APPEARANCE OF LH-RECEPTORS AND LH-STIMULABLE CYCLIC AMP ACCUMULATION IN GRANULOSA CELLS DURING FOLLICULAR MATURATION IN THE RAT OVARY

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SUMMARY

The number of receptors for human chorionic gonadotropin (hCG) was low in granulosa cells (GC) of follicles at 9.00 h on the day of metestrus (530 ± 150 sites/cell; mean ± S.E.) and gradually increased thereafter to reach a maximum at 21.00 h on the day of proestrus (24,300 ± 700). There was no significant difference in ^{125}I-hCG binding between GC collected before the preovulatory LH-surge and cells collected 7 h after the surge. LH-stimulable cyclic AMP accumulation was higher in GC collected 7 h after the surge than in GC collected before the surge or in GC from animals in which the LH-surge was blocked by treatment with Nembutal. Exogenous hCG (50 IU/rat) also failed to induce desensitization of LH-stimulable adenylate cyclase in GC collected 10 h after the injection. The results show (i) cyclic changes in the LH-receptor population of rat GC compatible with the concept that the receptor is induced by the estrogen and FSH surges of the preceding cycle, and (ii) failure of the preovulatory LH-surge to induce refractoriness of adenylate cyclase in rat GC *in vivo*.

The maturation of the Graafian follicle is associated with the appearance of receptors (1) for luteinizing hormone (LH), which prepares the follicle for the action of the ovulatory LH-surge. Induction of the LH-receptor is believed to be under the control of follicle stimulating hormone (FSH), since it has been shown that FSH induces LH binding sites in granulosa cells (GC) of rat preantral follicles both *in vivo* (2) and *in vitro* (3). Human chorionic gonadotropin (hCG) binds specifically to the LH-receptor of rat ovaries (4). Although previous studies of binding of labeled hCG to isolated porcine GC have shown that LH-receptor concentration increases with follicle size (1), a precise correlation between LH-receptor levels and follicular development has not hitherto been demonstrated.

Exposure of preovulatory follicles *in vitro* to a high dose of LH has been shown to render them refractory to further stimulation by LH (5-8).

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It has been reported that this process also occurs *in vivo* after the surge of LH (9-12). The mechanism underlying this desensitization of the follicle to LH is yet unclear, and opinions diverge whether or not a loss of receptor sites is involved (8,10,13).

In the present study, the appearance of the LH-receptors on GC of intact Graafian follicle was quantitatively analysed during follicular maturation. In addition, both the binding of ^{125}I-hCG and the activity of LH-stimulable cyclic AMP accumulation of GC were measured in relation to the LH-surge, in an attempt to resolve the question whether the LH-surge induces refractoriness to the hormone in GC *in vivo*.

MATERIALS AND METHODS

Animals. Three- to four-month-old Wistar-derived female rats (170-210 g) of the departmental colony were housed in air-conditioned quarters illuminated between 05.00 and 19.00 h. Only animals that had at least two consecutive normal 4-day cycles, as determined by daily vaginal smears, were used. Pentobarbitone sodium (Nembutal, Abbot, 6 mg/rat) or hCG (Pregnyl, Organon, 50 IU/rat) were administered intraperitoneally at 14.00 h on the day of proestrus where indicated.

Cell collection. Animals were killed at specified times by cervical dislocation, and the ovaries were excised and immersed either in ice-cold buffered sucrose solution for binding studies (14) or in Krebs-Ringer bicarbonate-glucose buffer for adenylate cyclase assay (15). Granulosa cells were isolated by puncture of the largest follicles (4-7 follicles per ovary) and gentle expression into medium using a dissecting microscope (15). Cells derived from 3 animals were combined, sedimented by centrifugation at 250 x g for 8 min at 4°C and resuspended in an appropriate volume of buffer for ^{125}I-hCG binding studies or for the assay of LH-stimulable cyclic AMP accumulation.

Assay of hCG binding. Iodination of hCG (13,500 units/mg; Serono, Italy) was carried out by the lactoperoxidase method described by Miyachi *et al.* (16). The biological activity of the labeled preparation was determined by assay in an adenylate cyclase system (17) and used to determine the mass of active hormone. Based on this mass, the specific radioactivity of labeled hCG was found to be 20-50 μCi/μg. Binding of ^{125}I-hCG to granulosa cells was assayed as described by Nimrod *et al.* (3). Briefly, cell suspensions (50 μl containing 5-8 x 10^4 cells) were incubated for 3 h at 37°C in a shaking incubator together with 50 μl of buffer containing 11 ng/ml of ^{125}I-hCG and varying amounts of unlabeled hCG. Nonspecific binding was determined by incubating cells in the presence of a 200-fold excess of unlabeled hCG, and this value was substracted from all values of bound radioactivity prior to the calculation of binding parameters (14).

