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Leydig Cell Heterogeneity as Judged by Quantitative Cytochemistry of 3β-Hydroxysteroid Dehydrogenase Activity in Individual Rat Leydig Cells

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3β-Hydroxysteroid dehydrogenase (3β-HSD) is one of the key enzymes involved in the steroidogenic pathway of Leydig cells. In this study, quantitative cytochemistry was used to detect the 3β-HSD staining intensity in individual rat Leydig cells. The measurement of the intensity of staining was a reliable method reflecting the relative amount of 3β-HSD activity. The objective was to determine the presence, basal and hCG-mediated effect of 3β-HSD activity in individual Leydig cells. 3β-HSD cytochemistry was performed in both, 8 and 12 μm diameter rat Leydig cells. The results showed that both populations of Leydig cells have different basal 3β-HSD activity. The 8 μm cells showed a greater basal 3β-HSD activity than the 12 μm cells when their optical density values were normalized to their size. A difference in regulation of the enzymatic activity by LH/hCG was observed in the two types of Leydig cells. Incubation of the whole population of Leydig cells with hCG (1IU), decreased the 3β-HSD activity in the 8 μm cells, but increased the activity in the 12 μm cells. The results describe for the first time that the 3β-HSD activity may be differentially regulated by LH/hCG in Leydig cells.

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INTRODUCTION

Steroid synthesis by Leydig cells relies on LH acting through the cAMP second messenger system. Based on the capacity of Leydig cells to produce testosterone, two types of LH/hCG-responsive cells have been found previously [1]. These cell populations have been classified according to the density of the cells and the presence of LH receptors as identified by radioligand binding assays. This Leydig cell heterogeneity has been discussed at length, and may be due in part to the heterogeneous cellular expression of steroidogenic enzymes within these cells [3, 4]. The conversion of pregnenolene to progesterone, which is catalyzed by the microsomal enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), is considered a key step in the steroidogenic pathway. LH is the main pituitary hormone required to maintain the level of 3β-HSD in testicular Leydig cells [5], providing evidence that *in vivo*, one of the trophic effects of LH may be to regulate the expression of testicular 3β-HSD [6]. Recently, studies with cultured steroidogenic cells have demonstrated that the expression of 3β-HSD is regulated at least in part at the level of gene transcription via a cAMP-dependent signalling pathway [7]. 3β-HSD is also modified by androgens [8–10], glucocorticoids [8], estrogens [11, 12] and opioids [13]. These effects have been observed either *in vivo* [5, 6] or in cell cultures of several steroidogenic cells [7–9, 14]. The use of a cytochemical reaction for the 3β-HSD has long been recognized as a judgment for Leydig cell identification, due to the blue coloration of formazan granules formed in the oxidation–reduction reaction [15]. Since individual cell methods provide advantageous conditions to evaluate responses not detected in a cell population, in this work, quantitative cytochemistry was used to detect the 3β-HSD staining intensity in individual Leydig cells. In rats, two different Leydig cell sizes (8 and 12 μm diameter) were found, which differed in their basal 3β-HSD activities. Treatment with hCG showed a differential regulation of enzymatic activity in these two cell types.

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EXPERIMENTAL

Chemicals

Medium 199 and Eagle's minimal essential medium (EMEM) were obtained from Grand Island Pharmaceuticals (NY, U.S.A.). Collagenase was obtained from Worthington Biochemicals Co. (Freehold, NJ, U.S.A.). Human chorionic gonadotropin (hCG 3000 IU/mg) and etiocholan-3β-ol-17-one (5β-androstan-3β-ol-17-one) and all other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Cyanoketone (Win 19,578) was a gift from Sterling Drug Inc. (U.S.A.).

Leydig cell preparation

Adult male Wistar rats (60–65 days old) from our colony were used. They were maintained under controlled conditions of 12 h light:12 h darkness and rat chow and water available *ad libitum*. Animals were first anesthetized and killed by decapitation. Testicular cells were dispersed by treating the decapsulated testis with collagenase, and Leydig cells were subsequently purified through a continuous gradient of 10–80% Percoll, as described previously [16, 17]. Leydig cell-enriched fractions (20 to 24) were pooled, washed and resuspended in appropriate volumes of EMEM containing 0.1% bovine serum albumin (BSA) pH 7.3.

