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RELATIONSHIP BETWEEN RECEPTOR OCCUPANCY AND STIMULATION OF ADENOSIN-3',5'-MONOPHOSPHATE (cAMP) AND PROGESTERONE PRODUCTION IN ISOLATED RAT GRANULOSA CELLS

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SUMMARY

In granulosa cells isolated from preovulatory follicles of PMSG-primed immature rats half-maximum steroidogenic response was seen when 3% of the available LH binding sites were occupied by hCG and maximum response was reached at 15–20% saturation. The response in cAMP was much less sensitive; a detectable response was seen at hCG concentrations which elicited about half-maximum progesterone production. Maximum response in cAMP was reached when more than 50% of the available binding sites were occupied. In the presence of the phosphodiesterase inhibitor IBMX the entire range of the progesterone response curve shifted to lower hCG concentrations indicating a sufficient cAMP level within an intracellular compartment for mediating steroidogenic response. Granulosa cells obviously differ from rat Leydig cells where maximum steroidogenic response is achieved when only 1% of the LH receptors are occupied.

INTRODUCTION

For isolated rat Leydig cells it has been reported that maximum steroidogenic response is achieved when only 1% of the available LH receptors are occupied by hCG [1]. The cAMP response was much less sensitive reaching a plateau at half-saturation of the LH binding sites. The results reported on collagenase dispersed rat ovarian cells are conflicting [2–4]. This may be due to both the heterogeneity of the cell population studied and differences in experimental conditions. We therefore were interested in studying the relation between receptor occupancy and biological responses in a homogeneous cell population at a defined developmental stage: granulosa cells of preovulatory follicles from PMSG-primed immature rats.

EXPERIMENTAL

Prepubertal female Sprague–Dawley rats were injected s.c. with 10 IU PMSG (pregnant mare's serum gonadotropin, Gestyl®, Organon) at 10.00 h on day 28 [5]. Forty-eight hours later the animals were killed by cervical dislocation and the ovaries were immediately removed and placed in chilled Hanks salt solution. In a typical experiment where the biological response and the receptor occupancy were determined in parallel, 24–26 ovaries were used. Per ovary 8–12 large follicles considered to be preovulatory were punctured with a fine hypodermic needle under a stereomicroscope (Wild, Heerbrugg, Switzerland) and the granulosa cells were released in Hanks solution by applying gentle pressure with a spatula [6]. The granulosa cells were collected by centrifugation at 110 *g* for 5 min at 4°C and resuspended in culture medium 199 supplemented with 0.4% BSA. Aliquots of the cell suspension corresponding to 3–4 × 10^5 cells were incubated (first incubation) in duplicate for the cAMP and progesterone response and in triplicate for the determination of the receptor occupancy in a final volume of 0.5 ml containing 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) and increasing concentrations of hCG (human chorionic gonadotropin, S.A. at least 3000 IU/mg, Calbiochem, U.S.A.). The incubation was performed in a metabolic shaker for 2 h at 37°C under an atmosphere of 5% CO_2 in air. To the incubation tubes for the cAMP and the progesterone response 2 ml ice cold ethanol was added to lyse the cells and to denature the proteins [6]. After centrifugation at 4°C for 10 min at 2100 *g* the supernatant was removed and stored at −30°C until analysed for cAMP and progesterone content.

The granulosa cells for the estimation of receptor occupancy were washed first with 2 ml ice cold phosphate buffered saline, pH 7.4, containing 0.1% gelatin and then with 2 ml medium 199. The cells were centrifuged each time at 490 *g* for 10 min. After careful aspiration of the supernatant the cells were incubated in 200 μl medium 199 containing about 400,000 c.p.m. ^{125}I-hCG at 37°C for 1 h under an atmosphere of 5% CO_2 in air. Unspecific binding was determined by the addition of 20 IU unlabelled hCG in 5 μl 10 min prior to the addition of the tracer. To ensure that the granulosa cells were incubated at a saturating

Table 1. Specificity of the antiserum raised against progesteron-11α-hemisuccinate-BSA

Steroid	Cross-reaction (%)
Progesterone	100
5α-Pregnane-3,20-dione	24.1
3α-Hydroxy-5α-pregnane-20-one	0.9
20α-Hydroxy-5α-pregnane-3-one	<0.1
5α-Pregnane-3α,20α-diol	<0.1
20α-Hydroxy-4-pregnene-3-one	0.2
20β-Hydroxy-4-pregnene-3-one	0.6
17-Hydroxyprogesterone	0.8
Cortisol	<0.1
Androstendione	<0.1
Cholesterol	<0.1

concentration of labelled hCG, incubations with two or three different concentrations of granulosa cells were performed in each experiment. The second incubation was terminated by the addition of 2 ml ice cold gelatin-PBS. The cells were centrifuged for 10 min at 2100 *g* at 4°C. The pellet was washed once with 2 ml gelatin-PBS and the radioactivity was counted in an automatic gamma counter. Specific binding was calculated as the difference between binding in the absence and presence of an excess unlabelled hCG.

