Journal of Cellular Biochemistry 21:209–217 (1983)
Evolution of Hormone-Receptor Systems 337–345

Epidermal Growth Factor and Gonadotropin-Releasing Hormone Inhibit Cyclic AMP–Dependent Luteinizing Hormone Receptor Formation in Ovarian Granulosa Cells

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The induction of luteinizing hormone (LH) receptors was studied in granulosa cells prepared from the ovaries of hypophysectomized diethylstilbestrol-treated immature rats. Incubation of granulosa cells for 48 h with increasing concentrations of follicle-stimulating hormone (FSH) or choleragen caused parallel rises in cAMP levels and LH receptors. These observations, with the finding that 8-Bromo-cAMP also induced LH receptor formation, indicate that hormonal stimulation of LH binding sites is mediated by cAMP. Peptide hormones that inhibited FSH-stimulated cAMP production, such as epidermal growth factor (EGF) and a gonadotropin-releasing hormone agonist (GnRHa), also prevented LH receptor formation. GnRHa and EGF had negligible effects on FSH-stimulated cAMP production from 0 to 24 h of culture, but reduced cAMP accumulation by 80% and 90%, respectively, from 24 to 48 h when the majority of LH receptors appeared. FSH-sensitive adenylate cyclase activity, as measured by the conversion of (^{3}H)-ATP to (^{3}H)-cAMP, was inhibited by GnRHa and EGF at 48 h of culture. EGF and GnRHa also reversed the inhibition of ectophosphodiesterase activity caused by FSH in granulosa cells between 48 and 72 h of culture. Both EGF and GnRHa inhibited induction of LH receptors by 8-Bromo-cAMP, suggesting that their effects are also on cAMP action. Addition of GnRHa, but not EGF, between 36 and 48 h of culture completely prevented further increases in LH receptors induced by 8-Bromo-cAMP, indicating that the inhibitory action of GnRHa can be initiated at later times during granulosa cell differentiation, whereas full expression of EGF action requires a longer period. These results demonstrate that EGF and GnRH inhibit FSH-induced LH receptor formation in the granulosa cell by reducing hormone-dependent cAMP production and also by impairing the ability of cAMP to stimulate LH receptor formation.

Key words: hormone receptors, cyclic AMP, epidermal growth factor, granulosa cells, gonadotropin-releasing hormone, follicle-stimulating hormone

Received April 22, 1982; accepted December 30, 1982.

The granulosa cell of the ovarian follicle provides a valuable model for studies on homone-induced cellular differentiation. During follicular maturation, the pituitary glycoprotein follicle-stimulating hormone (FSH) induces and maintains receptors for luteinizing hormone (LH), prolactin, and gonadotropin-releasing hormone (GnRH) [1–5]. These actions of FSH are expressed in vitro during culture of undifferentiated granulosa cells from ovaries of hypophysectomized estrogen-treated immature rats [3–5]. Since the release of the oocyte from the follicle depends on the responsiveness of granulosa cells to the preovulatory surge of LH at midcycle, the FSH-induced acquisition of LH receptors by the granulosa cell is a critical aspect of ovarian follicular maturation and ovulation [1,6]. Recently, inhibition of LH receptor formation by the hypothalamic peptide, gonadotropin-releasing hormone (GnRH) [5,7], and by epidermal growth factor (EGF) [8] has been shown to attenuate granulosa cell development. Similarly, in a cloned strain of Leydig tumor cells, EGF reduced LH receptors and gonadotropin-induced steroidogenesis [9].

FSH binds to homologous receptors on the surface of the immature granulosa cell and stimulates adenylate cyclase activity and cAMP production. Although this cyclic nucleotide has been generally accepted as the mediator of acute actions of FSH and possibly of its long-term effects [2], evidence in support of the latter action has been lacking. In studies on cultured granulosa cells, we have recently shown that FSH increases cAMP accumulation, and that expression of steroidogenic enzymes and hormone receptors, and morphological development are mediated by cAMP-dependent processes [5,10,11]. The present report provides evidence for the stimulatory role of cAMP in LH receptor development, and demonstrates that GnRH and EGF prevent granulosa cell differentiation by inhibiting cAMP production and action.

