Evidence for the Presence of Androgen Receptors in Purified Rat Leydig Cells

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Leydig cells, purified on two sequential Percoll gradients to purities of 89 ± 1%, were used to study the binding of 17-β-hydroxy-17α-methyl-estra-4,9,11-triene-3-one(^{3}H-R1881). The accumulation of ^{3}H-R1881 in the nuclear fraction of these cells was time- and temperature-dependent. Specific binding was saturable with an apparent K_a of 0.14 nM^{-1} and a single class of binding sites at a concentration of 721 fmol/mg DNA. A fraction of the bound radioactivity in the nuclear pellet could be extracted with 0.4 M KCl, and a portion of this extracted steroid was associated with macromolecular species. The nuclear accumulation was androgen-specific. These data are consistent with the presence of androgen receptors in rat Leydig cells.

Key Words: Leydig cells, Percoll gradient, androgen receptors, testosterone.

While there is some evidence for the existence of androgen receptors in Leydig cells, it is mostly indirect. Autoradiographic evidence for nuclear localization of androgen in interstitial cells of testes from hypophysectomized rats injected intravenously with ^{3}H-testosterone has been presented (Sar et al, 1975). Under similar conditions testicular fractions containing interstitial cells plus tubules accumulated more label than tubules alone (Wilson and Smith, 1975). In addition, Verhoeven (1980) presented evidence that cultures of interstitial cells from immature rat testes can accumulate ^{3}H-androgen. However, these studies did not demonstrate that Leydig cells were, in fact, the cells binding the androgens, nor did they rule out the possibility that the binding could be attributed to the presence of enzymes rather than receptors. The demonstration of specific androgen receptors in Leydig cells is of particular interest in light of recent studies suggesting that testicular androgen production may be locally autoregulated via ultrashort-loop negative-feedback mechanisms (Purvis and Hansson, 1978; Adashi and Hsueh, 1981; Darney and Ewing, 1981). In the present study evidence is presented that purified Leydig cells bind androgens in a manner that is consistent with the presence of androgen receptors.

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Materials and Methods

Materials

^{3}H-Testosterone (98.8 Ci/mmol) and ^{3}H-R1881 (17-β-hydroxy-17α-methyl-estra-4,9,11-triene-3-one; 87 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Medium 199 and Eagle's minimal essential medium (MEM) plus nonessential amino acids supplemented with penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) (MEMC) were purchased from GIBCO (Grand Island, NY). The hCG used was the WHO Second International Standard (Holly Hill, Hampstead, London), and Percoll was obtained from Pharmacia (Piscataway, NJ).

Preparation of Leydig Cells

Crude interstitial cells were isolated from the decapsulated testes of adult Sprague-Dawley rats (250–275 g; TIMCO, Houston, TX) by treatment with collagenase (Worthington CLS Type 1), as described by Grotjan and Steinberger (1978). Dispersed cells were further purified

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by centrifugation on continuous Percoll gradients by a modification of the method described by Browning et al (1981). Testicular cells (10^8/2 testes) were suspended in 2 ml of Medium 199 + 0.1% bovine serum albumin (M199 + BSA). This suspension was applied to a 35-ml continuous Percoll gradient and centrifuged at 800 × g for 30 minutes at 4 C in a SA-600 Sorvall rotor. The top 25-ml fraction was removed by aspiration, and the next 6 ml (26–31 ml) were collected, diluted with three parts of M199 + BSA, and centrifuged (150 × g, 15 minutes). The cells from three gradients were resuspended in 2 ml M199 + BSA, layered on a second 35-ml Percoll gradient, and centrifuged at 800 × g for 20 minutes at 4 C. The 26–31-ml fractions were collected, diluted, and centrifuged as described above. The cells were resuspended in MEMC and counted in a hemocytometer. The percentage of Leydig cells was determined by histochemical staining for 3-β-hydroxysteroid dehydrogenase (3β-HSD) (Browning et al, 1981). Under the staining conditions used, there was less than 5% positive staining without steroid substrate. Viability of cells was established by Trypan blue dye exclusion.

Response of Leydig Cells to hCG

Leydig cells were incubated for 3 hours at 34 C in MEMC plus hCG under 95% O_2, 5% CO_2 as indicated. Leydig cell homogenates and incubation media were assayed for testosterone by radioimmunoassay (Tcholakian et al, 1980).

