The Relationship between Androgen Receptors and the Hormonally Controlled Responses of Rat Ventral Prostate

By EDWARD VAN DOORN, SHARON CRAVEN and NICHOLAS BRUCHOVSKY
Department of Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2G3

(Received 15 March 1976)

1. The administration of dihydrotestosterone to rats orchidectomized 7 days previously stimulates the synthesis of nuclear receptor in prostatic cells several hours in advance of DNA synthesis and mitosis. 2. The synthesis of nuclear receptor is tightly coupled to cell proliferation; consequently, in resting cells, there is no further net synthesis of nuclear receptor above the maximum of approx. 8000 molecules/cell. 3. After orchidectomy a rapid decline in the concentration of free androgen in the nucleus and a slower decline in the concentration of nuclear receptor are observed. 4. Owing to the apparent scarcity of receptor-inactivating factors in the nucleus, and the inverse relationship between amounts of nuclear and cytoplasmic receptors, it is concluded that the nuclear receptor is discharged into the cytoplasm after orchidectomy. 5. The formation of the cytoplasmic receptor is an early event preceding the onset of cellular autolysis. 6. Regressing prostate develops the capacity to eliminate cytoplasmic receptor, and this capacity is retained by the regenerating prostate for at least 14 days. 7. The synthesis of nuclear receptor in early G1 phase may control the entry of cells into the cell cycle and the prolonged retention of receptor in the nucleus may prevent the activation of autophagic processes.

The physiological condition of the prostate ostensibly is determined by the binding of androgens to intracellular proteins, the commonly acknowledged androgen receptors found in the cytoplasm and nucleus of the prostatic cell (King & Mainwaring, 1974; Liao & Liang, 1974; Bruchovsky *et al.*, 1975a). Studies by Davies & Griffiths (1973, 1974, 1975), Mainwaring & Jones (1975) and Hu *et al.* (1975) have demonstrated a direct effect of androgen–receptor complexes on the transcription of chromatin by RNA polymerase. The work of Ichii *et al.* (1974) and of Liang & Liao (1975) has suggested that androgen–receptor complexes are further involved in the post-transcriptional regulation of protein synthesis. Also, Coffey (1974) and Rennie *et al.* (1975) have shown that certain features of DNA synthesis in regenerating prostate are probably mediated by androgen–receptor complexes. Notwithstanding this compelling evidence for the role of androgen–receptor complexes in regulating a broad spectrum of hormonal responses in the prostate, understanding of the function of receptors remains imprecise for several reasons. First, the response of prostate is finely tuned to the intracellular concentration of androgens and to the number of cells in the gland. These two variables apparently predetermine both the magnitude and the character of the response, independent of the concentration of androgen–receptor complexes (Lesser & Bruchovsky, 1974a,b; Bruchovsky *et al.*, 1975b; Bruchovsky & Craven, 1975). Secondly,

although it is generally assumed that the cytoplasmic androgen–receptor complex is the immediate precursor of the nuclear androgen–receptor complex, the direct relationship between these two molecules remains unconfirmed (Bruchovsky *et al.*, 1975b; Bruchovsky & Craven, 1975). Particularly in those instances where the cytoplasm is chosen as the source of the receptor molecule, any androgen–receptor effect on a nuclear biosynthetic mechanism must be regarded as tentative until the cytoplasmic and nuclear forms of androgen–receptor complex are shown to be equivalent. Ultimate proof rests on the number of different androgen–receptor complexes that are present in the cytoplasm (Rennie & Bruchovsky, 1972; Katsumata & Goldman, 1974; Bruchovsky *et al.*, 1975b). Thirdly, in assessing the importance of androgen–receptor complexes, considerable weight has been given to results obtained with anti-androgens, such as cyproterone acetate, known competitive inhibitors of androgen binding (King & Mainwaring, 1974; Liao & Liang, 1974). However, since high concentrations of anti-androgenic compounds are generally required for significant competition (Rennie & Bruchovsky, 1973; King & Mainwaring, 1974; Szalay *et al.*, 1975), it is doubtful that the action of anti-androgens at therapeutic doses is explained purely on the basis of interference with androgen binding (Szalay *et al.*, 1975).

The method of choice to clarify the relationship between androgen receptors and cellular responsive-

ness might be one furnishing a direct measurement of the concentration of cytoplasmic and nuclear receptors while the sensitivity of the cell to androgen is changing. As reviewed by Bruchovsky *et al.* (1975a) and Bruchovsky & Lesser (1976), there are three primary responsive states of the prostate: (1) if the number of cells in the prostate is below normal, DNA synthesis and cell proliferation are initiated by the administration of androgen; (2) when the number of cells is restored to normal, DNA synthesis is curtailed through a negative-feedback effect but secretion continues; (3) withdrawal of androgen, as by orchidectomy, is followed by autophagic involution of prostate.

The recent development in our laboratory of new methods permitting the unambiguous detection of specific cytoplasmic and nuclear receptors has enabled us to search for gross correlations between androgen receptors and the aforementioned basic responses of prostate in the rat. Such correlations are described below.

Materials and Methods

Experimental animals

Male rats of the Wistar strain, weighing 250–400g, were purchased from Woodlyn Laboratories, Guelph, Ont., Canada) and were maintained on a diet of Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO, U.S.A.) and water *ad libitum*. Orchidectomy was performed through a scrotal incision while the animals were under ether anaesthesia. Beginning 7 days after castration, various doses of dihydrotestosterone in 0.5ml of an aqueous medium containing 10% (v/v) ethanol and 10% (v/v) polyoxyethylene sorbitan monopalmitate were administered by subcutaneous injection. At appropriate times after treatment the rats were killed by decapitation and prostates quickly removed, stripped of connective tissue, placed on ice and weighed.