MATERIALS AND METHODS

Twenty-one-day-old female, hypophysectomized rats implanted with diethylstilbestrol in 10-mm Silastic capsules to stimulate granulosa cell proliferation [12] were obtained from Hormone Assay Laboratories (Chicago). Five days after surgery and diethylstilbestrol treatment, ovaries were removed and granulosa cells were isolated as previously described [10]. Cell viability was between 50% and 70%. Viable cells (2×10^5) were added to 35-mm tissue culture wells in a total volume of 1.0 ml of McCoy's 5A medium (modified, without serum) supplemented with 10 mM Hepes, pH 7.4, 4 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin sulfate. Ovine FSH (NIH-FSH-S13, National Pituitary Agency), 8-Bromo-cyclic AMP (8-Br-cAMP; Sigma, St Louis), choleragen (Sigma), (D-Ala^6)des-Gly^{10}-GnRH N-ethylamide (GnRHa; Peninsula Laboratories, San Carlos, California), and epidermal growth factor (EGF; Collaborative Research, Wortham, Massachusetts) were added in 10-μl volumes in sterile medium at the beginning of culture to produce the final concentrations indicated. In some experiments, GnRHa or EGF was added between 36 and 48 h of culture. Cells were cultured at 37°C in a humidified 95% air, 5% CO_2 incubator. For determination of cAMP levels, medium was removed at the indicated times of culture, boiled for 10 min, and stored frozen at −20°C until radioimmunoassay [10]. Extracellular levels of cAMP have been previously shown to be comparable to total amounts of the cyclic nucleotide at 48 h of culture [10]. Cell number was not altered by hormones during 48 h of culture.

For analysis of LH receptors, cells were scraped from the wells after 36 or 48 h of culture with a rubber policeman, washed twice, and incubated for 16 h with a saturating concentration of ^{125}I-human chorionic gonadotropin (hCG), which binds to the LH receptor in ovary and testes [1,13]. Nonspecific binding, determined in the presence of excess hCG, was subtracted from total binding to give specific binding.

Adenylate cyclase activity during granulosa cell culture was determined by measuring the conversion of (^{3}H)-ATP to (^{3}H)-cAMP as previously described [14,15]. This method is based on the uptake of (^{3}H)-adenine and the labeling of intracellular pools of ATP. Upon addition of hormone, adenylate cyclase is rapidly activated with the subsequent formation of (^{3}H)-cAMP, which in the presence of phosphodiesterase inhibitors is an indicator of cAMP synthesis. Briefly, after 48 h of culture, granulosa cells were washed twice to remove hormones and accumulated cAMP, and labeled with 20 μCi of (^{3}H)-adenine for 3 h. Cells were then washed twice and recultured with hormones for 1 h in the presence of 1.0 mM 1-methyl-3-isobutylxanthine (MIX) to inhibit phosphodiesterase activity. A previous report has indicated that 0.4 mM MIX is sufficient to prevent activation of phosphodiesterase activity by GnRHa as assessed by measurement of extracellular cAMP accumulation [14]. Radiolabeled ATP levels were similar after all hormone treatments. Measurement of cAMP catabolism was previously described and is indicative of phosphodiesterase activity in the plasma membrane [14]. Cyclic AMP catabolized from 48 to 72 h of culture was measured.

Statistical analyses were performed with Student's t-test for comparison of two groups, or analysis of variance with the Newman-Keul's multiple range test for comparison of more than two groups.

