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DETECTION OF TESTOSTERONE SECRETION FROM INDIVIDUAL RAT LEYDIG CELLS*

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Summary—The purpose of the present study was to analyze testosterone secretion from individual purified Leydig cells, using a reverse hemolytic plaque assay (RHPA) as an approach for identifying and characterizing subtypes of Leydig cells. Leydig cells from adult rats and protein A-coated ovine erythrocytes were mixed and incubated for appropriate lengths of time in the presence or absence of antitestosterone antibody, hormones or an analog of cyclic AMP. The slides from RHPA were histochemically stained for 3β-hydroxysteroid dehydrogenase (3β-HSD). Results show that testosterone secreting cells can be clearly identified by the formation of hemolytic plaques. The proportion of plaque-forming cells increases with incubation time, reaching a plateau at 60 min in the presence of gonadotropin. It was observed that not all 3β-HSD positive cells form plaques. It is concluded that the purified Leydig cell population has cells with differential steroidogenic and androgen-secretory activities.

INTRODUCTION

In the adult rat, Leydig cells are responsible for testicular steroidogenesis. The production of androgens by these cells is primarily under the control of LH from the pituitary, however an increasing amount of evidence indicates that local regulatory factors are also important for modulating androgen production [1]. Leydig cells, like other cell types identified by both morphological and cytochemical properties show heterogeneity, so that several populations have been identified which exhibit diversity of size, organelle composition, physicochemical properties and metabolic activity [2–4]. However, homogeneity among Leydig cells with respect to the steroidogenic activity and androgen secretion is a common assumption, mainly derived from the fact that individual cell secretion activity has not been analyzed and neither the structural nor biochemical details of the secretory process are known.

Recently an experimental approach has been developed that allows the detection of hormone secretion from individual cells in a mixed population, allowing the characterization of subtypes of endocrine cells. This method, the reverse hemolytic plaque assay (RHPA), is based on complement-mediated lysis of antibody-coated erythrocytes coincubated with antigen-secreting cells. Antigen secretion results in hemolysis of erythrocytes surrounding the secretory cells, so that clear areas of lysis (plaques) identify cells secreting antigen recognized by the antibody used to coat the erythrocytes [4–6].

Theoretically, the RHPA can be used to detect any cellular secretion for which an antibody is available [5]; however, until now all reports using this technique have been limited to proteins. Using the RHPA in combination with enzymatic staining for 3β hydroxysteroid dehydrogenase (3β-HSD), we show the first direct detection of androgen secretion in a Leydig cell population. Our results suggest that in a purified Leydig cell population, androgen secretion could be ascribed to a subset of the cells that have steroidogenic enzymatic activity.

EXPERIMENTAL

Chemicals

Staphylococcal protein-A, poly-L-lysine, bovine serum albumin (BSA), chromium chloride hexahydrate, Percoll, minimal essential medium (MEM), medium-199 (M-199), human

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chorionic gonadotropin (hCG), 8-bromo-adenosine 3′-5′-cyclic monophosphate (8Br cAMP) and 3-isobutyl-1-methyl xanthine (MIX) were purchased from Sigma (St Louis, MO). Collagenase was obtained from Worthington Biochemical Co. (Freehold, NJ).

Antiserum

Antitestosterone-11α-(succinyl) BGG was purchased from Steranti Research Ltd (St Albans, England), it cross-reacts 19.5, 17.6 and 6.5% with 5α-dihydrotestosterone, dehydroepi androsterone and androstenedione, respectively; all other steroids tested have cross-reactivity lower than 0.5%.

Animal, cell dispersion and purification

Adult male Wistar rats (65–75 days old) from our colony were used. Rats were housed under standard laboratory conditions with free access to food and water. The testes were decapsulated and enzimatically dispersed with collagenase, and Leydig cells were purified on a discontinuous gradient of Percoll [7]. The Leydig cell-enriched fractions were combined, washed and resuspended with appropriate volumes of MEM, containing 0.1% BSA.

RHPA

RHPA was performed as described by Neill and Frawley [5], with minor modifications. Fresh ovine red blood cells (oRBC) were obtained at 3 week intervals and coated weekly with protein-A. RHPA was conducted in Cunningham chambers constructed on poly-L-lysine coated glass microscope slides. The incubation chamber had an approx. volume of 30 μl. Purified Leydig cells and protein A-coated oRBC were mixed (1:260) in an appropriate volume of M-199–0.1% BSA, infused by capillarity into the chambers and incubated for 60 min at 34°C to promote adherence of both cell types. Unattached cells were discarded by washing with fresh M-199–0.1% BSA. Each chamber was then filled with a solution of M-199–0.1% BSA with or without antiserum (final dilution 1:10,000), hormones or an analog of cyclic AMP, as specified in Results. In some experiments albumin was ommitted from the cell suspension medium.