LH-stimulable cAMP accumulation. Cell suspensions (2-5 x 10^5 cells/ml) were incubated with shaking at 37°C for 20 min in Krebs-Ringer bicarbonate buffer (0.4 ml) containing 1 mg/ml glucose and 0.1 mg/ml of 3-isobutyl-1-methyl-xanthine (IBMX). Some samples also contained various amounts of ovine

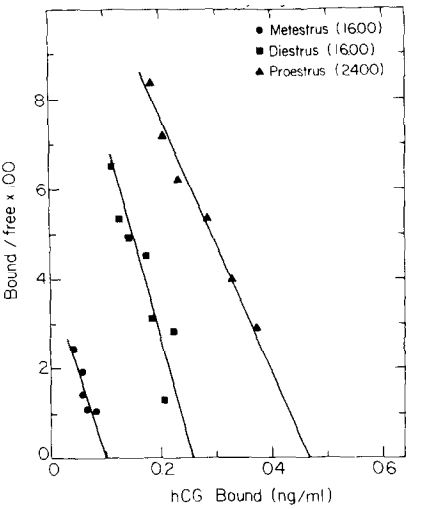

Figure 1. Scatchard-plot analysis of ^{125}I-hCG binding to granulosa cells obtained from rat follicles at different stages of the estrous cycle. Cell suspensions (0.1 ml; 5 x 10^5 cells/ml) were incubated in triplicate with ^{125}I-hCG and varying amounts of unlabeled hCG. Each line is derived from incubation of aliquots of a cell suspension obtained from 3 rats. The number of binding sites per cell, obtained from the intercept of the lines with the abscissa, was 2,500, 5,570 and 11,400 sites/cell for metestrous, diestrous and proestrous rats, respectively.

LH (NIH-LH-S18, kindly made available by the NIAMDD, N.I.H., Bethesda, Md.). The reaction was stopped by addition of 0.4 ml of 0.05 M sodium acetate buffer, pH 4.0 (5), and boiling for 5 min. Samples were then frozen and kept at -20°C. Cyclic AMP content was assayed by the method of Gilman (18).

RESULTS

Binding of ^{125}I-hCG to GC at various stages of the estrous cycle.. GC of Graafian follicles exhibited specific binding of ^{125}I-hCG, with an apparent association constant (Ka) of about 10^{10} M^{-1}, based on a molecular weight of 47,000 daltons (Fig. 1). The number of binding sites per cell, as determined from Scatchard plots (Fig. 1), was low in the morning of the day of metestrus (530 ± 150 sites/cell; mean ± S.E.; Fig. 2), gradually increased during metestrus and diestrus, then increased steeply on proestrus, to reach a maximum (24,300 ± 700) at 21.00 h. A small, yet significant ($p < 0.05$) decline in the number of binding sites (15,000 ± 3,200) was observed in GC collected at 24.00 h on the day of proestrus (Fig. 2).

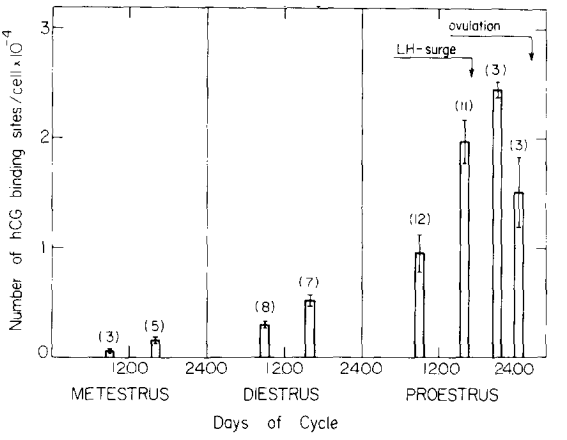

Figure 2. Appearance of hCG binding sites in granulosa cells of the developing ovarian follicles during the estrous cycle of the rat. The bars and vertical lines indicate the mean ± S.E. of the number of binding sites per cell obtained by Scatchard-plot analysis, as described in the Method section. The figures in brackets indicate the number of cell suspensions used, each obtained from 2-3 rats.

Rate of cyclic AMP accumulation in GC before and after the endogenous LH-surge. In two sets of experiments, cells were collected from preovulatory follicles on proestrus at 16.00 h (i.e. about one h before the peak of the LH-surge), and at 21.00 and 24.00 h, i.e. 3 h and 7 h, respectively, after the peak of the LH-surge (19). Both basal and LH-stimulable cyclic AMP accumulation increased after the LH-surge (Table 1).

In a third set of experiments, proestrous animals were divided into 3 groups assayed concurrently: one group served as a control, another group was injected at 14.00 h with Nembutal in order to block the LH-surge, and a third group was treated at 14.00 h with hCG (50 IU/rat; viz. 20-times the minimal ovulatory dose) in an attempt to induce refractoriness in the GC *in vivo*. The results, given in Table 1, confirmed the finding of increased cyclic AMP accumulation after the endogenous surge, since both the basal and LH-stimulable activities of GC adenylate cyclase were higher in control than in Nembutal-treated rats. Administration of hCG *in vivo* failed to desensitize LH-stimulable adenylate cyclase in follicular GC within 10 h after treatment with the hormone.