Nuclear Accumulation of ^{3}H-Androgen

Dispersed Leydig cells (0.6–1.0 × 10^6 cells), purified on two Percoll gradients, were washed with MEMC and incubated with ^{3}H-R1881 ± 200-fold molar excess of unlabeled steroid in 0.5 ml MEMC at 34 C for the intervals indicated. All subsequent operations were performed at 0 C. The cells were collected by centrifugation and washed once with MEMC (150 × g, 5 minutes). Following homogenization with a Teflon–glass homogenizer in 2 ml buffer A (0.32 M sucrose, 5 mM $MgCl_2$, 12 mM thioglycerol, 10 mM Tris HCl, pH 7.4), the homogenates were centrifuged (3000 × g, 10 minutes), and the resulting pellets were washed once with buffer A plus 0.3% Triton X-100 and twice with buffer A. In some studies the pellets were extracted with 2 ml ethanol; the ethanol extracts were evaporated and counted in toluene-based fluor at 46% efficiency, and the pellets were used for the determination of DNA (Burton, 1968).

In other studies the washed pellets were extracted with buffer B (0.4 M KCl, 1 mM EDTA, 0.01 M Tris HCl, pH 7.4; 0.25 ml/10^6 cells) at 4 C for 1.5 hours. The suspensions were centrifuged (20,000 × g, 5 minutes), and aliquots from the supernatants were counted to assess extracted radioactivity. The remaining radioactivity in the pellet was extracted with ethanol as described above. When KCl extracts were chromatographed on Sephadex G25 or G200, 0.4 M KCl was included in the column buffer (Sanborn et al, 1979).

Data are presented as mean ± SE, and n values refer to separate experiments unless otherwise indicated.

Results

Purification of Leydig Cells

After one Percoll gradient centrifugation, the purity of the Leydig cell fraction as judged by staining for 3β-hydroxysteroid dehydrogenase was 62 ± 3% (n = 8), and the yield was 4.0 ± 0.6 × 10^5 Leydig cells per testis (n = 5). Spermatozoa accounted for 5–10% of the cells, and other germinal cells constituted the rest of the fraction. Further purification on a second gradient gave a purity of 89 ± 1% Leydig cells (n = 6) (Fig. 1), with no spermatozoa and only a small decrease in yield (3.1 ± 0.6 × 10^5 Leydig cells per testis, n = 5). Leydig cells isolated by centrifugation through two Percoll gradients responded to stimulation by hCG in a dose-dependent manner, ie, 1.5, 11, and 16 ng testosterone produced/ml/3 hours (mean of duplicates) in response to incubation with 0, 0.1, and 10.0 ng/ml hCG, respectively.

Specific Nuclear Binding of ^{3}H-R1881 in Purified Dispersed Leydig Cells

In preliminary studies the authors attempted to obtain Leydig cells depleted of endogenous androgens by placing the partially purified cell fraction after one Percoll gradient in culture in MEMC in the absence of serum. However, after 120 hours in culture, the Leydig cells still exhibited a basal testosterone production rate of 1.9 ng/10^6 cells/3 hours compared with a rate of 7 ng/10^6 cells/3 hours when freshly isolated (mean of duplicates). This basal testosterone production would have resulted in steroid levels high enough to saturate a classic androgen receptor with a dissociation constant (K_d) in the nM range and presumably to promote translocation of putative receptors to the nucleus. Consequently, the culture system offered no advantage over the freshly isolated preparations, which were used in the subsequent studies.

The accumulation of ^{3}H-R1881 in the nuclear fraction of purified dispersed Leydig cells was time-dependent, reaching a maximum at 60 minutes at 34 C and declining after 75 minutes (Fig. 2). Nuclear accumulation was also temperature-dependent; at 0 C binding was only 21 and 9% of the values at 34 C after 20 and 60 minutes, respectively.

When purified Leydig cells were incubated at 34 C for 60 minutes with increasing concentrations (0.48–15 nM) of ^{3}H-R1881 ± 200-fold molar excess