Radioiodination of hCG (obtained from Calbiochem, 11,500 IU/mg) was performed by a lactoperoxidase method essentially as has been described [7]. However, the reaction was terminated by the addition of 300 μl PBS and the free iodine was separated from the labelled hormone by gel chromatography on Sephadex G-50. The specific activity of the hormone, calculated as described [8], ranged from 15–20 μCi/μg. The biological activity estimated as the specific binding in the presence of an excess of binding sites (rat testicular homogenate) varied between 38% and 45%.

Progesterone was determined by RIA after evaporation of 100–500 μl aliquots of the ethanolic supernatant. The antiserum used was developed by immunizing Chinchilla rabbits against progesterone-11α-hemisuccinate-BSA (Table 1). In order to evaluate the specificity of the direct assay 12 samples were determined in addition after thin-layer chromatography in benzene–ethyl acetate (75:25), a system which clearly separated progesterone from 5α-pregnan-3, 20-dione, and were corrected for procedural losses by an internal tritium-labelled standard. It was found that samples assayed by the direct method were overestimated by 8%.

Cyclic AMP was determined after evaporation of 10–50 μl aliquots of the ethanolic supernatant using a RIA kit from New England Nuclear Corporation, Boston, USA. To achieve high sensitivity, standards and unknowns were acetylated as described [9].

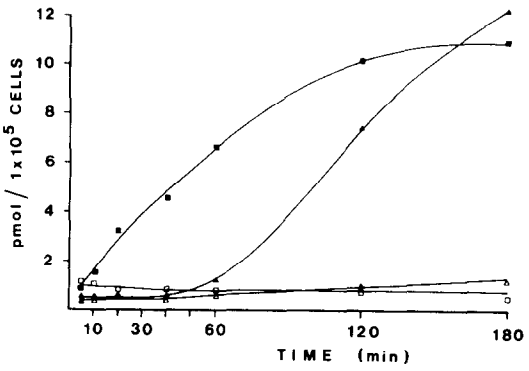

Fig. 1. Time course of progesterone and cAMP production by isolated rat granulosa cells from preovulatory follicles incubated in the presence of 0.02 IU/ml hCG (closed symbols) and in the absence of hCG (open symbols). All incubations were performed in the presence of the phosphodiesterase inhibitor IBMX (0.1 mM). Each symbol represents the mean of duplicates. Progesterone is indicated by triangles and cAMP by squares.

RESULTS

The time course of cAMP and progesterone production is shown in Fig. 1. In the presence of hCG (0.02 IU/ml) and IBMX (0.1 mM) a significant production of cAMP was seen within 10 min of incubation while progesterone did not increase earlier than 40 min. When granulosa cells were stimulated by a much higher dose (1.0 IU hCG/ml) a significant pro-

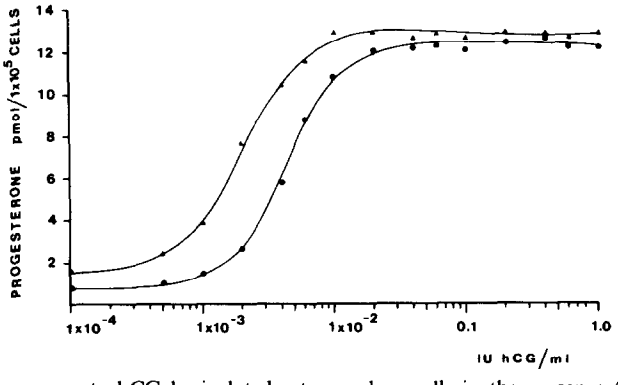

Fig. 2. Progesterone response to hCG by isolated rat granulosa cells in the presence (triangles, mean of duplicates) and absence (dots, mean of duplicates) of the phophodiesterase inhibitor IBMX (0.01 mM).