RESULTS

Incubation of granulosa cells for 48 h with FSH or choleragen induced a simultaneous rise in both cAMP accumulation and LH receptor formation (Fig. 1A, C). These stimulatory actions of FSH and choleragen were concentration-dependent, as increasing amounts of each ligand caused parallel rises in both cAMP production and LH receptor levels. Incubation with 8-Br-cAMP also stimulated a concentration-dependent elevation in LH receptors (Fig. 1B). These findings indicate that cAMP mediates the actions of FSH and other cAMP-inducing agents on LH receptor formation. Also, EGF and GnRHa inhibited FSH and choleragen-stimulated cAMP production (Fig. 2, top, $P < 0.05$), and prevented the cAMP-mediated effect on LH receptors at 48 h of culture (Fig. 2, bottom, $P < 0.05$). EGF and GnRHa had only minor effects on cAMP levels and LH receptors in the absence of FSH or choleragen. The inhibitory actions of EGF and GnRHa on cAMP production and LH receptor formation were primarily expressed after the first 24 h of culture (Table I). GnRHa and EGF had negligible effects on FSH-stimulated cAMP production in the first 24 h of culture, when few LH receptors appeared. However, from 24 to 48 h of culture, a marked rise in LH receptors occurred in FSH-treated cells, and this increase was prevented by both EGF and GnRHa due partially to an inhibition of cAMP production during that time.

Both EGF and GnRHa also attenuated the induction of LH receptors by 8-Br-cAMP (Fig. 3, $P < 0.05$), indicating that their inhibitory effects were exerted at a site distal to, as well as upon cAMP production. Studies on the temporal development

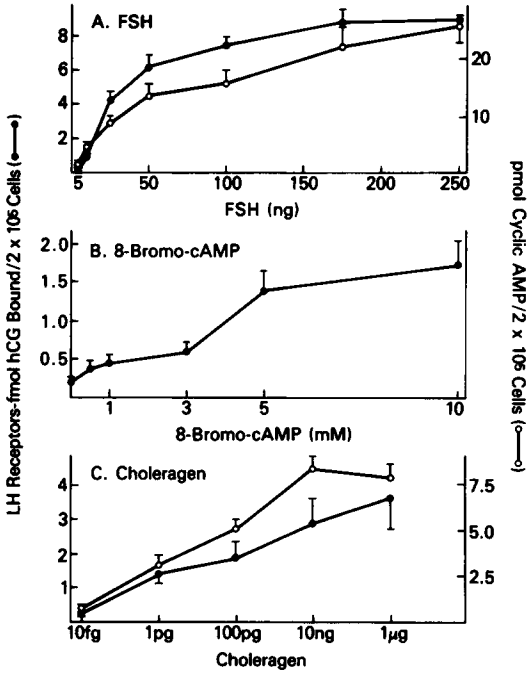

Fig. 1. Concentration-dependent elevation in cAMP accumulation and LH receptors by FSH, 8-Bromo-cAMP, and choleragen. Granulosa cells were cultured for 48 h with the indicated concentrations of hormone. Medium was then removed and saved for cAMP radioimmunoassay and cells were assayed for LH receptors. In this and subsequent figures, data are the mean ± SE of 3–10 samples.