Table 1. LH-stimulable cAMP accumulation by granulosa cells isolated from preovulatory follicles of proestrous rats. IBMX (100 µg/ml) was added.

Experiment No.	Treatment	Time of Sacrifice (h)	cAMP content* before incubation (pmol/tube)	cAMP accumulation* (pmol/tube/20 min) ovine LH (µg/ml) 0	0.1	0.4	10.0
1	Untreated	15.30	2.5 ± 0.3	2.5 ± 0.3			13.5 ± 2.5
		21.00	3.5 ± 0.3	4.0 ± 0.2			13.5 ± 2.0
		24.00	4.5 ± 0.3	6.0 ± 0.5			24.0 ± 2.0
2	Untreated	15.30	4.5 ± 0.2	4.2 ± 0.2		12.7 ± 1.7	14.8 ± 2.7
		21.00	6.8 ± 0.5	9.0 ± 0.1		30.9 ± 1.6	34.7 ± 1.8
		24.00	9.0 ± 1.4	13.4 ± 1.7		41.8 ± 5.8	46.5 ± 5.3
3	Untreated	24.00	2.0 ± 0.1	2.9 ± 0.4	9.6 ± 2.5		11.3 ± 3.5
	Nembutal**	24.00	1.3 ± 0.1	1.2 ± 0.1	3.8 ± 1.0		5.1 ± 1.0
	hCG**	24.00	2.2 ± 0.2	4.7 ± 0.8	11.6 ± 3.4		12.4 ± 4.8

* Values represent the mean ± S.E. of cAMP accumulation by 3 cell suspensions, each derived from 3 rats. Granulosa cell suspensions were incubated in duplicate, and cAMP content in each sample (cells plus medium) was assayed in duplicate as described in the Methods section.

** Nembutal (6 mg/rat) and hCG (50 IU/rat) were given intraperitoneally at 14.00 h.

DISCUSSION

Several authors have reported that during follicular growth there is an increase in the number of LH-hCG receptor sites in GC (1). The present results are in accordance with these observations and, in addition, provide a precise time-sequence of the appearance of GC binding sites during follicular maturation in the rat. Induction of LH-receptors in GC of immature rat follicles has been shown to be controlled by the sequential action of estrogen and FSH (2,3,20). A minimal time-lapse of 48 h is required after estrogen priming and exposure to FSH before LH-receptors are induced (17,20) and maximal number of binding sites are not attained before about 72 h (20). A similar hormonal control may take place during cyclic maturation of follicles in the adult rat: a steep rise in the number of ^{125}I-hCG binding sites is

observed (Fig. 2) in GC of follicles which have been exposed in the preceding estrous cycle to elevated estrogen levels (mid-diestrus to mid-proestrus; 21) followed by a FSH-surge (mid-proestrus to mid-estrus; 22). Likewise, a steep increase in ovarian adenylate cyclase activity in rat follicles was observed between 10.00 h of diestrus and 10.00 h of proestrus (11).

The number of available hCG binding sites was unchanged 3 h after exposure to the endogenous LH-surge (Fig. 2). An effect of bound LH, however, can be inferred from the rise in the rate of cyclic AMP accumulation in hormone-free medium (Table 1). The observed biological response to LH in the absence of a detectable decrease in available receptor sites *in vivo* is consistent with the existence of "spare" receptors described in several hormone-responsive tissues (23).

The present results indicate that GC do not become refractory to LH following exposure to the preovulatory surge and to exogenous hCG with respect to adenylate cyclase activity, nor is the number of receptor sites significantly decreased at that time: the number of available LH receptors in GC 7 h after the LH-surge, although lower than that observed 4 h earlier, was not significantly different from that measured just prior to the surge (Fig. 2). Moreover, LH-stimulable cyclic AMP accumulation markedly increased in GC following exposure to the endogenous surge of the hormone (Table 1). These findings are in agreement with preliminary data suggesting a lack of desensitization to endogenous LH in GC isolated from PMSG-treated rats (Prof. K. Ahrén, personal communication), but are in contrast with reports of preovulatory changes in LH sensitivity of rabbit (9,10) and rat (11,12) adenylate cyclase of intact Graafian follicles. It may thus be that refractoriness of the whole follicle reflects the behavior of follicular components other than the GC, or that follicular desensitization is dependent on the formation of an inhibitory factor, which has been washed away during cell collection.

An alternative explanation is suggested by the report of Conti *et al.* (13) that the rate of desensitization of adenylate cyclase activity by exo-

genous hCG in luteinized rat ovaries was dependent on the dose of hormone injected. *In vitro* desensitization of follicles to high doses of LH is maximal after 12-24 h, but 50% desensitization is attained within 6 h (8). It may well be that our failure in observing desensitization in GC 7 h after exposure to the LH-surge and 10 h after exposure to hCG derives from the fact that GC are exposed *in vivo* to lower hormone concentrations than those applied in the *in vitro* experiments and that therefore the onset of refractoriness *in vivo* is a later event. Extension of the time beyond that selected here would, however, complicate interpretation of the results, since the GC luteinize following ovulation, thus undergoing extensive differentiation.

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