androstane-3-one (5α -dihydro-testosterone) in the presence and absence of respiratory poisons and inhibitors of protein and RNA synthesis and also by isotope chasing of the radioactive androgen–receptor complexes. The androgen receptor in the prostate appears to go through a dynamic process of recycling between the cytoplasm and the nucleus as well as an inactivation process. The radioactive androgen–receptor complex, however, is maintained at a constant level for at least 2 h during incubation at 37°C , even in the absence of new protein synthesis, suggesting that early androgen actions may not require a depletion of a major portion of cellular receptor. In the presence of 2,4-dinitrophenol, the androgen receptor is rapidly deactivated (half life, 2 min). The inactive receptor can be reactivated efficiently by an energy-dependent process, even in the absence of protein synthesis. Receptor binding of androgen and nuclear chromatin binding of the androgen–receptor complex are fast processes; half-maximum binding can be achieved within 1 and 10 min respectively. On the contrary, the overall process of the release of the receptor complex from nuclear chromatin and its reappearance in the cytosol fraction has a long half life of about 70 min. This slow process may reflect the involvement of the steroid–receptor complex in a time-consuming mechanism that is essential for hormone responses. Actinomycin D can increase the nuclear receptor level by 50% or more. This increase is not due to a decrease in the rate of receptor release from nuclei or to inhibition of DNA degradation by the antibiotic.

In many target organs, the actions of steroid hormones appear to be dependent on binding of the hormones to specific cytoplasmic receptor proteins. The steroid–receptor complex can then migrate from the cytoplasm to the nucleus and interact with nuclear chromatin. It is generally assumed that this interaction can result in the alteration in the expression of specific genes (Liao, 1975; Gorski & Gannon, 1976; Jensen, 1977; O'Malley *et al.*, 1979). If this view is correct, it is important to understand the intracellular dynamics of steroid-receptor proteins and the factors that may control the steroid-binding activity and nuclear retention of receptor proteins.

For the 17β -oestradiol receptor of rat uterus, the

suggestion that the nuclear steroid–receptor complex is derived from the receptor protein in the cytoplasm is supported by the fact that the nuclear and cytosol receptors are antigenically related (Greene *et al.*, 1977), and that an increase in the amount of the nuclear oestrogen–receptor complex is accompanied by a decrease in the cytosol receptor level (Jensen *et al.*, 1969; Sarff & Gorski, 1971). With oestrogens such as 17β -oestradiol or diethylstilboestrol, only a small portion of the nuclear oestrogen receptor may reappear in the cytoplasm, and replenishment of cytosol receptor levels is believed to occur mainly by synthesis of new receptor protein (Little *et al.*, 1975; Mester & Baulieu, 1975; Cidlowski & Muldoon, 1978; Baudendistel *et al.*, 1978). Kassis & Gorski (1981), however, reported recently that, in the rat uterus, recycling of 16α -oestradiol–receptor complex occurs efficiently even in the presence of cycloheximide.

Abbreviation used: DNP, 2,4-dinitrophenol.

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Another important aspect of the receptor dynamics in the target cells is the possibility that steroid-binding activity of some cellular receptor proteins may be regulated by an energy-dependent process. In 1968, Munck & Brinck-Johnson showed that cortisol uptake by thymocytes was correlated with the ATP level in the cells. We also reported that NaCN or DNP can inhibit the nuclear retention of androgen in the rat ventral prostate (Liao & Fang, 1969). These and similar studies by others (Ishii *et al.*, 1972; Munck *et al.*, 1972; Auricchio *et al.*, 1981) suggested that the target cells of androgens, glucocorticoids and oestrogens contain inactive receptor proteins that can be activated by an energy-requiring process. In the present paper we report our study on the effects of several metabolic inhibitors on the dynamic status of the cytosol- and chromatin-bound androgen receptors during the incubation of minced ventral prostate of adult male rats. The results support the suggestion that, in this organ, the androgen receptor can go through inactivation-reativation processes as well as recycling between the cytoplasm and nucleus. The term 'activation' represents the intracellular process by which receptor that does not bind steroids is converted into a steroid-binding form.

Experimental

Materials

5 α -Dihydro[1,2,4,5,6,7,16,17-³H]testosterone (sp. radioactivity 200 Ci/mmol) was obtained from New England Nuclear. Non-radioactive 5 α -dihydrotestosterone (17 β -hydroxy-5 α -androstane-3-one) was a product of Steraloids. Actinomycin D, DNP and cycloheximide were purchased from Sigma. Eagle's minimum essential medium was a product of DIFCO laboratories. Other chemicals were of the best reagent grade available from commercial sources.