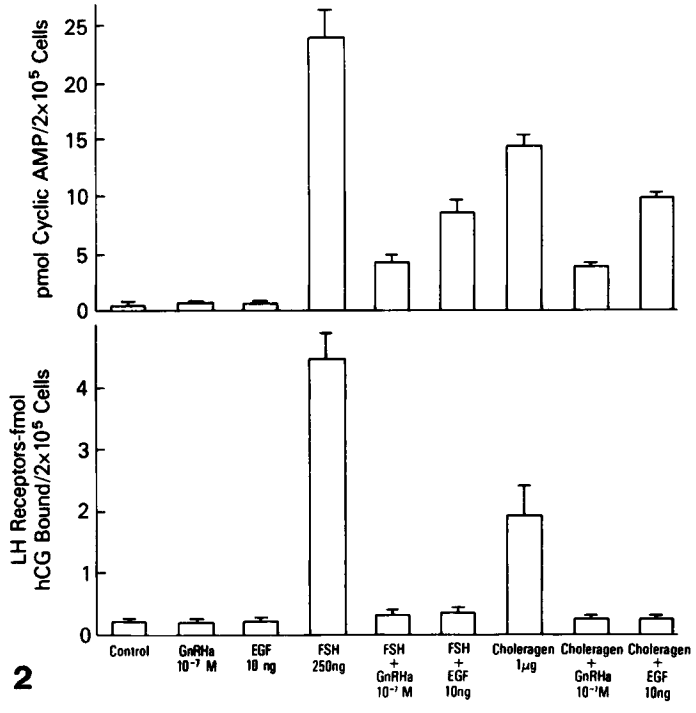

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TABLE I. EGF and GnRHa Inhibit cAMP Accumulation and LH Receptor Formation From 24 to 48 h of Granulosa-Cell Culture

Treatment	pmol cAMP (2×10^5 cells)	fmol hCG bound (2×10^5 cells)
A) 0 to 24 h		
Control	0.6 ± 0.1	0.1 ± 0.03
FSH, 250 ng	12.4 ± 0.5	0.4 ± 0.09
FSH plus EGF, 10 ng	12.7 ± 0.5	0.2 ± 0.02
FSH plus GnRHa, 10^{-7} M	11.0 ± 0.2	0.2 ± .03
B) 24 to 48h		
Control	0.5 ± 0.1	0.1 ± 0.03
FSH, 250 ng	11.4 ± 0.7	7.9 ± 1.3
FSH plus EGF, 10 ng	2.2 ± 0.1	0.3 ± 0.07
FSH plus GnRHa, 10^{-7} M	1.3 ± 0.1	0.1 ± 0.06

Granulosa cells were cultured for 24 h with the indicated reagents. A) Medium was then removed and saved for measurement of cAMP accumulation. Cells were washed once with fresh medium and recultured with the same hormones until 48 h, at which time medium was again removed and used for determination of cAMP levels (B). In separate wells, the LH receptor content of granulosa cells was measured after 24 h (A) and after 48 h (B). Values are the mean ± SE of 3–6 samples.

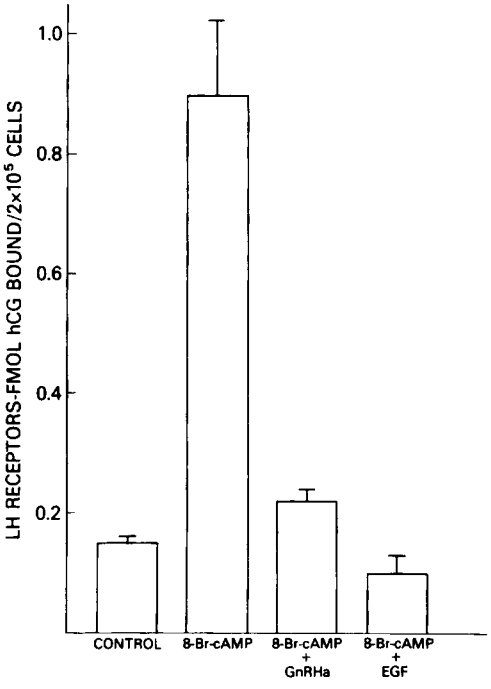

Fig. 3. Inhibition of 8-Bromo-cAMP–induced LH receptor formation by EGF and GnRHa. Final concentrations of 8-Br-cAMP (5mM), GnRHa (10^{-7} M), and EGF (10 ng) were added immediately prior to culture. Cells were assayed for LH receptors after 48 h of culture.

Fig. 2. Inhibition of FSH and choleragen-induced cAMP accumulation and LH receptor formation by EGF and GnRHa. All hormones were added immediately prior to culture, 48 h after which cAMP levels in the media and cellular LH receptor content were measured.

of the inhibitory actions of GnRHa and EGF indicated that addition of GnRHa after 36 h of 8-Br-cAMP treatment completely prevented any further rise in LH receptors, and partially reduced preexisting levels of receptors (Fig. 4). In contrast, EGF treatment caused only a partial inhibition of the 8-Br-cAMP–induced expression of LH receptors from 36 to 48 h of culture.

