Luteinizing Hormone Receptors and Gonadotropic Activation of Purified Rat Leydig Cells*

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Specific receptors and metabolic responses to luteinizing hormone (LH) were analyzed in testicular Leydig cells purified by centrifugation of collagenase-dispersed rat interstitial cells on density gradients of 14-32% Metrizamide. This procedure separated the interstitial cells into an upper, poorly responsive fraction and a lower, more dense population with high LH receptor content and prominent cyclic AMP and testosterone responses to gonadotropic stimulation. The upper layer consisted of morphologically heterogeneous and extensively vacuolated cells, of which relatively few were structurally identifiable as Leydig cells. The lower layer was comprised of almost homogenous Leydig cells when analyzed by electron microscopy and autoradiography, and sedimented as two adjacent bands with similar hormonal responses and densities of 1.085 and 1.105 g/cm3. The lighter and less responsive cell population appeared to result from the presence of damaged cells in the interstitial cell preparation and could be removed during density gradient isolation of the homogenous and biologically active Leydig cell fraction. The more dense and active Leydig cell population retained hormonal responsiveness during culture for 24 h and showed loss of LH receptors and diminished testosterone responses after exposure to low concentrations of gonadotropin in vitro. These findings emphasize the importance of appropriate fractionation of rat interstitial cells to isolate the structurally intact and functionally active population of Leydig cells during studies on LH receptors and action in the testis.

Studies on purified Leydig cells have been of considerable value in the analysis of hormonal regulation of gonadotropin receptors and the control of androgen biosynthesis (1-4). Purification of rat Leydig cells was first performed by centrifugal fractionation of collagenase-dispersed interstitial cells on density gradients of Metrizamide (1). This method gave a discrete fraction composed predominantly of Leydig cells that were morphologically intact and exhibited normal steroidogenic capacity and responses to gonadotropic stimulation. Such purified Leydig cells have been particularly useful for studies on specific aspects of gonadotropin action, including such hormone-induced responses as nuclear translocation of estrogen receptors (5) and cAMP-mediated events, including occupancy of protein kinase regulatory subunits (6) and phosphorylation of endogenous protein substrates (7). Such gradient-purified Leydig cells were also employed for comparison of specific hormonal actions and the generalized effects of choleragen upon target-cell responses with the demonstration of functional compartmentalization of the hormonal stimulus (8).

In more recent studies, we have employed 14-32% Metrizamide gradients to maximize the separation of interstitial cells into distinct Leydig-cell fractions (8). The cells obtained by this method were characterized by testosterone and cAMP responses, binding of 125I-hCG, and morphological analysis by electron microscopy and autoradiography. Selected Leydig cell fractions were also cultured for 24 h (9) and employed for in vitro studies on desensitization by gonadotropin.

MATERIALS AND METHODS

Intestinal cell suspensions were prepared from decapsulated testes of adult male rats (55-65 days old) by digestion with 0.25 mg/ml of collagenase for 12 min (1-9). After adjusting the total nucleated cell concentration of this preparation (methylene blue staining cells) to 40 X 106 cells/ml, 6 ml of the cell suspension was applied to 14-3276 Metrizamide gradients and centrifuged at 5000 X g for 10 min. The hCG-binding profile was obtained by incubating the initial cell suspension for 1 h with 125I-hCG (32 X 106 cells plus 106 cpm of labeled hCG), followed by washing to remove unbound tracer hormone before separation. After centrifugation, the gradient was collected as fractions of 1 or 2 ml. Binding of preincubated hCG was determined in each fraction by counting in a γ spectrometer; then fresh medium was added to each tube and the cells were spun down, washed, and resuspended in fresh medium. Histochemical staining for Δ, #-hydroxysteroid dehydrogenase, an enzymatic marker for functional Leydig cells, was performed in each fraction and cells were counted in a Levy ultraplane chamber (10). Only cells staining intensely with nitroblue tetrazolium were counted as positive, while nonstained or lightly stained cells were regarded as steroidogenically nonfunctional cells. Specifically bound counts/min were divided by the appropriate cell number to obtain a profile of relative LH/hCG receptor number/Leydig cell.

The major peaks from the gradient elution profile were evaluated for biological responses after collection of each of the five visible cell layers for analysis of cAMP and testosterone production. After washing with medium to remove Metrizamide, the cell bands were incubated with or without 100 ng of purified hCG at 35 °C for 2 h under 95% CO2, 5% O2 (8). Testosterone and cyclic AMP production were measured by radioimmunoassay of the incubation media as previously described (10).