Incubation of minced prostate

Male Sprague-Dawley rats (body weight, 250–300 g) were purchased from Sasco Co., Omaha, NE, U.S.A. Animals were castrated via the scrotal route under diethyl ether anaesthesia and, if not stated otherwise, killed 18–20 h later by cervical dislocation. The ventral prostates were dissected free of their capsules and their combined volume was measured by displacement in several millilitres of ice-cold 0.32 M-sucrose containing 1 mM-MgCl₂ and 20 mM-Tris/HCl, pH 7.5 (medium A). The ventral prostates (0.4–0.6 g) were minced with scissors and incubated in a 50 ml jar containing 5 ml of Eagle's minimum essential medium (Earle's base) under an atmosphere of O₂/CO₂ (19:1) at 37°C, in a Dubnoff incubator at a shaking rate of 35–45

oscillations per min (Anderson & Liao, 1968; Fang *et al.*, 1969). Incubations were carried out in the presence of radioactive 10 nM-5 α -dihydrotestosterone and/or metabolic inhibitors under the conditions indicated in the Figure legends for individual experiments. After the incubation, the jars were chilled on ice and the minced tissue was recovered by centrifugation at 400 g for 10 min. The minced tissue was washed three times with 5 ml of ice-cold medium A.

Preparation of cellular fractions and assay of the radioactive androgen-receptor complex

Washed prostate minces were homogenized in 3 vol. of medium A in an all-glass Potter-Elvehjem homogenizer at 2°C. The homogenate was centrifuged at 600 g for 15 min. The pellets from this centrifugation were used for the purification of nuclei, and the supernatant for the preparation of the cytosol fraction, by procedures described elsewhere (Liao *et al.*, 1965; Fang & Liao, 1971). The nuclear receptor complex was extracted with a medium containing 1.5 mM-EDTA, 20 mM-Tris/HCl, pH 7.5 (medium ET), and 0.6 M-KCl. The cytoplasmic supernatant was centrifuged at 130 000 g for 1 h. Radioactive androgen-receptor complex was precipitated from the cytosol fraction by the addition of solid (NH₄)₂SO₄ to 40% saturation and was then resuspended in medium ET containing 0.4 M-KCl. Both the cytosol and the nuclear androgen-receptor complexes were measured by gradient centrifugation. The linear 5–20% (w/v) sucrose gradient contained 5% (v/v) glycerol, 0.6 M-KCl, 1.5 mM-EDTA and 20 mM-Tris/HCl, pH 7.5. Centrifugation was performed for 20 h with a Spinco SW-60 rotor in a Beckman L5-65 ultracentrifuge. Detailed description of the above procedures can be found in our previous publications (Fang & Liao, 1971; Liao *et al.*, 1980a).

For preparation and assay of the radioactive androgen receptor protein in the cytosol fraction of the prostate that had not previously been exposed to radioactive androgen, the prostate homogenate was centrifuged at 10 000 g for 10 min. The supernatant fraction was then incubated with radioactive 10 nM-5 α -dihydrotestosterone at 2°C for 2 h. The radioactive androgen-receptor complex was isolated and assayed by gradient centrifugation (Shyr & Liao, 1978; Liao *et al.*, 1980a).

The methods described above measure high-affinity (K_d 0.1 nM) androgen-specific receptors in the cytosol and nuclear preparations (Fang & Liao, 1971; Liao *et al.*, 1973b). To estimate the receptor levels per cell, we determined the DNA content of the incubated prostate and of the isolated nuclei. The level of the nuclear radioactive receptor complex was corrected for the loss of nuclei during the nuclear isolation. To calculate the number of receptors per

cell, we assumed that each diploid cell contains 6 pg of DNA, and that one receptor protein binds only one molecule of androgen. In this study, the sum of the detectable receptor complexes in the nuclei and in the cytosol fraction was considered to be the total receptor complex in the prostate cells. In many experiments, we measured the receptor content of the microsomal fractions (pellets from centrifugation at 130000 g). Since the microsomal receptor levels were only about 10% of the total receptor content and did not affect the conclusions drawn in this report, the microsomal results are not shown here. It should be noted that the cytosol fraction may contain some receptor complexes that are originally in the nuclei but are not tightly bound to the macromolecular structures and can leak from nuclei during homogenization of the prostate.