The mechanism of action of EGF and GnRHa on cAMP production involved alterations in both the synthesis and breakdown of the cyclic nucleotide. FSH induced an approximate 10-fold increase in the conversion of (^{3}H)-ATP to (^{3}H)-cAMP in the presence of 1.0 mM MIX after 48 h of culture (Fig. 5A). FSH-stimulated adenylate cyclase activity was 80% inhibited by EGF and was reduced to basal levels by GnRHa. Likewise, the FSH-mediated fall in phosphodiesterase activity between 48 and 72 h of culture was completely reversed by EGF and GnRHa (Fig. 5B).

DISCUSSION

These results indicate that the induction of heterologous hormone receptors by FSH in the ovarian granulosa cell occurs through cAMP-dependent pathways, and that inhibitory peptides such as EGF and GnRHa prevent LH receptor formation [5,7,8] by impairing cAMP accumulation and cAMP action. FSH, choleragen, and 8-Br-cAMP all induced LH receptor formation in a concentration-dependent manner as shown here and elsewhere [5,16], and the former two agonists increased cAMP levels while elevating LH receptors. FSH increased adenylate cyclase activity and reduced cAMP catabolism to elevate cAMP levels optimally. In contrast, EGF and GnRHa prevented cAMP synthesis at 48 h of culture, as well as increasing the degradation of any formed cAMP. Since both EGF and GnRHa inhibited LH receptor formation induced by 8-Br-cAMP in granulosa cells, a post-FSH receptor site of action for these peptides is also suggested.

As shown here and elsewhere [17], FSH exerts a biphasic effect on granulosa cell differentiation in vitro. An early phase of FSH-stimulated cAMP production occurs during the first few hours of culture prior to the onset of LH receptor synthesis. Subsequently, a secondary rise in cAMP accumulation, concomitant with the rise in LH receptors, is observed from 24 to 48 h of culture. Both EGF and GnRHa had little effect on cAMP accumulation during the first 24 h of culture, but markedly reduced cAMP production and subsequent LH receptor formation from 24 to 48 h. Thus, the increase in cAMP after 24 h of culture is directly associated with LH receptor induction and provides a site of action for hormonal peptides, such as EGF and GnRH, to modulate granulosa-cell maturation. The importance of the initial rise in cAMP during the first 24 h of culture is unclear, but may be related to the multicellular aggregation, formation of gap junctions, and intercellular communication occurring in granulosa cells at that time [11,17]. Furthermore, the activation by GnRHa and EGF of an ectophosphodiesterase that reduces extracellular cAMP levels may, in combination with the decrease in cAMP production, be involved in the disruption of cellular aggregation previously observed during the later stages of culture [17]. The involvement of cAMP in cellular function and communication has also been shown in the amoeba, Dictyostelium discoideum [18].

It is interesting that two structurally different peptide hormones, EGF and GnRH, altered granulosa-cell differentiation in a comparable manner. GnRH is a