RESULTS AND DISCUSSION

After density gradient fractionation of 125I-hCG-labeled interstitial cells, five distinct bands (denoted I-V) were visible, of which three displayed specific binding of the labeled hormone (Fig. 1). Two main binding peaks of approximately equal magnitude (89 and 100 fmol/106 Leydig cells) were present in the lower portion of the gradient, with respective densities (measured by refractive index) of 1.085 and 1.105 g/cm3. The third peak with density of 1.078 g/cm3 contained

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Leydig cells. Of the three main Leydig cell bands (I, II, and IV), fractions, freshly collected bands which had not been preincubation of HCG (10 ng/ml) for 16 h at was determined from incubations in which 10 pg of hCG was added, and was always less than 1% of the total counts. Specifically bound counts expressed as hCG sites/Leydig cell confirmed that bands III and IV consisted of cells with 50 fmol/10^6 Leydig cells. In contrast, the lighter band II, with fewer hCG binding sites/cell, produced only 90 ng of testosterone and 60 fmol of cAMP/10^6 Leydig cells upon hCG stimulation. Band I showed no testosterone response to hCG, and only a minor cAMP response of 6.5 fmol of cAMP/10^6 cells, and no responses were detectable in band V. The lack of hormonal responsiveness in these two bands was correlated with the essential absence of hCG binding and morphologically intact Leydig cells. To confirm the hCG-binding profile of individual fractions, freshly collected bands which had not been preincubated with tracer hCG were assayed for specific hCG binding by incubating about 10^6 cells from each band with 10^5 cpm 125I-hCG in the presence or absence of a saturating concentration of HCG (10 ng/ml) for 16 h at 22 °C. Nonspecific binding was determined from incubations in which 10 µg of hCG was added, and was always less than 1% of the total counts. Specifically bound counts expressed as hCG sites/Leydig cell confirmed that bands III and IV consisted of cells with 23,000 and 30,000 receptor sites, respectively, whereas band II contained only 12,000 receptor sites/Leydig cell. The uppermost band of the gradient (band I) contained 4,400 sites/cell, while no LH receptors could be detected in the erythrocyte-rich band V.

To compare the steroidogenic capacity and binding properties with the morphological characteristics of the cells in these bands, the preparations were examined by light and electron microscopy. Autoradiographic analysis was also performed to assess receptor distribution among the cells of each layer. Of the three main Leydig cell bands (II, III, and IV), bands III and IV had similar densities and migrated close together and were also similar in LH-receptor content and steroidogenic activity. Because these adjacent bands had identical morphological characteristics, they were combined for further study. Specimens for microscopy and autoradiography were preincubated with tracer hCG and separated in the same manner as those fractionated for functional studies. The appropriate fractions from Leydig cell bands II and bands III and IV (combined) were pooled to obtain cell pellets which were fixed in 2% glutaraldehyde and processed for autoradiography and electron microscopy by standard procedures of dehydration, embedding in epon and microtomy. 1-µm thick sections were exposed to photographic emulsion for 1 week for autoradiography and stained with methylene blue azure II (1:1, v/v), while thin sections were stained with OsO4 and then uranyl acetate for electron microscopy. The autoradiograms (Fig. 3) clearly demonstrate differences in binding properties of band II, the “upper layer,” and bands III and IV, the “lower layer.” While nearly all cells in the lower layer are heavily labeled with 125I-hCG, the upper layer contains only a few cells labeled with grains. Also, the cells in the upper layer are markedly heterogeneous, and many are vacuolated or show other evidence of damage.

Electron micrographs further demonstrate the differences between the upper and lower layers. The upper layer appears heterogeneous, and most of the cells show extensive vacuolization (Fig. 4, top). Only 10% of these cells displayed typical Leydig cell morphology (10), as seen in the upper right. In contrast, the lower layer consists mainly of typical Leydig cells with cytoplasm densely packed with smooth endoplasmic reticulum and mitochondria, organelles involved in steroidogenesis (11) (Fig. 4, bottom). In addition, the high power autoradiogram of a cell from the lower layer demonstrates that such purified Leydig cells show preferential binding of 125I-hCG to their microvillous surfaces. The presence of gonadotropin receptors upon the microvilli of steroidogenic cells has been previously noted in the testis and ovary (12) and may be a general feature of peptide hormone-receptor sites (Fig. 5).
FIG. 3. Autoradiograms of $^{125}$I-hCG binding to Leydig-cell LH receptors in the presence (left) or absence (right) of excess hCG (10 μg). Top, the upper layer, band II. Bottom, the combined lower layer of adjacent bands III and IV.