Slides were incubated at 34°C for 60 or 90 min, the chambers were then washed and filled with fresh medium containing rabbit serum as the source of complement (final dilution 1:50), and incubated for another 45 min to allow plaque formation. The reaction was stopped by immersing the slides in 3% glutaraldehyde in PBS. After fixation overnight at 4°C, the slides were rinsed and stored in PBS at 4°C.

Leydig cells were identified by enzymatic staining to detect 3β-HSD [8]. Staining was performed on both fresh cell samples as well as after fixation of the cells, in the RHPA chambers. In both cases, 65 ± 3% of nucleated cells gave positive staining for 3β-HSD. Viability of the cells after incubation was better than 90%, as estimated by trypan blue exclusion.

Cells monolayers were viewed microscopically to identify and count plaque forming cells. Duplicated chambers were prepared for each determination; at least 200 nucleated cells were counted on each chamber. Results are the mean ± SD of the percentage of positively 3β-HSD stained cells that form hemolytic plaques; differences between groups were evaluated using Student's *t*-test.

RESULTS

The RHPA allows the identification of androgen-secreting cells in enriched Leydig cell samples; as shown in Fig. 1 plaques are identified microscopically as clear areas around the Leydig cells containing oRBC ghosts. Plaque size varied and it was appreciated that usually smaller cells formed larger hemolytic plaques. The plaque-forming cells are considered androgen-secreting cells because: (1) plaques did not form when testosterone antiserum was replaced with normal rabbit serum; (2) pre-incubation of the testosterone antiserum with testosterone, abolished plaque formation (not shown); and (3) plaque formation showed a clear dependence on hCG (Figs 2 and 3). Finally, omission of rabbit complement abolished plaque formation (Table 1).

Under these conditions, plaque formation can be attributed to Leydig cells, since only positive 3β-HSD staining cells developed plaques. Plaque first formed within 15–30 min after the initiation of incubation and the number of secreting cells increased with time, this was more remarkable in the presence of hCG (Fig. 2). Furthermore, a clear dose dependent effect of the gonadotropin on the number of plaque-forming cells was observed (Fig. 3).

Table 1 summarizes the results of several experiments performed either as controls, or to analyze whether the composition of the

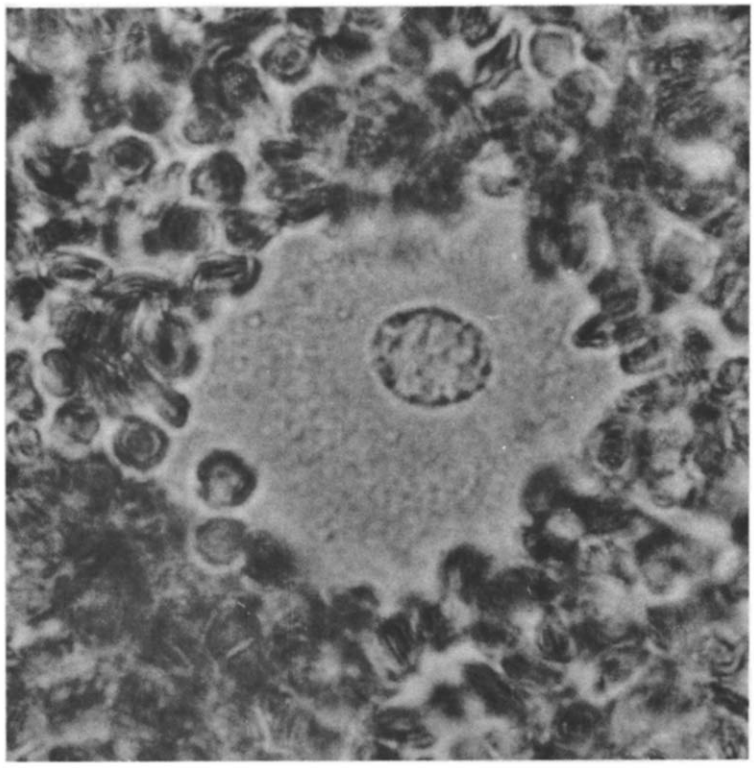

Fig. 1. RHPA of androgen secreting cell. oRBCs surrounding the Leydig cell show hemolysis, after 90 min incubation in the presence of hCG. 100 × magnification.

incubation medium and/or receptor function were limiting the detection of androgen secretion. Results show that neither substrate availability nor receptor function seem to restrain secretion, as measured by RHPA.