Nuclear retention of the radioactive androgen-receptor complex in cell-free system

Nuclear retention studies were carried out in 2 ml of medium A containing 30000 c.p.m. (about 1 mg of protein) of 5 α -dihydro[³H]testosterone-receptor complex and cell nuclei (75–150 μ g of DNA equivalent). This mixture was incubated at 0°C for 15 min, then at 20°C for 20 min, and finally at 2°C for 10 min (Shyr & Liao, 1978; Chen *et al.*, 1982). After incubation, 5 ml of ice-cold medium A was added to the mixture. The nuclei were re-isolated by centrifugation at 1000 g for 10 min and washed, first with 5 ml of medium A, then with 5 ml of medium A containing 0.25% (w/v) Triton X-100, and finally with 5 ml of 20 mM-Tris/HCl at pH 7.5, containing 1.5 mM-EDTA. The nuclear chromatin aggregate was then extracted with 3 vol. of 0.8 M-KCl containing 20 mM-Tris/HCl, pH 7.5, and 1.5 mM-EDTA for 45 min. The radioactive receptor content of the KCl extract was determined and expressed as the nuclear (or chromatin-bound) receptor complex.

Other methods

The radioactive androgens were identified and their purity was assessed by t.l.c. (Anderson & Liao, 1968). Radioactivity was measured in a Packard liquid-scintillation spectrometer, with a scintillation fluid containing Triton X-100 and toluene (1:3, v/v), 0.4% (w/v) diphenyloxazole and 0.005% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene. The counting efficiency was about 30%. Each radioactive sample contained 5000–20000 c.p.m.; counting errors, therefore, were well below 5%. DNA was measured by the diphenylamine test, with calf thymus DNA as standard (Burton, 1956). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. All values are results of a representative experiment. Three or more replicate experiments gave similar results. Variation between experiments was within 20%. Each value in the

Figures shown is the mean of results from duplicate or triplicate samples. Individual values were within 10% of each other.

Results

Cytosol and chromatin-bound androgen receptor levels during the incubation of minced prostate with radioactive 5 α -dihydrotestosterone

Fig. 1 shows that, when the minced prostates of castrated rats were incubated with radioactive 10 nM-5 α -dihydrotestosterone, the cytosol receptor protein was labelled very rapidly, and that the labelling reached a maximum level within the first 2 min. As reported previously (Fang *et al.*, 1969), the nuclear chromatin-bound radioactive receptor complex increased gradually and reached a plateau value within 30–40 min. This increase was accompanied by a stoichiometric loss of the cytosol radioactive receptor complex, suggesting that the nuclear complex originated in the cytoplasmic soluble fraction. After the plateau was reached, the nuclei and cytosol fraction contained about 55%

Fig. 1. Changes in the cytosol and nuclear receptor content during incubation of the prostate with radioactive androgen

The ventral prostates of castrated rats were minced and incubated with 5 α -dihydro[³H]testosterone at 37°C, under the conditions described in the Experimental section, for the times shown on the abscissa. The prostates were then analysed for the amounts of radioactive androgen-receptor in the cytosol and nuclear fractions. For comparison, the sum of the receptor contents in the two cellular fractions of the prostates (8500 receptor molecules per cell) incubated for 80 min was taken as 100%. Abbreviation: AR, androgen receptor.

and 45% respectively of the total detectable receptor complexes.

In the experiments described in the present paper, we generally found that the amount of the radioactive receptor complexes in the prostate was about 6000 ± 1500 fmol/g of fresh organ or about 8000 ± 2000 receptor molecules per prostate cell. The total receptor level in the individual cells might actually be higher if the isotope dilution due to endogenous androgen was also taken into consideration. In many of the experiments described below, we also employed as much as $0.3 \mu\text{M}$ of radioactive androgen and obtained essentially identical results.

Effects of respiratory poisons on the androgen-binding activity of the receptor proteins and on the nuclear retention of the receptor complex

We found that DNP, KCN and NaN_3 , at concentrations (1–10 mM) known to affect cellular

respiration and ATP production in mammalian tissues, were capable of reducing the retention of 5α -dihydro- $[\text{H}]$ testosterone–receptor complex by nuclei during incubation of minced prostate. By gradient centrifugation, no radioactive androgen–receptor complex was detectable in the nuclear extract or in the cytosol fraction of the prostates incubated with 1 mM-DNP, 1 mM-KCN or 10 mM- NaN_3 .