These observations have demonstrated that the interstitial cell fractions separated by density gradient centrifugation differ both functionally and morphologically. The upper and lighter layer was heterogeneous, with reduced hCG binding and testosterone response/Leydig cell, and showed morphological damage to the point that it was difficult to determine the origin of these vacuolated cells. The denser two-band lower layer was almost homogeneous and showed the highest hCG binding and testosterone production, as well as being structurally intact by morphological criteria. Because the Metrizamide gradient separates cells according to their densities, it is interesting to note that the functionally active Leydig cells migrate to the lower layer, while those with reduced responses and binding are lighter and remain in the upper layer. Electron microscopy suggests that the functional Leydig cells’ higher density is related to their extensive smooth endoplasmic reticulum and mitochondria, both sites of steroid biosynthesis. The cells in the upper layer with reduced response are heavily vacuolated and thus appear to be damaged as well as lighter than the highly responsive cells of the lower layer.

In earlier studies on interstitial cell fractionation in Metrizamide gradients (1, 2), we obtained a broad layer of Leydig cells just above the red blood cell zone. This consisted almost entirely of Leydig cells with optimal testosterone responses and hCG binding, while lighter layers showed little hCG binding activity or steroidogenic activity. Similar results were described in the mouse testis (13), where a dense band of highly responsive Leydig cells with increased binding migrated just above the red blood cells and below other lighter bands with little steroid response or gonadotropin binding. Recent reports by Payne et al. (14, 15) also indicate the existence of
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FIG. 4. Electron micrographs of cells from the heterogeneous upper layer, band II (top) and the lower layers enriched in Leydig cells, bands III and IV (bottom).

FIG. 5. EM autoradiogram of a Leydig cell from the lower layer (band III + IV) prepared by centrifugation after preincubation of the interstitial cell preparation with $^{125}$I-hCG. Magnification, $\times 5000$. Most of the label is localized at the cell membrane, in association with microvilli. A small proportion of the labeled hormone shows internalization and is associated with endocytic vesicles.

A lighter, less "Population I" that probably corresponds to this upper layer, and a heavier, responsive "Population II" corresponding to our lower cell layer (bands III + IV) consisting of pure Leydig cells. The present method of purification has resolved the lower layer into two visibly distinct bands of Leydig cells that are similar in terms of the functional characteristics discussed above. The presence of these adjacent bands of highly active Leydig cells may explain the variability of the steroidogenic responses attributed to the upper cell layer, which was reported to be almost unresponsive to hCG (14), and subsequently, to be about one-half as responsive as the lower cell layer (15). The latter partially responsive band probably contained cells from the denser band III that were not completely resolved from the damaged cell layer.

The nature of the Leydig cells which migrate in the upper band of heterogeneous cells has not been determined with certainty in previous studies. It now appears very likely that their presence is due to damage occurring during dispersion of the interstitial tissue. The Leydig cells of the rat testis are highly susceptible to damage during physical dispersion, possibly by shearing forces that breach the plasma membrane upon disruption of the interstitium. In this regard, it has been noted in earlier studies that physical dispersion of the rat testis does not yield a satisfactorily responsive cell preparation for in vitro analysis of hormone action (16). It is also possible that the presence of damaged and fragmented cells in the upper layer is responsible for the moderate amount of $^{125}$I-hCG binding observed in this region in the absence of a
corresponding testosterone response, though with a minor rise in cyclic AMP production (Fig. 1). The presence of two Leydig cell populations with markedly divergent responses to hCG was also noted in rat interstitial cells fractionated on Percoll gradients (17), a procedure earlier applied to the purification of mouse Leydig cells (12). The upper band of poorly responsive cells has been suggested to represent less mature Leydig cells, since repeated treatment with ovine LH appeared to cause an increase in responsiveness of the upper layer to hCG stimulation in vitro (15). However, density gradient analysis of interstitial cells from 24-month-old rats still revealed the two distinct Leydig cell populations, of which only the more dense cells gave significant testosterone responses to LH stimulation in vitro, though the lighter cells showed a small increase in cyclic AMP production (18).