Preparation of subcellular fractions

Prostatic nuclear and cytosol fractions were prepared as described by Bruchovsky *et al.* (1975b) with two minor changes: (1) the prostatic tissue was rinsed with 0.25M-sucrose in 0.01M-Tris buffer, pH 7.0, containing 0.05 mM-EDTA, 5 mM-MgCl₂ and 0.5 mM-mercaptoethanol (Tris/EDTA buffer), as well as 1.5 mM-CaCl₂ instead of tissue-culture medium, and (2) tissue was minced with scissors and forced through a stainless-steel wire screen (30 mesh; 0.55 mm grid) instead of being chopped with an automatic tissue slicer before homogenization with a Dounce apparatus.

Recovery of cytoplasmic and nuclear androgen-binding proteins

Nuclear and cytoplasmic androgen-binding pro-

teins were recovered from purified nuclei and cytosol preparations as described by Bruchovsky *et al.* (1975b).

Labelling of androgen-binding proteins in vitro

(a) *Cytoplasmic receptor*. Minced prostate tissue was transferred to a 15 ml test tube and suspended in 2 ml of tissue-culture medium containing 750 nM-[1,2-³H]dihydrotestosterone. This sample was then incubated at 37°C for 2 min with gentle shaking in an atmosphere of O₂+CO₂ (95:5). The incubation was terminated by the addition of 5 vol. of ice-cold tissue culture medium. The tissue was washed three times with 5 vol. of Tris/EDTA buffer containing 0.25M-sucrose and 1.5 mM-CaCl₂ to remove unincorporated steroid, and then processed as described previously (Bruchovsky *et al.*, 1975b) to obtain the cytoplasmic androgen-binding protein.

(b) *Nuclear receptor*. The nuclear extract was incubated with [1,2-³H]dihydrotestosterone (0–20 nM) at 20°C for intervals up to 18 h, and analysed by gel-exclusion chromatography.

Labelling of androgen-binding proteins in vivo

[1,2-³H]Testosterone was injected intravenously into rats orchidectomized 24 h previously. At appropriate intervals after the injection, the rats were killed and the prostates removed.

Gel-exclusion chromatography

Cytoplasmic and nuclear receptors were isolated by using a dual-column gel-exclusion chromatography system. The apparatus consisted of a column (0.9 cm × 30 cm) of Sephadex G-25 (Pharmacia, Montreal, Que., Canada) connected in series with a column (0.9 cm × 100 cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden). The sample (less than 2 ml) was introduced into a sample loop and subsequently pumped into the short column. The sample was then eluted in an upward direction with Tris/EDTA buffer containing 600 mM-NaCl at a flow rate of 2–3 ml/h. Fractions (1.2–1.4 ml) were collected and assayed for radioactivity and u.v.-absorbing materials.

Sucrose-density-gradient centrifugation

Samples (200 µl) were layered on linear 5–20% (w/v) gradients of sucrose in Tris/EDTA buffer (3.6 ml) containing 600 mM-NaCl. The gradients were centrifuged at 246000g for 17 h (SW 56 rotor, average radius 8.8 cm) in a Beckman-Spinco ultracentrifuge. Thirty five fractions from each gradient were collected and counted for radioactivity. Bovine serum albumin and haemoglobin were used as reference standards to estimate the approximate sedimentation coefficients of androgen-binding components (Martin & Ames, 1961).

Because of the low sedimentation coefficient of the nuclear receptor, it was necessary to remove free

radioactive androgen by charcoal adsorption before centrifugation. The charcoal-treated sample was concentrated to a volume of 500 μ l in a Minicon B15 macrosolute concentrator (Amicon Corp., Lexington, MA, U.S.A.) before application to the sucrose gradient.

Radioactive materials

[1,2- 3 H]Testosterone (40 Ci/mmol) and [1,2- 3 H]-dihydrotestosterone (44 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Purity was checked by t.l.c. (Bruchovsky, 1971) and the steroids were considered acceptable only if they were 95–100% pure.

Solutions for intravenous injections were prepared as follows. Radioactive steroid, shipped and stored in 10% (v/v) ethanol/benzene, was dried under N_2 and dissolved in a small volume of ethanol. Distilled water containing 5% (v/v) polyoxyethylene sorbitan monopalmitate was then added to adjust the steroid solution to the desired concentration. For administration *in vivo*, 250 μ l of such a solution was injected into each rat.

For incubations *in vitro*, radioactive steroid in ethanol/benzene was dried under N_2 , dissolved in a small amount of ethanol, and diluted with Tris/EDTA buffer so that the final concentration was 100 pmol/40 μ l. This solution was used for incubation of both tissue minces and nuclear extracts.

Liquid-scintillation counting

Aqueous radioactive samples were counted in a diphenyloxazole/toluene solution containing 1 litre of toluene, 6g of 2,5-diphenyloxazole, 75 ml of water and 116g of Bio-Solv (BBS-3, Beckman Instruments, Fullerton, CA, U.S.A.).

The incorporation of radioactive androgen into prostatic cells is computed in terms of molar concentration as previously described (Bruchovsky *et al.*, 1975b). The binding of radioactive androgen is expressed in terms of radioactivity/ E_{260} unit or in terms of radioactivity/mg of total protein recovered from the columns. For purposes of calculation, it was determined that there are $1.88 \times 10^{-7} \pm 0.11 \times 10^{-7}$ (mean $\pm$ S.E.M., $n = 43$) E_{260} units/nucleus and 11×10^7 cells/g of tissue (Lesser & Bruchovsky, 1973; Bruchovsky *et al.*, 1975b).