The effect of the DNP appeared to be very rapid. In the absence of DNP, the loss of the androgen-binding activity of the receptor protein was less than 10% during a 5 min incubation, but incubation of prostates with 1 mM-DNP for 5 min eliminated 90% of the binding activity (Fig. 2). Cycloheximide, at a concentration known to inhibit at least 95% of protein synthesis in the prostate (Liang

Fig. 2. *Effect of androgen, cycloheximide and DNP on the total receptor content in the ventral prostate*

The ventral prostates of castrated rats were minced and incubated at 37°C in the absence (▲) or presence of cycloheximide ($50 \mu\text{g/ml}$) (×), DNP (1 mM) (△), 5α -dihydro $[\text{H}]$ testosterone (10 nM) (●) or DNP (1 mM) and 5α -dihydro $[\text{H}]$ testosterone (10 nM) (○) for the length of time indicated on the abscissa. 5α -Dihydro $[\text{H}]$ testosterone (10 nM) was then added to the tubes that did not contain the radioactive androgen. The prostates were then homogenized in the incubation medium, and their nuclear and cytosol androgen receptors were analysed. For comparison of results, the total amount (cytosol receptor and nuclear receptor) of androgen receptor in the prostates at zero time was taken to be 100%.

Fig. 3. *Reversibility of the DNP effect on androgen receptor*

Ventral prostates of castrated rats were minced and incubated at 37°C in the absence (●) or presence (○) of 0.3 mM-DNP. After incubation for 20 min, DNP was removed from two jars (△, ×) that contained DNP by washing of the prostate with the incubation medium three times. Cycloheximide ($50 \mu\text{g/ml}$) was added to one (×) of the two jars containing washed prostates. After all prostates were incubated for the times indicated on the abscissa, 5α -dihydro $[\text{H}]$ testosterone was then added to all jars. Incubation was then continued for another 30 min, and the radioactive androgen–receptor complexes in the nuclear fraction were assayed. For comparison, the amount of the nuclear receptor complex in the prostate that was not incubated before the addition of radioactive androgen was taken to be 100%.

et al., 1977), did not affect the loss of androgen-binding activity. Therefore, the effect of DNP on the receptor was probably not due to a decrease in the synthesis of new receptors.

The results shown in Fig. 3 indicated that the amount of the nuclear radioactive androgen-receptor complex decreased during incubation of prostates with 0.3 mM-DNP. After DNP was removed from the incubation medium, the level of the receptor complex recovered gradually to about the same level exhibited by the prostate that had been incubated in the absence of DNP for the same length of time. The androgen-binding activities of the cytosol receptor preparations exhibited the same recovery pattern (results not shown). Since these patterns could be seen even in the presence of cycloheximide (Fig. 3), the recovery is apparently not dependent on the synthesis of new receptor protein molecules. If the prostates were first incubated with radioactive 5 α -dihydrotestosterone at 37°C for 30 min to allow formation of the radio-

active receptor complex and the nuclear retention of the complex, and if they were then incubated with different concentrations of DNP, the nuclear complex was more resistant than the cytosol complex to the effect of DNP (Fig. 4).

In a cell-free system the retention of the radioactive androgen-receptor complex by isolated prostate cell nuclei was not affected by DNP. DNP also did not prevent binding of the radioactive androgen to the receptor protein in the cytosol fraction, nor did it alter the stability of the isolated androgen-receptor complex. The inhibitory effect of DNP was apparently not due to a decrease in uptake, nor to an increase in the metabolism of the radioactive 5 α -dihydrotestosterone during the incubation. The amount of radioactive androgen associated with the incubated prostate was not changed by the poison, and more than 80% of the radioactive steroids associated with the incubated prostate, or in the incubation medium, could be recovered as 5 α -dihydrotestosterone at the end of the incubation. In fact, during the 60 min incubation period we consistently found more (about 10%) radioactive 5 α -dihydrotestosterone in the minced prostate incubated with 1 mM-DNP than in that without the poison. This might have been due to a decrease in the rates of reduction of 5 α -dihydrotestosterone to androstane diols when the cellular energy production was inhibited by DNP.

The above studies suggest that, in prostate cells, the androgen receptor protein is rapidly inactivated to a form incapable of binding androgen. The inactive form, however, can be reactivated by an energy-dependent process that can be impaired by DNP. This energy-dependent reactivation is not dependent on synthesis of new protein.