Analysis of the staining intensity for the 3β-HSD by cytophotometry

Leydig cells contained in the purified suspension (1×10^6 cells) were incubated with a staining solution for 3β-HSD, containing 1.5 mM nicotin amide adenine dinucleotide (NAD), 0.25 mM nitroblue tetrazolium (NBT) and etiocholan-3β-ol-17-one (0.2 mM in DMSO) as substrate in phosphate-buffered saline (PBS)–0.1% BSA. The number of Leydig cells was established by counting the percentage of stained Leydig cells. Usually, the purified Leydig cells were 85–90% 3β-HSD positive, and the viability after the whole procedure estimated by either trypan blue or pyridine nucleotide exclusion, was 90%. Previously counted Leydig cells were incubated for variable times with the staining solution. After, cells were fixed in formalin–acetic acid (1:1, v/v), centrifuged and washed twice with 1 ml buffer PBS. Leydig cells were extended over a slide, previously cleaned with ethanol–eter solution, and dried at room temperature. Afterwards, fixed cells were mounted in Permount and put in a cytophotometer Carl Zeiss equipped with an inscriptor Servegor RE 511. Transmission lectures of individual cells were converted to absorbance by the formula: A (Optical Density) = log 1/T. The apparatus was calibrated to 100% transmittance (0% absorbance), with a non-stained Leydig cell. Non-stained Leydig cells were distinguished from macrophages, by incubation with India ink (1:100, v/v in PBS) for 15 min at 35°C [18]. India ink is specifically incorporated to testicular macrophages that are frequently contaminating the purified Leydig cell suspensions [19]. The cytophotometer contains diaphragms with different diameters. Most of the stained Leydig cells fitted exactly into the 400 and 630 μm diameter diaphragms, corresponding to a true cell diameter of 8 and 12 μm, respectively. Therefore, optical density values were measured in cell areas of 50.2 and 113 μm^2 for the 8 and 12 μm diameter cells, respectively.

For experiments with hCG, 0.5×10^6 cells were preincubated for 15 min, and then incubated for 3 h with 1 IU of hCG at 34°C with 95% O_2/5% CO_2. After this time, cells were incubated for 1 h in the staining solution, then, observed at the cytophotometer and individual cell transmittance was measured as described.

Statistics

Data are expressed as means $\pm$ SD and differences between groups were evaluated by the Student's t-test. Differences were considered significant if $P < 0.05$. Chi-square tests were performed to compare observed population distributions with expected normal distributions.

RESULTS

When purified Leydig cells were incubated with the staining solution for 3β-HSD, a blue coloration of formazan granules was observed (Fig. 1). Cell permeabilization was not necessary for the 3β-HSD staining reaction to occur (unpublished data). A low percent of non-stained cells could be observed, corresponding to

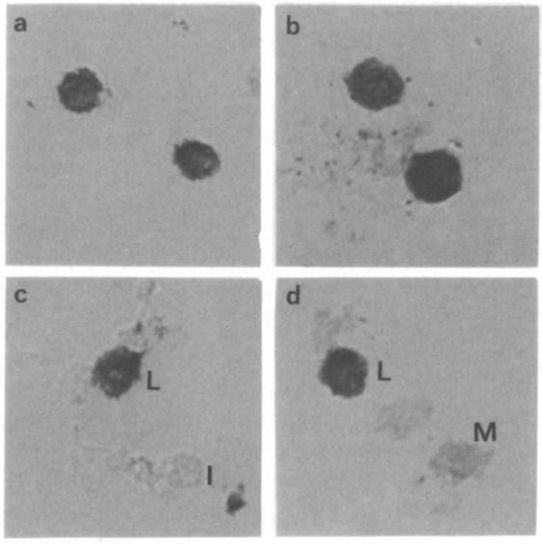

Fig. 1. Purified Leydig cells were incubated with a staining solution for 3β-HSD for 60 min at 37°C. Stained Leydig cell, 3β-HSD (+) showing a blue coloration of the formazan granules; (a) 8 μm diameter cells, and (b) 12 μm diameter cells, (c) Leydig cell 3β-HSD (+) (L), and Leydig cell 3β-HSD (−), non-stained (l), (d) Leydig cell 3β-HSD (+) (L), and a macrophage (M). 600×.