Other analytical procedures

Protein was measured by differential u.v. absorption (Layne, 1957). Assay of receptor-inactivating factors was carried out by a tissue-mixing procedure (Bruchovsky & Craven, 1975).

Chemicals

Reagents used to prepare buffers and other solutions were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and were of the highest available

purity. All steroids were obtained from Steraloids, Pawling, NY, U.S.A. The histidine-reactive proteinase inhibitor 7-amino-1-chloro-3-L-tosylamidoheptan-2-one hydrochloride was supplied by Sigma. Deionized glass-distilled water was used in making up all solutions.

Results

Partial isolation and properties of nuclear receptor

Gel-exclusion chromatography of nuclear extract labelled with [1,2- 3 H]dihydrotestosterone yields a characteristic profile of which an example is shown in Fig. 1. The radioactivity is eluted in three peaks; the first, recovered in fractions 20–30, corresponds to the void volume and probably represents a fraction of chromatin-bound androgen that is not salt-extractable; the second, recovered in fractions 40–50, is the nuclear androgen-receptor complex; the third (not shown) is recovered in later fractions and represents free steroid. A number of properties of the nuclear androgen-receptor complex are known (King & Mainwaring, 1974) allowing for its precise identifica-

Fig. 1. Partial isolation of nuclear receptor

A sample of nuclear extract was prepared from prostates of non-orchidectomized rats as described by Bruchovsky *et al.* (1975b). The sample was incubated for 18 h at 20°C in the presence of [1,2- 3 H]dihydrotestosterone at a concentration of 20 nM. After incubation the sample was divided into two equal parts. One part was analysed for binding by Sephadex G-25/G-200 dual-column chromatography. The other was treated with dextran-coated charcoal, concentrated and analysed for binding by sucrose-density-gradient centrifugation (see inset). The column effluent was collected in 1.2 ml fractions. O, E_{260} ; ●, radioactivity recovered. Arrow shown in inset marks position of haemoglobin-sedimentation standard (4.3S).

tion. The receptor is characterized by a sedimentation coefficient of 3.3S (Fig. 1, inset), and displays a marked preference for dihydrotestosterone and testosterone relative to that for oestradiol-17 β , progesterone and cortisol. Maximal binding of dihydrotestosterone occurs between 9 h and 18 h of incubation at 20°C. When the concentration of dihydrotestosterone in the incubation mixture is gradually increased the binding of dihydrotestosterone to nuclear receptor reaches an apparent plateau at a concentration of 20 nM. Above this concentration there is a marked increase in non-specific binding to the receptor. A Scatchard plot of the nuclear binding data yields an association constant of $8.3 \times 10^7 \text{ M}^{-1}$, in accordance with the value published by Rennie & Bruchovsky (1972). The theoretical number of binding sites afforded by this analysis is 12000/nucleus, comparing moderately well with a value of 9000/nucleus obtained with the labelling procedure *in vivo* (Fig. 7).

In selecting the best conditions for the determina-

Fig. 2. Partial isolation of cytoplasmic receptor

Prostatic tissue was obtained from rats orchidectomized 24 h previously, minced and incubated for 2 min at 37°C in the presence of [1,2- ^3H]dihydrotestosterone at a concentration of 750 nM. The cytosol fraction was treated with $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation, and the protein precipitate was analysed for binding by Sephadex G-25/G-200 dual column chromatography and by sucrose-density-gradient centrifugation (see inset). The column effluent was collected in 1.4 ml fractions. The amount of radioactivity in the receptor peak was calculated by doubling the area under the most clearly defined half-portion of the peak, and was normalized by dividing the amount of radioactivity in each fraction by the total amount of protein recovered from the column. $\circ$, Protein; $\bullet$, radioactivity. Arrow shown in inset marks position of haemoglobin-sedimentation standard.

tion of nuclear receptors, practical considerations dictated that we adopt a one-point assay, a concentration of radioactive androgen of 20 nM being used. Since at this concentration 60% of the theoretical binding sites and 80% of the physiological binding sites are labelled, one obtained a result acceptably close to what may be the actual value, without encountering difficulties due to non-specific binding.

Partial isolation and properties of cytoplasmic receptor

The brief incubation of whole prostatic cells at 37°C with radioactive dihydrotestosterone (750 nM) results in the maximal labelling of a cytoplasmic androgen-binding protein identified by gel-exclusion chromatography (Fig. 2). Although more than one peak of radioactivity is sometimes observed, the major peak (Fig. 2, fractions 30–45) represents a specific form of androgen-receptor complex identical with the form labelled *in vivo*; however, the configuration of this molecule may differ from that of the receptor labelled by incubating isolated cytosol at 4°C with radioactive androgen (King & Mainwaring, 1974; Bruchovsky *et al.*, 1975b). The androgen-receptor complex depicted in Fig. 2 has a sedimentation coefficient of 4.4S in 600 mM-NaCl (Fig. 2, inset). In competition experiments, the binding of radioactive dihydrotestosterone is inhibited by a 100-fold excess of dihydrotestosterone, testosterone, progesterone and oestradiol-17 β , but a similar excess of cortisol is less effective. Other aspects of the biochemical properties and functional significance of this receptor are considered in reports by Rennie & Bruchovsky (1972, 1973) and Bruchovsky *et al.* (1975b).