Chasing of receptor-bound radioactive androgen by non-radioactive androgen

Steroid hormones bind to their cellular receptors non-covalently. Therefore, studies of the dynamic status of the radioactive steroid-receptor complex in target cells by chasing the complex with non-radioactive steroid can be complicated by steroid exchange on the receptor surface (Rennie & Bruchovsky, 1973). This is especially true for several oestrogen receptors systems in which the steroid exchange seems to take place very rapidly (Zava *et al.*, 1976; deBoer & Notides, 1981). In contrast, steroid exchange has been found to proceed rather slowly for the androgen receptors of human and rat prostates (Bonne & Raynaud, 1976; Mobbs *et al.*, 1979; Shain *et al.*, 1979; Walsh & Hicks, 1979). Our experiments also showed that less than 10% of the radioactive androgen bound to the isolated receptor of rat ventral prostate could be exchanged by non-radioactive 5 α -dihydrotestosterone within 1 h at 25°C and 37°C. To study the translocation of the

Fig. 4. Effect of the different concentrations of DNP on the cytosol and on nuclear androgen-receptor complexes

The ventral prostates of castrated rats were minced and incubated with 5 α -dihydro[³H]testosterone at 37°C for 30 min to allow formation of the cytosol androgen-receptor complex and retention of the complex by nuclei. The prostates were then washed with the incubation medium for removal of the excess amount of androgen and were incubated for another 30 min in the presence of various concentrations of DNP. The radioactive-receptor complexes in the nuclei (○) and cytosol (●) fractions were then assayed. The results were compared with the receptor contents in the individual cellular fractions of the prostates incubated without DNP.

androgen-receptor complex in the cellular compartments of the rat ventral prostate by chasing, we incubated minced prostates with 10 nM-5 α -dihydro[3 H]testosterone at 37°C for 30 min to allow the receptor to bind the radioactive ligand and to allow the radioactive androgen-receptor complex to interact with nuclear chromatin. The prostate tissue was then rinsed and incubated again, for various periods of time, in a new medium that contained non-radioactive 1 μ M-5 α -dihydrotestosterone. The levels of radioactive androgen-receptor complex in the cytosol and in the nuclear fractions of the prostates were then analysed.

Fig. 5 shows that, in the absence of additional non-radioactive androgen, the amounts of radioactive androgen-receptor complex in the cytosol and in the nuclear fractions were maintained at constant levels during 90 min of incubation (cf. Fig. 1). When non-radioactive androgen was added, the radioactive receptor complex disappeared from the two compartments with a half-life ($t_{1/2}$) of about 90 min. The levels of the radioactive receptor complexes in the nuclear and cytosol fractions (without chasing) and their rates of disappearance in the presence of non-radioactive androgen were not altered when cycloheximide was added to the incubation medium (Fig. 5). In several other experiments, we found that the cellular androgen receptor was maintained at a constant level for at least 2 h during the incubation of minced prostate even in the presence of cycloheximide. These observations suggest that androgen receptor protein was not synthesized or irreversibly degraded to any significant extent during this incubation period.

When DNP was added to the incubation medium, the amount of the radioactive receptor complex in the cytosol fraction fell off very rapidly ($t_{1/2}$, 20 min) (Fig. 5a), whereas loss of the complex from nuclei proceeded at a slower rate ($t_{1/2}$, 70 min) (Fig. 5b). The rates of these losses were not increased when non-radioactive androgen was added to the medium. Such increases would be expected if the non-radioactive androgen could exchange with the radioactive androgen bound to the cytosol or to the nuclear receptor at a rate significantly greater than the rate of inactivation or release of the receptor complex from nuclei.

Actinomycin D-dependent increase in the nuclear retention of androgen-receptor complex

The loss of oestradiol-receptor complex from nuclei was prevented when rat uterus (Schoenberg & Clark, 1979) and human cancer cells (MCF-7 line) (Horwitz & McGuire, 1978b) were incubated in media containing actinomycin D. These observations are interesting since we have hypothesized that various steroid-receptor complexes may bind to

Fig. 5. Chasing of radioactive androgen by non-radioactive 5 α -dihydrotestosterone in the presence and absence of cycloheximide or DNP

The ventral prostates of castrated rats were minced and incubated with 5 α -dihydro[3 H]testosterone (10 nM) at 37°C for 30 min. The prostates were then incubated further for the times shown on the abscissa in the absence (●) or presence of cycloheximide (50 μ g/ml) (○), non-radioactive 1 μ M-5 α -dihydrotestosterone (▲), non-radioactive 1 μ M-5 α -dihydrotestosterone and cycloheximide (50 μ g/ml) (△), 1 mM-DNP (■) or non-radioactive 1 μ M-5 α -dihydrotestosterone and 1 mM-DNP (□). The cytosol (a) and nuclear (b) radioactive-receptor complexes were then assayed. For comparison of results, the radioactive receptor levels in the individual cellular fractions of the prostates incubated with the radioactive androgen only for the initial 30 min were taken to be 100%.