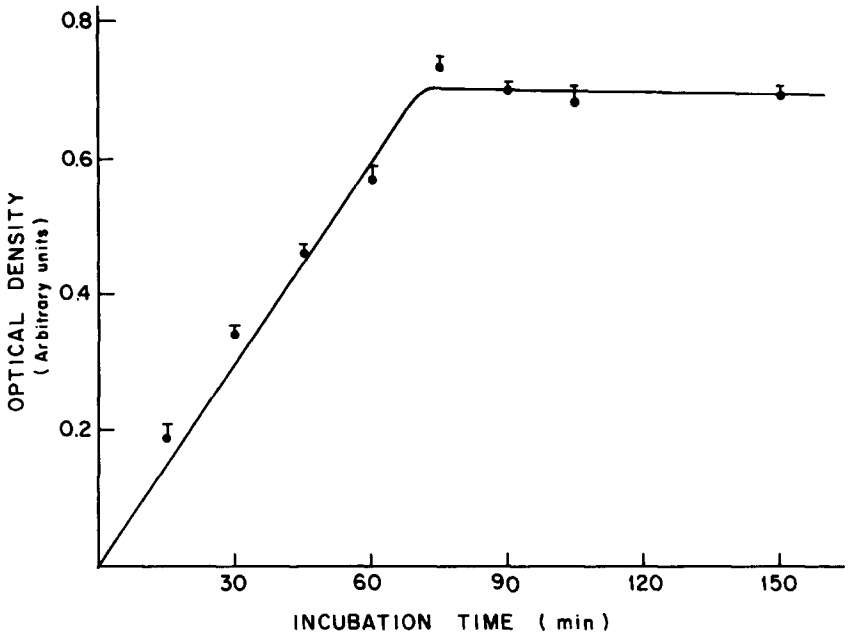

Fig. 2. Time-course of the cytochemical reaction for 3β-HSD. Purified Leydig cells were incubated with the staining solution at 37°C for different period of time. Results are expressed in optical density (arbitrary units) and represent the mean ± SD of 737 cells of 8 μm diameter obtained from three independent experiments.

Leydig cells lacking the 3β-HSD activity and/or other types of cells, which are usually located in the interstitium of the testes [Fig. 1(c)]. Macrophages were distinguished because they showed a black coloration resulting from the selective incorporation of the black ink [Fig. 1(d)]. Stained cells corresponded to an 89% of the total population (counted cells). Among the stained cells, 67% fitted exactly within the 400 μm diameter diaphragm, and 12% fitted within the 630 μm diameter diaphragm. Thus, 8 and 12 μm diameter cells, respectively were observed [Fig. 1(a and b)]. Approximately 10% of the stained cells showed diameters different to those already established, therefore these cells were not included in this study. To establish a relationship between optical density and 3β-HSD activity of individual Leydig cells, the staining reaction was performed in different experimental conditions. A time-course study of the staining reaction showed a progressive increase of the optical density until 75 min of incubation time (Fig. 2). Successive experiments were carried out by incubating the cells in the staining solution during 60 min.

The effect of the presence of substrate and cofactor for the 3β-HSD staining reaction was evaluated (Fig. 3). As expected, maximal absorbance was obtained when both, the substrate etiocholan-3β-ol-17-one and the cofactor NAD^+ were present. Without substrate and/or cofactor, the optical density was very low (0.1 arbitrary units or less). When different increasing substrate concentrations were used (0.01, 0.05, 0.1, 0.2 and 0.5 mM), gradual increasing values of optical density were obtained (results not shown). Although the values were not significantly different, a maximal optical density of 0.52 ± 0.02 arbitrary units was obtained when 0.2 mM of etiocholan-3β-ol-17-one was used. These results suggest that the 3β-HSD is already saturated with substrate concentrations lower than 0.01 mM.

When Leydig cells were incubated with 1 or 10 μM cyanoketone (Win 19,578), an inhibitor of the 3β-HSD activity, a marked decrease of the optical density in both types of cells was obtained (Fig. 4). Although in control cells the staining intensity was similar in both types of cells, different values were obtained when optical densities were normalized to their size. Eight μm diameter cells showed more than twice higher optical density per μm^2 when compared with the 12 μm diameter cells ($8.96 \pm 1 \times 10^{-3}$ vs $3.54 \pm 0.44 \times 10^{-3}$ arbitrary units).

Staining intensity values for each size of Leydig cell were normalized to their sample mean by assigning them a z-score based on a standard normal curve. The observed frequency distributions for the 8 μm diameter cells matched a distribution, which was significantly different from a normal distribution [Fig. 5(a); Chi-Square = 28.09, $P < 0.006$]. However, the 12 μm diameter cells showed a normal distribution, as evaluated by the Chi-Square test [Fig. 5(b); Chi-Square = 11.94, non-significant].