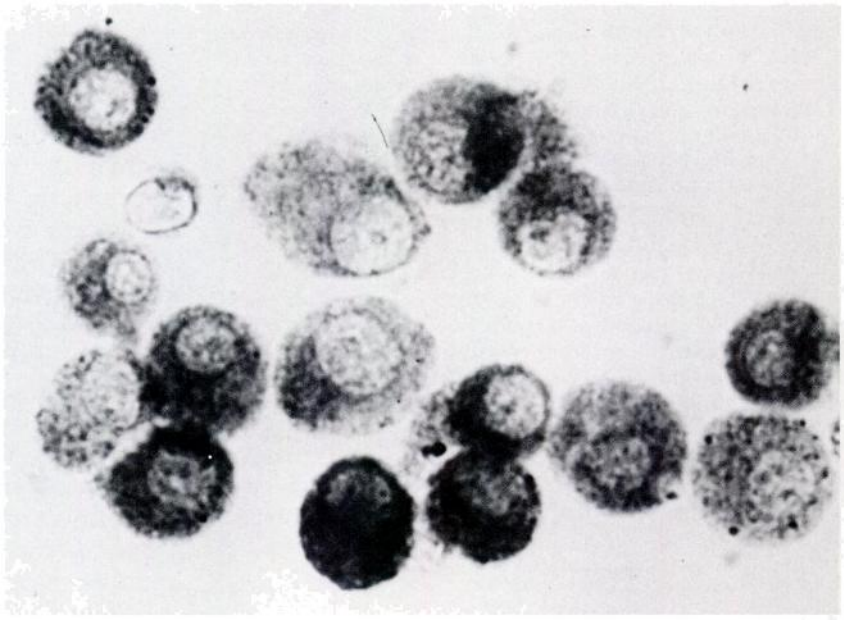

Fig. 1. Light micrograph of rat Leydig cells after purification on two Percoll gradients. Leydig cells stain positive with 3β-hydroxysteroid dehydrogenase (3β-HSD). Note heavy deposits of formazan in cytoplasm and differential degree of staining in individual cells (original magnification ×640).

of unlabeled steroid, the specific nuclear uptake of label was found to be saturable (Fig. 3). A Scatchard plot of the binding data revealed a single class of high-affinity binding sites, with an apparent association constant (K_a) of 0.14 nM^{-1}. The concentration of binding sites was estimated to be 721 fmol/mg DNA by extrapolation. Specific binding ranged from 63 to 81% of total binding over the concentration range employed. Endogenous testosterone levels were determined to be 0.34 ± 0.17 ng/10^6 cells (n = 3).

A fraction (62 ± 19%, n = 3) of the radioactivity bound to the washed nuclear pellet was extracted with 0.4 M KCl within 1.5 hours. Approximately 22 and 26% of this material was associated with macromolecules as judged from Sephadex and

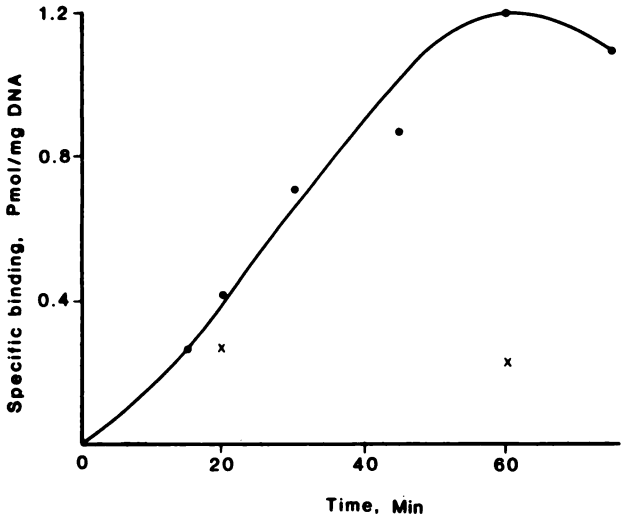

Fig. 2. Time course of specific nuclear accumulation of 16 nM R1881 ± 200-fold molar excess of unlabeled R1881 in purified rat Leydig cells at 34 C (●) and 4 C (×).

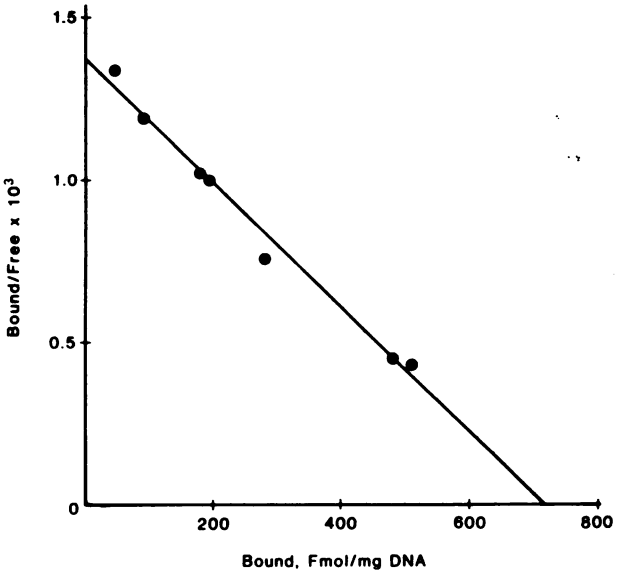

Fig. 3. Scatchard plot of specific nuclear-bound ^{3}H-R1881 in purified rat Leydig cells following incubation for 1 hour at 34 C with 0.4–15 nM ^{3}H-R1881 ± 200-fold excess of unlabeled hormone.

TABLE 1. Competition for Nuclear Accumulation of ^{3}H-R1881 by Unlabeled Steroids

Steroid	Relative Competitive Ability
R1881	1.000
Testosterone	0.128
Diethylstilbestrol	0.000
Triamcinolone acetonide	0.002

Leydig cells were purified on two Percoll gradients. The cells were incubated at 34 C for 1 hour with 10 nM ^{3}H-R1881 (alone) or 10 nM ^{3}H-R1881 plus 4- to 300-fold molar excess of the steroids listed. Relative competitive ability was calculated as the ratio of the concentrations of added steroids to the concentration of R1881 required to achieve a 50% decrease in bound ^{3}H-R1881.