Receptor flux in the regenerating prostate

Beginning 7 days after orchidectomy rats were treated with daily injections of dihydrotestosterone (400 $\mu\text{g}/100 \text{ g}$ body wt.) to stimulate DNA synthesis and cell proliferation and restore the prostate to its normal size. The receptor flux was observed over a 14-day period of growth, and the results are presented in Fig. 3(a). Within 12 h of the first injection, the number of molecules of nuclear receptor (open circles) increases from 0 to 5000/cell and continues to increase to a maximum of 12000/cell at 2 days. This value is maintained for the next 3 days and then falls to 9000/cell by day 7 of treatment. There is a further gradual decline to about 8000/cell over the remaining period of 7 days.

During the same time-course of tissue regeneration, the apparent number of cytoplasmic receptors (closed circles) remains constant at 1000/cell. It should be stressed that this result is probably an overestimate of the number of cytoplasmic receptors owing to our method of calculation. Baseline radioactivity was included even when the chromatogram

Fig. 3. Receptor flux in the regenerating prostate

Groups of 3–12 rats, orchidectomized 7 days previously, were treated with daily subcutaneous injections of dihydrotestosterone ($400 \mu\text{g}/100 \text{g body wt.}$). Prostatic tissue was recovered at the times shown and analysed for the presence of cytoplasmic and nuclear receptors by the methods described in the legends to Figs. 1 and 2. In calculating the number of molecules of receptor it was assumed that there is only one androgen-binding site per receptor. (a) Concentration of nuclear receptor ($\circ$) and of cytoplasmic receptor ($\bullet$) expressed as molecules/cell; (b) concentration of nuclear receptor ($\circ$) expressed as molecules/prostate. Broken line in (a) indicates rate of DNA synthesis and in (b) indicates the number of cells in the regenerating prostate, as replotted from the data of Lesser & Bruchovsky (1973).

suggested that no receptor peak was present. In essence, the lower limit of sensitivity of the assay for cytoplasmic receptor is about 1000 molecules/cell or approx. 3000 d.p.m./mg of protein, but above this limit the characteristic receptor peak, if present, is well-defined.

The results in Fig. 3(a) show that the flux in the amount of nuclear receptor is not in phase with the changes in rate of DNA synthesis (broken line). DNA synthesis is initiated 12 h after the number of nuclear receptors at 9000/cell is almost at a maximum. More significantly, the negative-feedback effect on DNA synthesis occurring between days 3 and 4 of treatment happens in the presence of a constant amount of

nuclear receptor. These two observations indicate that any effect of nuclear receptor on DNA synthesis must be indirect, and that other factors besides nuclear receptor are probably responsible for switching DNA synthesis on and off.

The decline in the number of nuclear receptors between days 5 and 14 of treatment parallels the decline in the rate of DNA synthesis, probably because the nuclear receptor is synthesized in dividing cells. Between days 2 and 5 of treatment, 50–60% of the total cell population of the prostate is progressing through the cell cycle at all times (Lesser & Bruchovsky, 1974b). In preparing for division, these cells double their complement of nuclear receptor, giving rise to the marked elevation in the average number from 8000/cell to 12000/cell. It is evident that the number of receptors from the second day of regeneration onwards is approximated by the relationship

$$N_t = N_s + G.F. (N_s)$$

where N_t is the number of nuclear receptors in the regenerating cell, N_s is the number of nuclear receptors in the non-dividing cell after replication and G.F. is the growth fraction.

The dependence of receptor synthesis *de novo* on cell proliferation is demonstrated further by the results shown in Fig. 3(b). The product of the number of receptors/nucleus (Fig. 3a) and the number of cells/prostate (Fig. 3b, broken line) yields the number of nuclear receptors/prostate (Fig. 3b, open circles). Comparison of the curves in Fig. 3(b) reveals a distinct parallelism between the number of nuclear receptors/prostate and the number of cells/prostate. On day 5 of treatment when cell proliferation is curtailed by the negative-feedback effect, there is no further net synthesis of nuclear receptor. It is notable that the decrease in the number of nuclear receptors/cell between days 5 and 14 of treatment is offset by a slight increase in the number of cells/prostate, allowing the number of nuclear receptors/prostate to remain constant. This suggests that the prostate contains a small population of cells which is slow to complete mitosis in spite of a double complement of nuclear receptor.

Perhaps the most unexpected feature of these data is the lack of coherence between the synthesis of nuclear receptor and the period of cell differentiation between days 5 and 14 of treatment when protein synthesis is still stimulated by androgens (Lesser & Bruchovsky, 1973).

Effect of dose of dihydrotestosterone on the formation of nuclear receptor in regenerating prostate

Since the rate of DNA synthesis in the regenerating prostate is proportional to the number of cells entering the S phase (Lesser & Bruchovsky, 1973), a correlation between the rate of DNA synthesis and

Fig. 4. Effect of dose of dihydrotestosterone on the formation of nuclear receptor in regenerating prostate

Groups of 11–13 rats, orchidectomized 7 days previously, were treated with daily subcutaneous injections of various doses of dihydrotestosterone ranging from 0 to 500 µg/100g body wt. On day 3 after the onset of therapy, the rats were killed and the prostatic tissue was analysed for the concentration of nuclear receptor (○, expressed as molecules/cell). ----, Rate of DNA synthesis as replotted from the data of Lesser & Bruchovsky (1973).