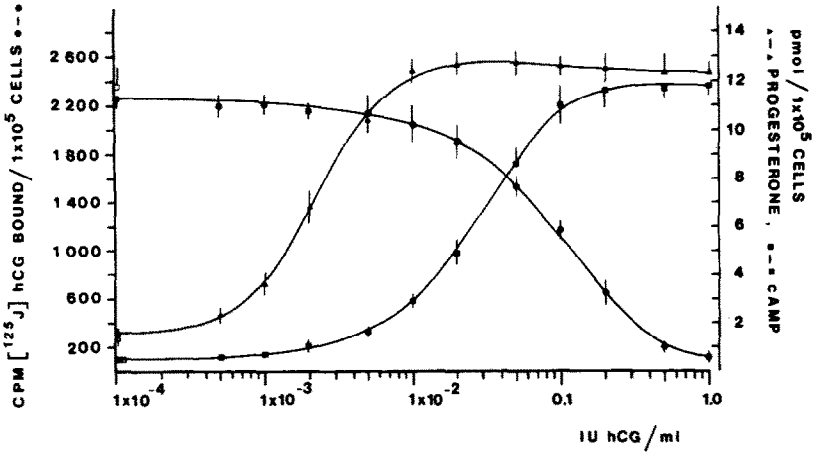

Fig. 3. Relation between receptor occupancy and responses in progesterone and cAMP. Granulosa cells isolated from preovulatory rat follicles were incubated in the presence of 0.1 mM IBMX and either in the absence (open symbols) or in the presence (closed symbols) of increasing concentrations of hCG for 2 h. After two washings the unoccupied binding sites were saturated by radioiodinated hCG. The results of three different experiments are combined. Means ± 1 SD are given; $n = 9$ for receptor occupancy and $n = 6$ for progesterone and cAMP respectively.

duction of progesterone was seen as early as 20–40 min after the onset of incubation. In the absence of hCG only progesterone showed a small but significant increase.

The sensitivity of the granulosa cell response to hCG was considerably increased in the presence of IBMX (Fig. 2). Half-maximum response was seen at 1.7×10^{-3} IU/ml in the presence of IBMX and at 4.8×10^{-3} IU/ml in the absence of IBMX.

The relation between receptor occupancy and responses of progesterone and cAMP is shown in Fig. 3. The results of three different experiments were combined. From these data it was calculated that only 3% of the available binding sites were occupied at the half-maximum progesterone response which occurred at 2.0×10^{-3} IU hCG/ml. At 2.6×10^{-2} IU/ml where half-maximum cAMP was seen and progesterone response approached the maximum, 19% of the available binding sites were occupied.

DISCUSSION

A wide range of relationships between receptor occupancy and specific cellular responses has been observed in various target tissues [10]. In rat Leydig cells maximum steroidogenic response was achieved when only 1% of the LH receptors were occupied by hCG. However, for maximum cAMP production half-saturation of the LH receptors was required [1]. With respect to the relation between cAMP response and receptor occupancy granulosa cells resemble rat Leydig cells. In granulosa cells, in contrast to Leydig cells, maximum steroidogenic response was achieved when 15–20% of the LH receptors were occupied. Our results are restricted to granulosa cells of PMSG-induced preovulatory follicles which were not yet exposed to the endogenous LH surge. Under conditions leading to receptor desensitization [11, 12] the relation between receptor saturation and biological response may be quite different from that seen in our study. There is increasing evidence that even for a given cell type no simple relation between receptor occupancy and cAMP response may be found. Due to previous hormone exposure the LH/hCG stimulable adenylate cyclase may show a partial refractoriness [11–13]. Moreover, the responsiveness of the adenylate cyclase is modulated by prostaglandin E_2 and guanosine triphosphate [12].

For the rat Leydig cell it has been questioned whether the formation of cAMP plays a direct role in the gonadotropin-induced steroidogenesis since testosterone production was maximal in the absence of detectable amounts of cAMP [1]. In our experiments with granulosa cells progesterone production became apparent at hCG concentrations which did not induce a detectable production in cAMP. The shift of the entire range of the response curve to lower hCG concentrations in the presence of the phosphodiesterase inhibitor IBMX strongly suggests that progesterone synthesis is mediated by cAMP.

For obvious technical reasons we used preovulatory follicles from PMSG-primed immature rats rather than from cycling rats. The validity of the PMSG-primed immature female rat preparation as a model for the estrous cycle of the adult rat has been demonstrated [5, 15, 16]. Recently a concomitant increase in the activity of the LH-sensitive adenylate cyclase and in the number of LH/hCG binding sites in ovaries of immature rats following a single injection of PMSG has been reported [14].

In conclusion, we have shown that in rat granulosa cells obtained from preovulatory follicles a considerable proportion of the available LH/hCG receptors must be occupied to evoke maximum steroidogenic response. This finding differs markedly from that seen in rat Leydig cells where maximum steroidogenic re-

sponse is achieved when only about 1% of the available receptors are occupied.

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