When purified Leydig cells were previously incubated with hCG (1 IU) and subsequently stained for 3β-HSD activity, a differential hormone-induced effect on the optical density was observed. In 8 μm diameter cells the hormone caused a decrease of the optical density when compared to the control

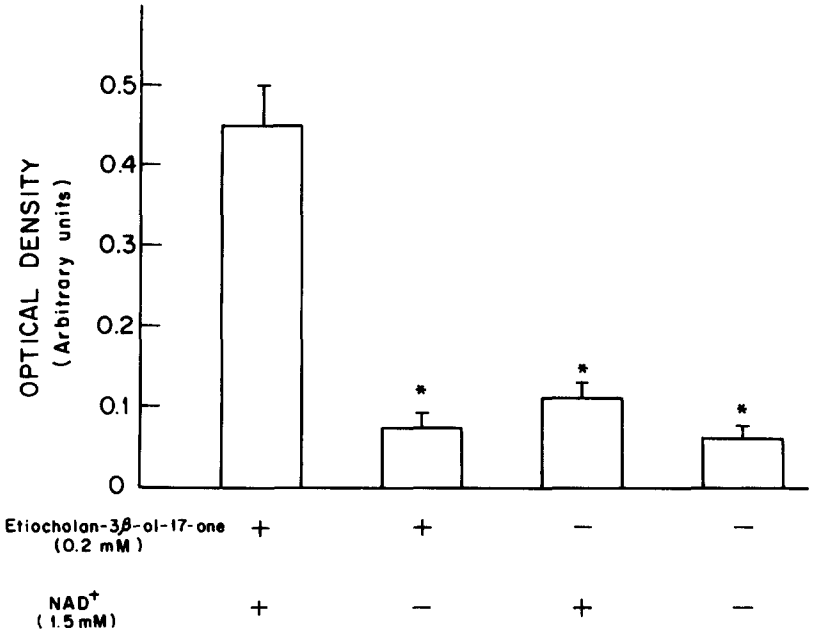

Fig. 3. Effect of the presence of the substrate etiocholan-3β-ol-17-one and the cofactor NAD^+ in the cytochemical reaction for 3β-HSD. Results are expressed in optical density (arbitrary units) and represent the mean ± SD of 464 cells of 8 μm diameter (approx. 100 cells per each experimental condition) obtained from three independent experiments. *$P < 0.005$ compared with the control value (with substrate and cofactor).

untreated cells. Conversely, hCG increased the optical density in the 12 μm diameter Leydig cells (Table 1). This slight but significant effect was also observed when values were expressed per unit of area (μm^2).

DISCUSSION

The results demonstrate that measurements of the intensity of the staining reaction for 3β-HSD in individual cells, can be a realiable method for detecting the

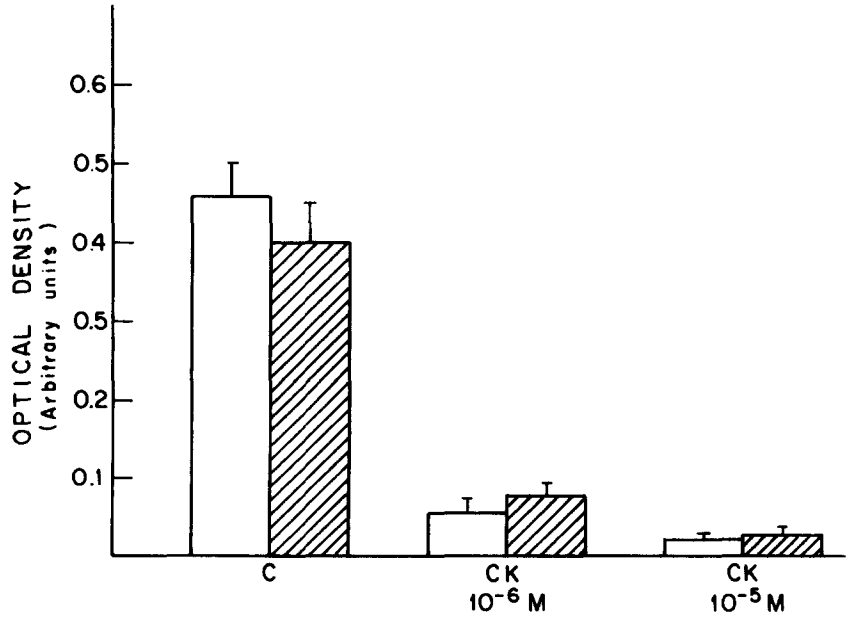

Fig. 4. Cytochemical reaction for 3β-HSD in presence of the inhibitor cyanoketone (Win 19,578). Purified Leydig cells were incubated with 10^{-6} and 10^{-5} M of cyanoketone (Win 19,578) for 3 h. After washing, cells were incubated with the staining solution for 3β-HSD for 60 min at 37°C. Results are expressed in optical density (arbitrary units) and represent the mean ± SD of three independent experiments. Approximately 150 cells of 8 μm diameter with each inhibitor concentration were measured.