Fig. 5. Receptor flux in the involuting prostate

Prostatic tissue was recovered from orchidectomized rats at intervals after surgery, and analysed for the presence of cytoplasmic and nuclear receptors as described in the legends to Figs 1 and 2. Concentration of nuclear receptor (○) and of cytoplasmic receptor (●) expressed as molecules/cell. ----, Number of cells in the involuting prostate as replotted from the data of Lesser & Bruchovsky (1973).

the formation of nuclear receptor would affirm that nuclear receptor is produced as cells enter the cell cycle. The effect of increasing doses of dihydrotestosterone on the rate of DNA synthesis and on nuclear-receptor formation in prostates of orchi-

dectomized rats is presented in Fig. 4. Both parameters increase as a function of dose of dihydrotestosterone and maximal stimulation in each case is obtained at a dose of about 400 µg/100g body wt. Therefore these results indicate that the synthesis of nuclear receptor is indeed coupled to cell proliferation.

Receptor flux in the involuting prostate

One of the first effects of lowering the concentration of androgens in the adult male is a rapid decline in the basal rate of DNA synthesis to 25% of normal within 24h (Lesser & Bruchovsky, 1973). In contrast with the rapid effects on DNA synthesis, both prostatic weight and the number of cells in the prostate (Fig. 5, broken line) do not fall below normal until day 4 after orchidectomy, declining to about 30 and 15% of normal respectively by day 7.

As shown in Fig. 5 (open circles), the number of nuclear receptors decreases from a value of 7000/cell to 500/cell within 24h after orchidectomy. No nuclear receptor is detected by day 7. Initially, the number of cytoplasmic receptors (closed circles) increases from less than 1000/cell in the normal animal to a maximum of 14000/cell at 16h. Following this increase, the number of cytoplasmic receptors falls rapidly, reaching the threshold value of 1000/cell by day 5. Thus the regression of the prostate is preceded by a marked decrease in the number of both nuclear and cytoplasmic receptors.

Receptor-inactivating factors

In an attempt to explain the disappearance of cytoplasmic receptor after orchidectomy, and its apparent absence from regenerating prostate, we examined the possibility that binding to this molecule is abolished by factors produced by the cell. Experiments were performed in which tissue samples were prepared from prostate of rats orchidectomized 24h previously and minced with an equal weight of prostate being tested for the presence of inactivating agents. The results of these experiments are listed in Table 1. It is clear that involuting tissue acquires the capacity to eradicate binding to receptor on day 3 after orchidectomy just before the autophagic response is manifested (Fig. 5). The agents responsible continue to be highly active during the 7 days after orchidectomy. Daily treatment of animals, orchidectomized 7 days previously, with dihydrotestosterone (400 µg/100g body wt.) produces a steady decrease in activity beginning on day 1 of treatment; however, the destructive agents are still active even after 14 days of treatment. The addition of a proteinase inhibitor, 7-amino-1-chloro-3-L-tosyl-amidoheptan-2-one hydrochloride to the tissue-incubation mixture has no effect in preventing the loss of binding activity.

Three conclusions can be drawn from the above

Table 1. *Eradication of the binding of [1,2-³H]dihydrotestosterone to cytoplasmic receptor by factors emanating from the cell*

Tissue samples were prepared from 500mg of prostate of rats orchidectomized 24h previously and 500mg of the prostate being tested for the presence of receptor-modifying factors. The combined sample was minced and incubated for 2 min at 37°C in the presence of [1,2-³H]dihydrotestosterone at a concentration of 750 nM. The incubation was stopped by the addition of 5 vol. of ice-cold tissue-culture medium, and the tissue was washed three times to remove unincorporated radioactivity. Cytosol protein was recovered as described by Bruchovsky *et al.* (1975b) and the amount of binding in the expected position of the receptor peak was determined by Sephadex G-25/G-200 dual-column chromatography. Results were normalized by dividing the amount of radioactivity in the receptor peak by the total amount of protein recovered from the columns. The decrease in the amount of binding is measured relative to the amount of binding found in prostates of 1-day-orchidectomized animals, and is expressed in terms of percentage of receptor inactivated. Where indicated, 7-amino-1-chloro-3-L-tosyl-amidoheptan-2-one hydrochloride (TLCK) was added at a concentration of 1 mM.

Interval of experiment (days)		Radioactivity in area of receptor peak (d.p.m./mg of protein)			Receptor inactivated* (%)
Orchidectomy	Treatment	Actual†	Expected‡	Found	
1		14600	14600	14600	0
3		5500	10100	2100	79
5		2700	8700	3100	64
7		3400	9000	2400	73
7	1	2600	8600	3600	58
7	7	1500	8100	4200	48
7	14	2300	8500	6900	18
7	7(+TLCK)	1500	8100	3300	59
Normal control		2000	8300	8700	0

* Calculated from relationship $[(\text{Expected} - \text{Found})/\text{Expected}] \times 100$.

† Radioactivity recovered in assays described in Figs. 3(a) and 4, expressed in d.p.m./mg of protein.

‡ Calculated from relationship $(\text{Actual} + 14600)/2$.

observations. First, the failure to detect cytoplasmic receptor in the regenerating prostate is not entirely due to the occupancy of receptor sites by the exogenous androgen used in treating the animals. Secondly, even though the regenerating prostate grows to a normal size in terms of cell number after 7 days of treatment, the tissue does not simultaneously revert to its pre-castration state. The complete suppression of receptor-inactivating factors appears to be a trait of differentiation acquired at a much later stage in the maturation of the prostatic cell. Thirdly, although intense proteolytic activity has been noted in the regressing prostate (Paris *et al.*, 1972; Helminen *et al.*, 1972; Dahl & Kjaerheim, 1973), the failure of 7-amino-1-chloro-3-L-tosylamidoheptan-2-one hydrochloride to prevent the disappearance of receptor suggests that the mechanism of receptor inactivation is not necessarily dependent on proteolysis. Further testing with other proteinase inhibitors would be essential to confirm this point.