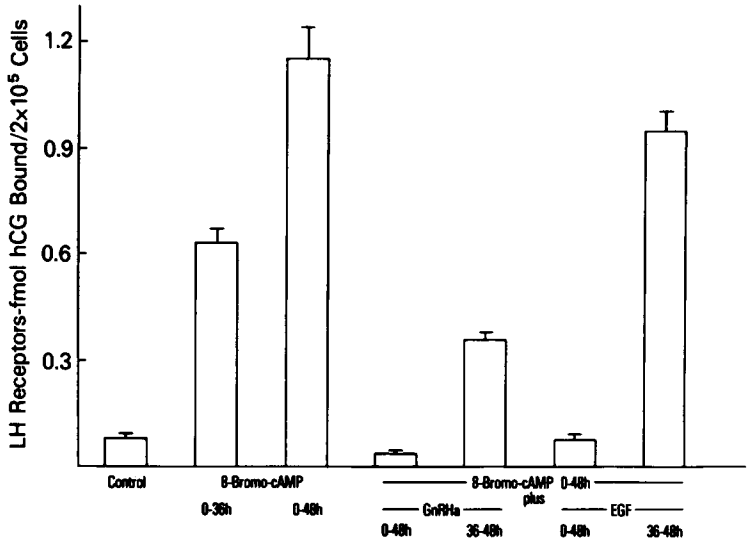

Fig. 4. Effect of GnRHa and EGF on LH receptor formation when added during granulosa-cell development. Cells were incubated with 8-Bromo-cAMP (5mM) for 36 h. EGF (10 ng) or GnRHa (10^{-7} M) was added from 0 to 48 h or from 36 to 48 h of culture. Cells were assayed for LH receptors after 48 h of culture, except for 8-Bromo-cAMP–treated cells, which were also utilized at 36 h.

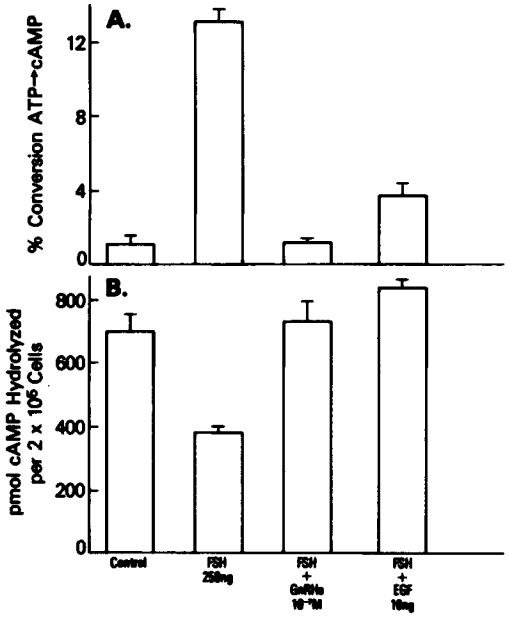

Fig. 5. Effect of FSH, EGF, and GnRHa on adenylate cyclase (A) and phosphodiesterase (B) activities. Granulosa cells were cultured for 48 h in the presence of the indicated agents and then assayed for cAMP biosynthesis and catabolism.

small hypothalamic peptide with a molecular weight of approximately 1,100, whereas EGF, originally isolated from the mouse submaxillary gland, is a larger polypeptide of approximately 6,000 molecular weight [19–21]. However, both EGF and GnRHa bind to specific receptor sites in the ovary and thus initiate their actions through receptor-dependent mechanisms [4,22]. In certain cell types, binding of EGF to its receptor has been shown to stimulate phosphorylation of membrane proteins, presumably related to EGF action [23]. Such phosphorylated substrates may directly affect FSH-sensitive adenylate cyclase, phosphodiesterase, and steroidogenic enzymes, or may indirectly alter ovarian function as modifiers of gene expression. Whether GnRH also activates protein kinase(s) is currently unknown.

In addition to similarities between the inhibitory actions of EGF and GnRHa, certain distinguishing characteristics were detected. GnRHa caused a larger reduction in FSH and choleragen-stimulated cAMP accumulation than did EGF, probably because of the more marked inhibition of adenylate cyclase activity by 48 h of culture. Also GnRHa action can commence when added late during granulosa-cell development, whereas EGF must be present during earlier stages of granulosa-cell culture to inhibit LH receptor formation. Furthermore, GnRHa has been shown to inhibit FSH-induced progesterone production markedly, and to partially prevent steroidogenesis stimulated by 8-Br-cAMP [5,7,10]. However, preliminary data suggest that EGF enhances choleragen and 8-Br-cAMP-induced progestin formation, and inhibits FSH-stimulated progesterone production owing to increased metabolism of progesterone to 20α-dihydroprogesterone. Total progestin levels are comparable in FSH and FSH plus EGF-treated cells, similar to the finding of Mondschein and Schomberg [8]. Further studies on the roles of EGF and GnRH in the hormonal control of follicular maturation are necessary to clarify their contributions to the normal processes responsible for cyclical ovarian function.

ACKNOWLEDGMENTS

Ovine FSH was a gift from the National Pituitary Agency.

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