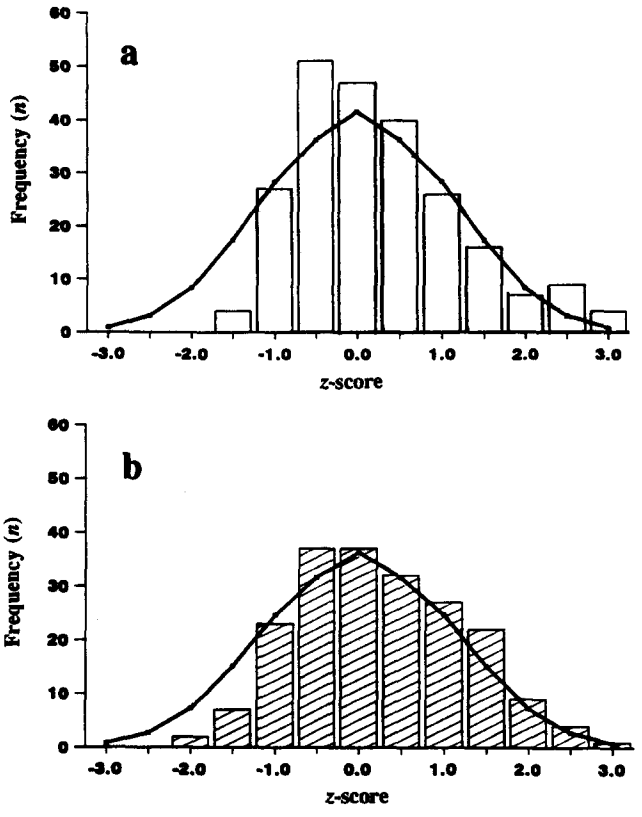

Fig. 5. Staining intensity values for the 3β-HSD reaction in 8 and 12 μm diameter Leydig cells were normalized to their sample mean by assigning them a *z*-score. The distribution of the *z*-scores was compared to an expected normal distribution using Chi-Square. Values are expressed as frequency of distribution of the staining intensity of 232 and 203 cells of 8 and 12 μm diameter, respectively. (a) Observed frequency for 8 μm diameter cells. Chi-Square = 28.09, $P < 0.006$. (b) Observed frequency for 12 μm diameter cells. Chi-square = 11.94, NS.

individual cell expression of such enzymatic activity. Previous studies [20] reported a correlation between quantitative histochemical and biochemical assays for 3β-HSD in primary cultures of normal rat adrenal cells.

In the present report, cell permeabilization for the entrance of nicotinamide adenine dinucleotide (NAD^+) was not necessary (unpublished data). In contrast, Marrone and Sebring [21] did not observe a positive 3β-HSD cytochemistry in non-permeabilized granulosa cells. They used a digitonin-permeabilized cell model as previously shown by Molenaar *et al.* [22]. The different type of cells and the higher concentration of NAD^+ used in this study could be an explanation for this discrepancy.

Leydig cell heterogeneity shown in this study has been observed previously [1, 2, 23]. Some authors suggested that the heterogeneity of the steroidogenic capacity of isolated adult rat Leydig cells could be dependent, at least in part, upon the procedures used for cell isolation [23]. In this work, Leydig cells were purified through a continuous Percoll gradient [24] and several types of heterogeneity were found. Two populations of cells based on size were observed in the purified Leydig cells. The 8 μm diameter cells constituted 67% of the stained cells. Leydig cell sizes found in this study, agreed with those described by Bergh *et al.* [25, 26].

Heterogeneity was also related to cell 3β-HSD enzymatic activities, since different staining intensities were observed. Stained Leydig cells showed similar basal enzymatic activities; but, when their optical densities were normalized to their size, 8 μm diameter cells expressed a greater basal enzymatic activity than the 12 μm cells. Twelve μm diameter cell population fitted in a normal distribution of the staining intensities. However, 8 μm cells showed a distribution significantly skewed to the right. The observed frequency distribution for the 8 μm Leydig cells shows that they are preferentially constituted by a subpopulation of darkly stained cells, and thus suggesting an increased steroidogenic capacity compared with the 12 μm cells [20, 27, 28].