Unmasking of receptor binding sites

The results in the previous section indicate that androgen binding to receptor sites can be destroyed by factors released from the androgen-stimulated cell. Therefore the failure to detect receptor during

certain phases of growth is not explained entirely by the occupancy of binding sites by intracellular androgen. However, since the normal prostate is free of inactivating factors, the pronounced increase in cytoplasmic receptor concentration after orchidectomy (Fig. 5) could be attributed to effects other than the unmasking of receptor binding sites, such as the discharge of nuclear receptor into the cytoplasm, or the formation of new receptor.

The possibility that receptors are unmasked was examined by measuring cytoplasmic receptor concentration after administering a single dose of dihydrotestosterone (400 µg/100 g body wt.) to animals orchidectomized 7 days previously. As shown in Table 2, an unequivocal receptor peak appears only on day 10 after the injection. The late appearance of the peak suggests that receptor-binding sites do not become accessible simply through the loss of endogenous androgen.

Under the conditions of this experiment, androgen-withdrawal effects are first noticeable at 7–10 days after a single dose of dihydrotestosterone (Lesser & Bruchovsky, 1973). Since the appearance of cytoplasmic receptor occurs within the interval of these early autophagic changes, its appearance may be elicited strictly by castration or an equivalent manipulation.

Table 2. *Appearance of cytoplasmic receptor after a single treatment of orchidectomized rats with dihydrotestosterone*

Rats orchidectomized 7 days previously were treated with a single subcutaneous injection of dihydrotestosterone (400 μ g/100 g body wt.). At the times shown groups of 3–12 rats were killed and prostates removed. Minces of tissue were prepared and incubated for 2 min at 37°C in the presence of [1,2- 3 H]dihydrotestosterone at a concentration of 750 nM. Cytosol protein was recovered as described by Bruchovsky *et al.* (1975b) and analysed for binding by Sephadex G-25/G-200 dual-column chromatography. Results were normalized as described in the legend to Table 1.

Time after injection (days)	Radioactivity in area of receptor peak (d.p.m./mg of protein)	Receptor peak well-defined?
1	3500	—
2	1700	—
5	2300	—
10	7500	+
14	2700	—
Untreated control	3400	—

Efflux of androgens and of androgen receptors from the nucleus

To obtain greater insight into the events leading up to the autophagic response, we investigated the process of hormone release from the nucleus. The concentration of androgens was adjusted to a physiological value of 250 nM (Bruchovsky *et al.*, 1975b) by injecting animals orchidectomized 24 h previously with a suitable dose of androgen. As shown in Fig. 6(a) this concentration is very closely approximated to 1 h after the intravenous administration of a dose of [1,2- 3 H]testosterone (40 Ci/mmol) of 300 μ Ci or greater. Such high doses also assure that the concentration of active androgens in the cytoplasm is within the physiological range of 5–20 nM. The subsequent decrease in the concentration of radioactive androgens in the cytoplasm and nuclear compartments is shown in Fig. 6(b). Within 12 h the cytoplasmic concentration of androgens (open circles) is decreased by 95% from an initial value of 50 to 5 nM, but thereafter no obvious change is observed. By comparison the nuclear concentration of androgens is decreased by 75%, from an initial value of 225 to 60 nM within the first 8 h and by another 20%, to 10 nM, during the subsequent 16 h. To clarify the reason for the biphasic release of androgens from the nucleus, we measured the disappearance of radioactive androgens from the three principal nuclear fractions which are separated by gel-exclusion chromatography (Fig. 7a, inset). Peak I represents binding of androgens to a high-molecular-weight component of the nucleus, presumed to be chromatin,

Fig. 6. *Efflux of androgens and of androgen receptors from the nucleus*

Groups of two or three male rats were injected 24 h after orchidectomy with doses of [1,2- 3 H]testosterone (40 Ci/mmol) ranging from 25 to 500 μ Ci. In the experiment on androgen incorporation (a), the rats were killed 1 h after injection. In the experiment on androgen efflux (b), the rats were injected with a dose of 500 μ Ci of [1,2- 3 H]testosterone only and killed at intervals lasting from 1 to 24 h. In both experiments, appropriate cytoplasmic and nuclear fractions were prepared and assayed for radioactivity. (a) Incorporation of radioactivity into cytoplasm and nuclei; (b) efflux of radioactivity from cytoplasm and nuclei. Radioactivity recovered: cytoplasm, $\circ$; nuclei, $\bullet$.

peak II represents binding to nuclear receptor and peak III represents free androgen, which originates from a pool of free androgen as such, or by dissociation from low-affinity-binding sites (Rennie & Bruchovsky, 1973). The results in Fig. 7 suggest that the rate of loss of androgen from both the chromatin fraction (Fig. 7a) and the receptor fraction (Fig. 7b) is lower than the rate of loss from the free fraction (Fig. 7c). This difference probably accounts for the biphasic kinetics of androgen efflux from the nucleus described in Fig. 6(b).