To investigate differences between the Leydig cell bands during gonadotropin-induced desensitization, rats were treated with 1.0 and 2.5 µg of hCG subcutaneously 48 h prior to separation of their interstitial cells on Metrizamide gradients as described above. Preliminary data indicate that band IV, containing the heaviest cells, is most sensitive to this treatment, since about 80% of these cells migrated to higher positions. In contrast, bands II and III showed only slight changes in cell number at the highest dose, with a decrease in the number of receptor sites/cell to 43.4 and 37.6% for band II, and to 86.1 and 62% for band III, after treatment with 1.0 and 2.5 µg of hCG, respectively. Alterations in Leydig cell density after desensitization with hCG could be due to changes in cell volume, smooth endoplasmic reticulum, and/or cholesterol/cholesterol ester pools following stimulation with gonadotropin.

Additional information about the properties of the Leydig cells in the individual layers can be derived from in vitro studies on desensitization of cultured Leydig cells. By this approach, the characteristic responses of these cells to hormone-induced desensitization can be examined without the interference of cell redistribution within the gradient. For this purpose, Leydig cells from the damaged band II and those from the responsive band III + IV were cultured in multiwell Petri dishes and incubated for 24 h with doses of hCG (20 and 50 ng) that were previously shown to cause down-regulation of LH receptors and impaired steroidogenic responses (9). The majority of hormone binding in untreated cells was present in the functional and intact band III + IV, and marked reduction of LH-binding capacity by 76 and 92% was observed at the 20- and 50-ng hCG doses, respectively (Fig. 6). Also, desensitization of testosterone responses to hCG stimulation in vitro due to the previously described “early” and “late” steroidogenic defects was observed (8, 9). The results obtained by culture of the upper band (II) containing poorly responsive Leydig cells confirmed the low receptor number and insensitivity of the cell population to trophic stimulation in vitro. Furthermore, the cells of this band also exhibited down-regulation of receptors and loss of testosterone responses after hCG treatment. The changing responsiveness of the lighter cells described by others (13, 14) could result from the variable presence of active Leydig cells at a higher position in the gradient. This could be caused by redistribution of cells from band IV to a higher position after in vivo stimulation, and possibly by incomplete resolution of band III from band II in the earlier studies.

The impairment of gonadotropin-stimulated testosterone production in the cultured Leydig cells after exposure to 50 ng of hCG was accompanied by a fall in pregnenolone production, as shown in Table I. This finding indicates the presence of the early lesion in steroid biosynthesis that occurs after over-stimulation of the Leydig cell with hCG in vivo or in vitro (8, 9). This defect has been shown to result from impairment of biosynthetic steps prior to pregnenolone formation (19, 20). LH receptors following occupancy and down-regulation due to interaction with saturating concentration of hCG in vitro. These results clearly demonstrate that the active Leydig cell population undergoes desensitization and indicate the value of cell culture to analyze the susceptibility of indi-

![Graph]( attachment:graph.png)  

**Fig. 6.** Gonadotropin-induced desensitization of LH/hCG receptors and testosterone responses to hormonal stimulation of Leydig cells in culture. 2 × 10⁵ interstitial cells or Metrizamide-purified Leydig cells from bands II and III + IV were cultured as previously described (9). Cells were incubated for 24 h in the absence of hormone and with 20 or 50 ng of hCG in Medium 199, 0.1% bovine serum albumin at 37°C under 5% CO₂/air. After removal of medium from the individual wells, the cells were washed and 4 wells from each treatment group (n = 4) were further incubated for 3 h with fresh media in the presence or absence of a maximal dose of hCG (100 ng) to determine the degree of desensitization of steroidogenesis caused by the initial treatment with trophic hormone (9).

| Table 1 | LH receptor sites and steroid production in cultured Leydig cell band III isolated on 18-24% Metrizamide gradients (mean ± S.E., n = 3) |
|-----------------|------------------------------|-----------------|-----------------|
| hCG in culture | LH receptors (sites/Leydig cell) | Testosterone | Pregnenolone* |
| ng | ng/10⁵ Leydig cells | ng/10⁵ cells | ng/10⁵ cells |
| 0 | 834 ± 148 | 7.9 ± 1.0 | 60.8 ± 3.2 | 8.2 ± 2.4 | 25.6 ± 1.0 |
| 50 | 259 ± 19 | 20.6 ± 1.7 | 24.9 ± 1.3 | 8.4 ± 2.1 | 11.7 ± 1.6 |

* Measured in the presence of cyanoketone and spironolactone (4) to inhibit pregnenolone metabolism.
individual populations to trophic hormone-induced changes in receptors and responses.

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