Fig. 6. Nuclear androgen receptor levels during the incubation of actinomycin D with minced prostates or isolated nuclei

Minced prostate (●) or nuclei (○) isolated from the castrated rats were incubated with different concentrations of actinomycin D at 37°C for 20 min. The minced prostates were then incubated with 5α-dihydro[³H]testosterone (10 nM) at 37°C for 40 min, whereas the prostate nuclei were incubated with radioactive 5α-dihydrotestosterone receptor complex under the conditions described in the Experimental section, to allow the nuclei to retain the radioactive receptor complex. The nuclear receptor levels were then estimated. For comparison of results, the nuclear receptor level of the prostate or nuclei incubated without actinomycin D was taken to be 100%.

Fig. 7. Effect of actinomycin D on androgen receptor levels in the nuclei and in the cytosol fraction

The ventral prostates of castrated rats were minced and incubated with 5α-dihydro[³H]testosterone at 37°C for 30 min. The prostates were then incubated further for the times shown on the abscissa in the presence of actinomycin D (20 μg/ml) (●) or of non-radioactive 1 μM-5α-dihydrotestosterone and actinomycin D (20 μg/ml) (○). The cytosol (----) and nuclear (—) radioactive receptor complexes were then assayed. For comparison of results, the radioactive receptor levels in the individual cellular fractions of the prostates incubated with the radioactive androgen only for the initial 30 min were taken to be 100%.

newly synthesized RNA and are released from chromatin as ribonucleoprotein particles (Liao & Fang, 1969; Liao *et al.*, 1972). In our study we found that the retention of radioactive androgen-receptor complex by prostate cell nuclei increased when minced rat ventral prostate was incubated with actinomycin D (Fig. 6). This effect could also be seen when isolated nuclei and radioactive 5α-dihydrotestosterone-receptor complex were incubated in the presence of actinomycin D. The effect of actinomycin D in the cell-free system, however, was not as significant as that seen with the prostate incubated in the culture medium.

To study the kinetics of the actinomycin D-induced alteration of receptor retention by nuclei, we first incubated minced prostate with radioactive androgen for 30 min to allow the radioactive androgen-receptor complex to reach a steady state distribution pattern, as seen in Fig. 1. Actinomycin D was then added with or without non-radioactive

5α-dihydrotestosterone. As shown in Fig. 7, actinomycin D gradually increased the amount of the nuclear radioactive receptor complex during the incubation to a plateau after about 90 min. This increase in the level of nuclear receptor complex was accompanied by a reduction in the amount of the radioactive receptor complex in the cytosol fraction.

The presence of non-radioactive 5α-dihydrotestosterone in the incubation medium completely abolished the effect of actinomycin D, apparently by isotope dilution of the cytosol radioactive receptor complex that could be retained by nuclei after the addition of actinomycin D. As shown in Fig. 7, the rate of loss of the nuclear radioactive androgen-receptor complex ($t_{1/2}$, 90 min) during chasing was similar to that occurring in the absence of actinomycin D (Fig. 5b). It appears that the actinomycin D-dependent increase in the nuclear receptor complex was not due to a decrease in the rate of disappearance of the receptor complex from nuclear fraction.

We did not detect any effect of actinomycin D on the sedimentation pattern of the radioactive 5 α -dihydrotestosterone-receptor complexes prepared from the prostate cytosol or extracted from the prostate nuclei; this indicated that actinomycin D may not interact with the receptor complex directly. To test the possibility that actinomycin D increases the amount of the nuclear steroid-receptor complex by preventing degradation of the bulk of nuclear DNA, we incubated the minced prostate with different concentrations of actinomycin D and 5 α -dihydrotestosterone. Nuclei were isolated from the incubated prostate, and DNA was extracted. Using gradient centrifugation, we were unable to find differences in the sedimentation patterns of DNA preparations from the control and those from actinomycin D-treated prostates. The effect of actinomycin D, therefore, was not due to its influence on the degradation of the bulk of nuclear DNA as was suggested for the oestrogen-receptor system in rat uterus (Schoenberg & Clark, 1979).