We conclude that the effects of androgen depriva-

Fig. 7. Loss of androgens from intranuclear fractions

Groups of two or three male rats were injected 24h after orchidectomy with a dose ($500 \mu\text{Ci/animal}$) of $[1,2\text{-}^3\text{H}]\text{-testosterone}$ (40Ci/mmol). At intervals of 1–24h after injection, the rats were killed and nuclear extracts were prepared from the prostatic tissue recovered. The extracts were analysed by Sephadex G-25/G-200 dual-column chromatography. Fractions (1.4ml) were collected and assayed for radioactivity. The results shown in (a) inset, indicate that the radioactivity is distributed among three peaks identified by the symbols I, II and III (see text for details). The rates of loss of radioactivity from peaks I, II and III are illustrated in (a), (b) and (c) respectively.

tion are first reflected in a rapid decline in the concentration of free androgens within the nucleus followed by a slower decline in the concentration of androgens that are bound both to chromatin and to receptor.

Scarcity of receptor-inactivating factors in the nucleus

The disappearance of receptor from the nucleus described in Fig. 5 and Fig. 7(b) presumably results either from its discharge into the cytoplasm or from its inactivation within the nucleus. To check the latter, various nuclear extracts, alone and in combination, were tested for androgen-binding activity as shown in Table 3. Since a mixture of nuclear extract from non-orchidectomized and orchidectomized animals yields the expected amount of androgen binding, nuclear extract from prostate of orchidectomized animals does not contain receptor-inactivating factors. Consequently, the most plausible explanation for the disappearance of nuclear receptor is that the receptor is released into the cytoplasm.

Discussion

In the regenerating prostate, the appearance of receptors in the nucleus precedes the initiation of DNA synthesis and cell proliferation by several hours (Fig. 3a). Because cytoplasmic receptor is undetectable, the appearance of nuclear receptor can be accounted for by the synthesis *de novo* of receptor protein, as demonstrated by two lines of evidence. First, the average number of nuclear receptors is proportional to the growth fraction in the regenerating prostate, signifying that nuclear receptor is synthesized during the proliferative response at the beginning of the G1 phase of the cell cycle; this deduction is consistent with the observed lag period between the appearance of receptor in the nucleus and the onset of DNA synthesis. Secondly, since the number of nuclear receptors/prostate parallels the number of cells/prostate (Fig. 3b), the synthesis of nuclear receptor must proceed during cell proliferation. This conclusion is also confirmed by the finding that the synthesis of nuclear receptor and the rate of DNA synthesis in regenerating prostate are both dependent on dose of dihydrotestosterone (Fig. 4). Under these conditions of cell replication, the cytoplasmic synthesis of nuclear receptor is not in synchrony with either the onset or the shutdown of DNA synthesis (Fig. 3a). Hence it is extremely unlikely that nuclear receptor contributes directly to the initiation of DNA synthesis or to the negative-feedback effect. A more general correlation between receptor synthesis and cell proliferation is nevertheless obvious, and explains our failure to detect any net synthesis of nuclear receptor in resting cells (Fig. 3b). Once cell division stops there is no net synthesis of nuclear receptor, despite androgen stimula-

Table 3. *Scarcity of receptor-inactivating factors in the nucleus*

Nuclear extracts were prepared from prostates of non-orchidectomized animals and from those of animals orchidectomized 24 h previously. The extracts alone and in combination were incubated for 2 min at 37°C in a final volume of 2 ml, and then for 18 h at 20°C in the presence of [1,2-³H]dihydrotestosterone at a concentration of 20 nM. The extracts were incubated for 2 min at 37°C to simulate the conditions of incubation for detecting the cytoplasmic receptor in the event that a more physiological temperature is required for the action of receptor-inactivating factors. After incubation, the extracts were analysed for binding by Sephadex G-25/G-200 dual-column chromatography. Results were normalized by dividing the amount of radioactivity in the receptor peak by the total number of E_{260} units recovered from the column. The expected amount of binding is the mean of the amounts of radioactivity found in the samples from the non-orchidectomized and orchidectomized animals.

Source of nuclei	Radioactivity in receptor peak (d.p.m./ E_{260} unit)	
	Found	Expected
Non-orchidectomized animals	10500	—
Orchidectomized animals	900	—
Non-orchidectomized + orchidectomized animals	5300	5700

tion of protein synthesis during cell differentiation.

The synthesis of nuclear receptor at a very early stage in G1 phase implies that the entry of cells into the cell cycle is dependent on receptor synthesis. Cessation of synthesis and the subsequent decrease in the number of cells entering S phase would account for the negative-feedback effect on DNA synthesis shown in Fig. 3(a).

The withdrawal of androgens is followed by a chain of events ultimately resulting in autophagia. The first step in this chain is a rapid efflux of free androgen from the nucleus followed by a slower efflux of androgen receptors (Figs. 5 and 7). In the absence of receptor-inactivating factors within the nucleus, the loss of androgen receptors from the nucleus should be attended by an increase in the concentration of such receptors in the cytoplasm. We estimate that the actual number of receptors discharged from the nucleus immediately after orchidectomy is 9000–12000/cell (Figs. 5 and 7b). This number agrees with the observed concomitant increase of 10000–14000 receptors/cell in the cytoplasm (Fig. 5). Since the cytoplasmic receptor is characterized by physical properties and binding affinities which are different from those of the nuclear receptor, it appears that the nuclear receptor (distribution coefficient 0.5–0.7, sedimentation coefficient 3.3S) is transformed into the larger cytoplasmic receptor (distribution coefficient 0.2–0.4, sedimentation coefficient 4.4S) in leaving the nucleus; during efflux its reactivity appears to be changed as well.