DISCUSSION

This study extends the application of the RHPA to the detection of steroid-secreting cells. The method described here has sensibility and specificity similar to that found for the detection of protein and peptide-secreting cells [4–6]. Furthermore, as used here the RHPA in conjuction with cytochemical staining, allows for the detection of cells that have steroidogenic enzymatic activity but do not secrete the steroid under study.

Thus, we observed that there is heterogeneity among Leydig cells in their capacity to secrete androgens, since only a subset (50%) of Leydig cells developed hemolytic plaque, under maximal stimulation with hCG. The gonadotropin

Table 1. Effect of changes in the composition of the incubation medium on the percentage of plaque-forming Leydig cells

Medium	Percentage of plaque-forming Leydig cells	
	Without hCG	With hCG
Normal Medium (N.M.)	25.7 ± 10 (6)*	51.8 ± 13 (10)
N.M. without antibody	ND	4.3 ± 1.8 (11)*
N.M. without complement	ND	6.1 ± 3.8 (12)*
N.M. without albumin	ND	61.8 ± 7 (3)
N.M. + pregnenolone, 10^{-5} M	ND	55.3 ± 12 (3)
N.M. + (8Br cAMP + MIX) 1 mM	ND	46.2 ± 3 (3)

Cells were incubated for 90 min as described in Experimental. Results are the mean ± SD; values in parenthesis indicate the number of different experiments. *$P < 0.01$ as compared with N.M. with hCG. ND: not determined.

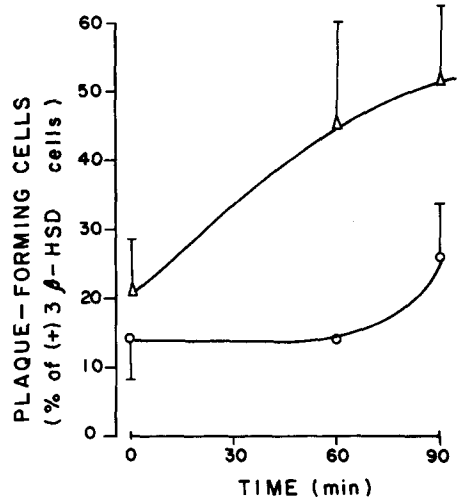

Fig. 2. Rate of plaque formation by Leydig cells untreated (○) or treated with hCG (25 IU/ml) (△). Each point corresponds to the fraction of Leydig cells forming plaques and represent the mean ± SD of 8 experiments.

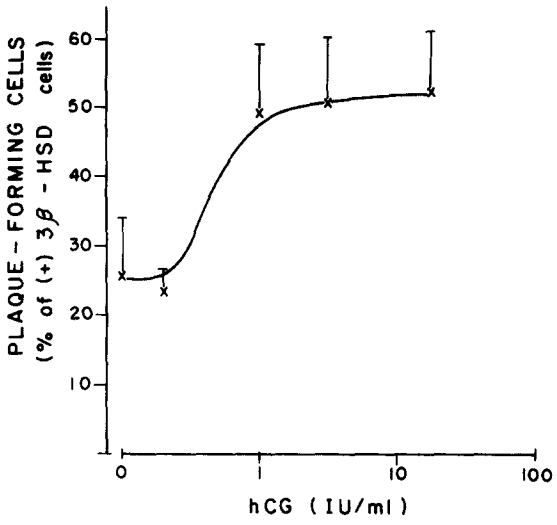

Fig. 3. Dose–response curve of hCG effect on plaque-forming cells. RHPA were read at 90 min. Each point represents the fraction of Leydig cells forming plaques and corresponds to the mean ± SD of 8 experiments.

both recruits new cells into the population of secreting cells and increases the rate of secretion, since plaques were larger in the presence of the hormone. The recruitment of cells was established early and was maximal at 60 min, even though plaque size enlarged with extended incubation.

The Leydig cell heterogeneity found cannot be ascribed to membrane receptor alteration during purification or incubation of cells, since there was no increase on the fraction of secreting cells when an analog of the second messenger of hormone action, 8Br cAMP, was added during incubation.

We further investigated other factors that could limit detection of the secretory activity. Here we show that neither substrate availability nor testosterone binding to proteins in the medium [9] are limiting, since no increase in the proportion of secreting cells was observed either in the presence of pregnenolone or the absence of albumin.

Further studies on the mechanism controlling the cellular heterogeneity described here might define whether the subset of Leydig cells that do not form plaques are secreting other steroids (not androgens), or though capable of synthetizing androgens, as dispersed cells they lack some local signals (auto or paracrine) necessary for regulating androgen secretion.

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