The retention of 5 α -dihydro[3 H]testosterone-receptor complex by DNA-cellulose was not altered by actinomycin D (Liao *et al.*, 1980a). Actinomycin D added before, simultaneously with, or after application of the receptor complex to the DNA-cellulose column did not change the retention or elution (by KCl solution) pattern of the receptor complex.

To insure that the actinomycin D effect was not due to superinduction (Tomkins *et al.*, 1972) of the receptor protein, we incubated minced prostate with actinomycin D in the presence or absence of cycloheximide. The results indicated that the effect of actinomycin D was not influenced by cycloheximide, and that the total receptor content of the prostate cells was not changed, regardless of whether the incubation was carried out in the presence or absence of the two antibiotics. In the presence of cycloheximide, the increase in the nuclear receptor complex was accompanied by a quantitative loss of the complex in the cytosol fraction, as was the case without cycloheximide.

Discussion

Our attempts to show directly that the disappearance of the nuclear receptor complex was accompanied by an increase in the cytosol receptor level had not been successful. This might be due to the rapid replenishment of the nuclear receptor and the comparably slow rate of release of the receptor from the nucleus into the cytoplasm. The large quantity of receptor (about 50% of the total) remaining in the cytoplasm when the level of nuclear receptor reached a plateau might also mask the gradual increase in the cytosol receptor level due to recycling. This is in contrast with the uterine system,

in which as much as 80% of the cytoplasmic oestrogen receptor can be taken up by nuclei and, therefore, the subsequent release of the receptor from nuclei can be seen by measuring the increase in the cytosol receptor level (Kassis & Gorski, 1981).

The ability to detect the recycled radioactive androgen-receptor complex (AR) in the cytosol fraction is also dependent on the rate of the release of the complex from chromatin (r_1) and the rate of the inactivation of the released complex (r_2) into androgen (A) and the inactive receptor (R^0) that does not bind androgen:

The accumulation of cytosol AR can be seen only if $r_1 > r_2$. Our failure to show accumulation of cytosol AR may imply that, with radioactive 5 α -dihydrotestosterone, $r_1 < r_2$. Since these rates may vary with different radioactive steroids, a direct demonstration of the recycling phenomenon may be possible with another androgen. This view may be in line with a recent report by Kassis & Gorski (1981), who found that, in the rat uterus, 16 α -oestradiol (but not 17 β -oestradiol) receptor complex can be recycled efficiently from the nucleus into cytoplasm. Regardless of whether receptor recycling does occur in the rat prostate, our studies support the suggestion that certain early actions of steroid hormones in target cell nuclei, such as increases in RNA synthesis, which can be observed within 1 h (Gorski, 1964; Liao *et al.*, 1965), may not require a mechanism that involves a major depletion of nuclear receptor and reduction in total cellular receptor. Such a mechanism, referred to as 'processing', has been correlated with some oestrogenic responses (Horwitz & McGuire, 1978a).

The rates of loss of the radioactive receptor complexes from the cytosol and nuclear fractions in the presence of DNP are not affected by the addition of a large quantity of non-radioactive 5 α -dihydrotestosterone to the incubation medium (Fig. 5). This is in agreement with our contention that the direct exchange of androgen on the receptor is a very slow process. Even considering the sensitivity and the error in our assay, direct isotope exchange appears to account for no more than 15% of the loss of these complexes during the 90 min incubation. If this assumption is correct, the loss of the cytosol and nuclear radioactive receptor complexes in the presence of non-radioactive androgen and in the absence of DNP (Fig. 5) must be due to another isotope-dilution process. We believe that, in prostate cells, both losses occur mainly through the same steps: (a) release of the radioactive-receptor complex from the nuclear acceptor site; (b) inactivation of receptor and release of radioactive androgen; (c) reactivation of receptor and binding of radioactively diluted androgen; and (d) retention of the radio-

actively diluted receptor complex by nuclear chromatin. This view is in line with the fact that, in the presence of non-radioactive androgen, the apparent rate of loss of the radioactive-receptor complex from the cytosol is about the same as that from nuclei ($t_{1/2}$, 90 min) and that this rate is somewhat lower than the rate of release of the radioactive receptor complex from nuclei in the presence of DNP ($t_{1/2}$, 70 min). It is necessary, however, to explain why the cytosol radioactive androgen-receptor complex can disappear much more rapidly (Fig. 5) in the presence of DNP ($t_{1/2}$, 20 min) than in the presence of non-radioactive androgen (but without DNP) ($t_{1/2}$, 90 min). One of the plausible explanations is that transformation and binding of the receptor complex to nuclei are also affected by DNP, thus causing the receptor complex to remain in the cytoplasm and to be inactivated rapidly.