Between the days 1 and 5 after orchidectomy, the decrease in the amount of cytoplasmic receptor (Fig. 5) is presumably a result of protein degradation. Not only does the initial decline in the concentration of cytoplasmic receptor foreshadow the onset of prostatic involution 2 days later, but also the near disappearance of cytoplasmic receptor on day 5 roughly coincides with the termination of the acute

phase of the autophagic response. These findings suggest that prostatic involution is influenced by the concentration of cytoplasmic receptor.

The eradication of the binding of dihydrotestosterone to cytoplasmic receptor in the regressing prostate is logically attributed to the action of proteolytic enzymes. However, our evidence is not complete enough to allow us to decide whether the agents that we have termed 'receptor-inactivating factors' cause the destruction of cytoplasmic receptor or merely prevent its formation. It is difficult to rationalize the necessity for the regenerating prostate to retain the capacity to eliminate cytoplasmic receptor, but such capacity is indeed preserved for a number of days (Table 1). However, if the formation of cytoplasmic receptor triggers autophagia, the cell must prevent the transformation of nuclear receptor into the cytoplasmic form. The need for resisting transformation would be most acute during the period when nuclear receptor is synthesized in the cytoplasm, and during mitosis when breakdown of the nuclear membrane exposes the nuclear receptor to cytoplasm. Once cell proliferation is completed and there is no more net synthesis of nuclear receptor, the requirement for inactivating factors would expire. Such a mechanism is consistent with the results presented in Table 1.

In summary, the evidence described in the present paper suggests that the synthesis of nuclear receptor at the beginning of G1 phase controls the entry of cells into the cell cycle and that the retention of androgen receptor in the nucleus stabilizes the cell against the onset of autophagia. Autophagia begins after nuclear receptor is apparently released into the cytoplasm where it is transformed into a configuration susceptible to elimination. Whether the transformed receptor can be recycled before being irreversibly degraded remains uncertain. However, the existence of a recycling mechanism has been predicted

(see review by Liao, 1975) and would be advantageous to the survival of the cell.

These studies were supported by grants from the Medical Research Council of Canada (M.T. 3729) and the National Cancer Institute of Canada. E. V. D. is a recipient of a Medical Research Council of Canada Fellowship.

References

- Bruchovsky, N. (1971) *Endocrinology* **89**, 1212-1222
- Bruchovsky, N. & Craven, S. (1975) *Biochem. Biophys. Res. Commun.* **62**, 837-843
- Bruchovsky, N. & Lesser, B. (1976) *Adv. Sex Horm. Res.* **2**, 1-55
- Bruchovsky, N., Lesser, B., Van Doorn, E. & Craven, S. (1975a) *Vitam. Horm. (N.Y.)* **33**, 61-102
- Bruchovsky, N., Rennie, P. & Vanson, A. (1975b) *Biochim. Biophys. Acta* **394**, 248-266
- Coffey, D. S. (1974) in *Structure and Function of Male Accessory Sex Organs* (Brandes, D., ed.), pp. 307-328, Academic Press, New York
- Dahl, E. & Kjaerheim, A. (1973) *Z. Zellforsch. Mikrosk. Anat.* **144**, 167-178
- Davies, P. & Griffiths, K. (1973) *Biochem. J.* **136**, 611-622
- Davies, P. & Griffiths, K. (1974) *J. Endocrinol.* **62**, 385-400
- Davies, P. & Griffiths, K. (1975) *Mol. Cell. Endocrinol.* **3**, 143-164
- Helminen, H. J., Ericsson, J. L. E. & Arborgh, B. (1972) *Acta Endocrinol.* **69**, 747-761
- Hu, A.-L., Loor, R. M. & Wang, T. Y. (1975) *Biochem. Biophys. Res. Commun.* **65**, 1327-1333
- Ichii, S., Izawa, M. & Murakami, N. (1974) *Endocrinol. Jpn.* **21**, 267-274
- Katsumata, M. & Goldman, A. S. (1974) *Biochim. Biophys. Acta* **359**, 112-129
- King, R. J. B. & Mainwaring, W. I. P. (1974) *Steroid-Cell Interactions*, Butterworths, London
- Layne, E. (1957) *Methods Enzymol.* **3**, 447-454
- Lesser, B. & Bruchovsky, N. (1973) *Biochim. Biophys. Acta* **308**, 426-437
- Lesser, B. & Bruchovsky, N. (1974a) *Biochem. J.* **142**, 429-431
- Lesser, B. & Bruchovsky, N. (1974b) *Biochem. J.* **142**, 483-489
- Liang, T. & Liao, S. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 706-709
- Liao, S. (1975) *Int. Rev. Cytol.* **41**, 87-172
- Liao, S. & Liang, T. (1974) in *Hormones and Cancer* (McKerns, K. W., ed.), pp. 229-260, Academic Press, New York
- Mainwaring, W. I. P. & Jones, D. M. (1975) *J. Steroid Biochem.* **6**, 475-481
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372-1379
- Paris, J. E., Brandes, D., Anton, E. B. & Barnard, S. (1972) *J. Natl. Cancer Inst.* **49**, 1685-1696
- Rennie, P. & Bruchovsky, N. (1972) *J. Biol. Chem.* **247**, 1546-1554
- Rennie, P. & Bruchovsky, N. (1973) *J. Biol. Chem.* **248**, 3288-3297
- Rennie, P. S., Symes, E. K. & Mainwaring, W. I. P. (1975) *Biochem. J.* **152**, 1-16
- Szalay, R., Kreig, M., Schmidt, H. & Voigt, K. D. (1975) *Acta Endocrinol.* **80**, 592-602