G200 chromatography, respectively, in two separate experiments.

The nuclear accumulation of ^{3}H-R1881 was shown to be steroid-specific in competition experiments (Table 1). R1881 was a more effective competitor than testosterone. Diethylstilbestrol and triamcinolone acetonide, which bind with high affinity to estrogen and to glucocorticoid and progesterone receptors, respectively, were essentially ineffective.

Discussion

Leydig cells are one of the constituents of the interstitial cell fraction obtained from the testis by collagenase treatment. The purity of the Leydig cell fraction can be enhanced by Percoll gradient separation (Schumacher et al, 1978; Browning et al, 1981) and by further washing (Schumacher et al, 1978). In the present study a second Percoll gradient was used to improve purity greatly without sacrificing yield. This simple modification, therefore, allows the isolation of Leydig cells in purities previously accomplished only by cell culture.

The data presented herein are consistent with the interpretation that Leydig cells possess androgen receptors. Accumulation of androgen into the nuclear fraction of freshly isolated Leydig cells was temperature- and time-dependent, saturable, and androgen-specific. Reports based on data obtained by autoradiographic studies (Sar et al, 1975), uptake studies in interstitial cells plus tubules versus tubules alone (Wilson and Smith, 1975), and retention of ^{3}H-R1881 by interstitial cells in culture (Verhoeven, 1980) support this conclusion. Due to the limited amount of material available, it was not possible to measure such parameters as sedimentation coefficient to establish further the presence of a classic steroid receptor. Nonetheless, the binding was found in the Triton-X100 washed nuclear fraction, whereas most steroid metabolic enzymes are found in mitochondria and the smooth endoplasmic reticulum. Also, the K_d (apparent) is smaller than K_m (Michaelis-Menten constant) for most enzymes. It therefore seems unlikely that this binding represents interaction with enzymes in the steroid metabolic pathway. The proportion of KCl-extractable and macromolecule-bound label found in Leydig cells is comparable with results in Sertoli cells (Sanborn et al, 1977, 1979).

The nuclear binding of ^{3}H-R1881 was highly specific in that androgens were considerably more effective as competitors than an estrogen or a glucocorticoid. The order of specificity was similar to that observed in Sertoli cells (Sanborn et al, 1977) and a variety of androgen-dependent tissues (Fang et al, 1969; Verhoeven et al, 1975). Although Leydig cells contain estrogen receptors (Nozu et al, 1981) and androgens can interact at high concentrations with such receptors (Rochefort and Garcia, 1976), the lack of competition by diethylstibestrol in the Leydig cells makes it unlikely that ^{3}H-R1881 is binding primarily to estrogen receptors. A similar conclusion was reached by Verhoeven (1980) using estradiol as competitor in interstitial cell cultures.

Given the cellular concentration of testosterone in the purified Leydig cells (0.3 ng/10^6 cells) and the basal production rate of 2.3 ng/10^6 cells/hour (Gulizia, Sanborn, and Steinberger, unpublished observations), nuclear-bound androgen should already be present in these cells at the time of exposure to ^{3}H-R1881. It is most probable that the observed nuclear binding of ^{3}H-R1881 is the result of an exchange reaction that takes place during the 1-hour incubation at 34 C. The apparent affinity may therefore by underestimated. In any event, the number of nuclear binding sites measured in the present study is greater than that found in Sertoli cells (Sanborn et al, 1977), in general agreement with the findings of Verhoeven (1980).

Several recent studies have suggested a possible function for Leydig cell androgen receptors. Purvis and Hansson (1978) found evidence for autoregulation of Leydig cell function *in vivo*. Adashi and Hsueh (1981) recently demonstrated autoregulation of testosterone production in testicular cell cultures, and Darney and Ewing (1981) observed a similar phenomenon in perfused testes. The

presence of androgen receptors in purified Leydig cells suggests that such autoregulation may be mediated via receptor mechanisms.

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1983 Testis Workshop

The 1983 Testis Workshop on the topic "Hormonal Regulation of Testicular Function" will be held at the National Institutes of Health on October 14–17, 1983. An introductory lecture on the evening of Friday, October 14th will be followed by 2-1/2 days of sessions on the following topics: peptide and steroid hormone action, sperm motility; and immunological, developmental and clinical aspects of testis function. There will also be a section on new techniques in reproduction research, and two poster sessions. The local organizing committee members are Drs. Kevin Catt, Richard Sherins, and Maria Dufau.

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