Although 3β-HSD activity is not rate limiting for testosterone synthesis, previous studies have indicated a significant correlation between circulating testosterone levels and 3β-HSD activity [20, 28]. If this hypothesis is correct, one could expect a greater testosterone production by the 8 μm cells in comparison to the 12 μm cells. Previous studies from our laboratory have demonstrated that individually tested Leydig cells

Table 1. Effect of hCG (1 IU) in vitro in the staining intensity for 3β-HSD in 8 and 12 μm diameter Leydig cells

	Type of cell			
	Optical density		Optical density/μm² × 10^{-3}	
Condition	8 μm	12 μm	8 μm	12 μm
Control	0.45 ± 0.05^a	0.40 ± 0.05^a	8.96 ± 1.00^a	3.54 ± 0.44^b
hCG (1 IU)	0.37 ± 0.01^b	0.51 ± 0.01^c	7.37 ± 0.20^c	4.51 ± 0.09^d

Purified Leydig cells were incubated in the presence or absence of hCG (1 IU) for 3 h at 34°C. After washing, cells were incubated with the staining solution for 60 min at 37°C. Results are expressed in optical density (arbitrary units) and represent the mean ± SD of three independent experiments. For each expression of the results, values with different superscripts are significantly different (Student's *t*-test). For the 8 μm diameter cells, $n = 1070$; for the 12 μm diameter cells, $n = 187$.

showed differential steroidogenic activities and that the small Leydig cells constituted the higher percentage of the androgen-secretory cells [27].

When both types of cells were incubated with hCG, 3β-HSD activity was decreased in the 8 μm cells and increased in the 12 μm cells. It has been demonstrated that in rat gonads and adrenals, type I is the predominant 3β-HSD transcript, and type II is a minor species [7]. If the two Leydig cell populations expressed different types of 3β-HSD, then it might account for the difference in regulation by LH/hCG. In addition, to our knowledge, the 3β-HSD activity of steroidogenic cells has not been reported to be modified acutely by LH/hCG. Only trophic effects of LH/hCG on 3β-HSD activity have been described previously [5, 29]. Perhaps acute effects of LH/hCG are masked when biochemical methods are performed utilizing whole cell populations to evaluate the enzyme activity.

A previous report has shown that increased testosterone secretion—obtained after LH/hCG-treatment—inhibits 3β-HSD activity in rat Leydig cells cultures via an androgen receptor-mediated mechanism [10]. The inhibitory effect of LH/hCG on the 3β-HSD activity of 8 μm Leydig cells may be explained in part by the observed negative regulatory effects of testosterone on 3β-HSD activity. The possibility that LH/hCG treatment could induce receptor down-regulation and/or desensitization cannot be completely ruled out. Nevertheless, the fact that LH receptor are internalized very slowly [17] precludes any detrimental effect on LH mediated 3β-HSD activity increase.

It has been demonstrated that LH/hCG may also exert receptor-mediated effects on the 3β-HSD and other steroidogenic enzymes [14], attempting to maintain the levels of several enzymes involved in testosterone biosynthesis [29]. In the 12 μm cells, the hCG-mediated increase of the 3β-HSD activity may be explained by the presence of a large population of functional LH receptors. Thus, different populations of functional LH/hCG receptors on each type of cells, may be another factor related to the differential response in the 3β-HSD activity.

In other studies related to the 3β-HSD activity of LH/hCG-treated cultured Leydig cells, LH produced little effects on the activity and levels of immunoreactive 3β-HSD in some experiments, but it had inhibitory effects in others [7]. This is paradoxical with the LH/hCG-cAMP-mediated stimulatory effect on the cellular levels of 3β-HSD mRNA [7]. This effect occurs 12 h after LH-treatment. Since our experiments were performed for 3 h, an increase of the 3β-HSD activity in 12 μm cells, due to increased transcriptional activity, is unlikely.

In summary, these results not only demonstrate a differential expression of basal 3β-HSD activity in Leydig cells, but also a differential response when they are treated with hCG. The opposite effects of the hormone on the 3β-HSD activity in both types of cells suggest the existence of true Leydig cell heterogeneity, which may be related to different individual steroidogenic capacities.

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