The mechanism involved in the receptor inactivation is not clear. Inactivation is reversible (Fig. 3) by a DNP-sensitive process. The regeneration of the androgen-binding activity of the receptor is carried out rapidly and efficiently, without significant loss of the total receptor in the prostate cells (Fig. 5). Androgen appears to protect the receptor from inactivation to some extent. The apparent half lives of the receptor in the absence and presence of androgen (added to the medium at the same time as DNP) are 2 min and 8 min respectively (Fig. 2). Nuclear androgen-receptor complex is inactivated much more slowly than is the cytosol receptor complex (Figs. 4 and 5). Whether this is due to a difference in the two complexes or to stabilization of the nuclear complex by a nuclear component is not clear. As described above, receptor inactivation may occur only after its release from the nuclear acceptor site.

Receptor inactivation and reactivation can be very important in the regulation of the growth and functions of target cells. We have observed that the rate of receptor inactivation (measured in the presence of DNP) in the ventral prostate of rats is gradually reduced within 10 days after castration to less than 10% of the rate in the prostate of normal rats. These processes should also be considered when one evaluates whether the lack of receptors in cells that are insensitive to steroid hormones is due solely to a specific defect in the genes regulating the production of receptor proteins for steroid hormones.

As described in the introduction to the present paper, the energy-dependent control of the steroid-binding activity of glucocorticoid receptors has been studied by several investigators. Our preliminary experiments showed that there may be a similar energy-dependent process involved in activation of the oestrogen receptor in rat uterus. However, the

effect of respiratory poisons on the steroid-binding activity of the oestrogen receptor was not as remarkable as that on receptors for androgens and glucocorticoids. ATP has been shown to stabilize the prostate androgen-receptor complex (Liao *et al.*, 1975) and to interact with hen oviduct progesterone-receptor complex (Toft & Nishigori, 1979; Moudgil *et al.*, 1981). More recently, Pratt and his associates concluded that the glucocorticoid receptors of L-cells or thymocytes could be deactivated by a dephosphorylation step (Sando *et al.*, 1979a,b; Wheeler *et al.*, 1981). The inactive receptor was also shown to be activated in the presence of ATP and a heat-stable factor. There is, however, no direct evidence that the steroid-binding activity is related to phosphorylation or dephosphorylation of the receptor proteins.

Actinomycin D does not appear to affect the rate of release of the receptor complex from nuclei (Figs. 5b and 7). The actinomycin D-dependent increase in the nuclear receptor level, therefore, may be due to an increase in the receptor-binding sites in the nuclei. The newly created sites may not be identical with those that were present originally. Under the conditions of our study, and with 10–20 μ g of actinomycin D/ml in the medium, we found that as much as $(3-7) \times 10^8$ molecules of actinomycin D were bound to each prostate cell nucleus. The nuclear receptor-binding sites, however, increased only by about 2000 sites per nucleus. Whether the actinomycin D effect is due to an alteration of chromatin structure or related to RNA chain elongation is not clear. Alternatively, actinomycin D might inhibit the synthesis of a rapidly-turning-over phosphatase, which inactivates the receptor (cf. Okulicz *et al.*, 1981; MacDonald *et al.*, 1982). With rifamycins that inhibit the initiation of RNA synthesis, we found some reduction (10–20%) rather than an increase in chromatin binding of the receptor complex (results not shown).

Very little is known about the way in which the steroid-receptor complexes interact with the nuclear components in the target cells. The receptor complexes may bind to different acceptor sites (Tymoczko & Liao, 1971; Liao *et al.*, 1980a; Barrack & Coffey, 1980) simultaneously or sequentially. The low rate of release of the receptor complex from nuclei may reflect the involvement of the receptor complex in a slow process that is essential for hormone responses. In this regard, our suggestion (Liao *et al.*, 1972, 1973a, 1980a,b) that certain steroid receptors may bind to specific RNA or ribonucleoproteins in the nuclei and play important roles in the post-transcriptional processing and utilization of RNA may be worth exploring further.

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