The Regulatory Role of Cyclic AMP in Hormone-induced of Granulosa Cell Differentiation

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The induction of ovarian granulosa cell differentiation by follicle-stimulating hormone (FSH) was accompanied by increased cAMP production, expression of steroidogenic enzymes and hormone receptors, and cell clustering into epithelioid aggregates. These biochemical and morphological changes were reproduced by 8-Bromo-cAMP (8-Br-cAMP) and were inhibited by a gonadotropin-releasing hormone (GnRH) analog which impaired cAMP production. 8-Br-cAMP induced a concentration-dependent increase of up to 5-fold in luteinizing hormone (LH) and prolactin receptors of cultured granulosa cells from hypophysectomized diethylstilbestrol-treated rats. Scatchard analysis of gonadotropin binding data indicated that the affinity constants of hormone binding were similar after FSH or 8-Br-cAMP treatment, and that 8-Br-cAMP induced at least 50% of the receptor concentration present in FSH-treated granulosa cells. Agents elevating cAMP production, such as cholera and prostaglandin E1, also induced LH receptor formation and steroidogenesis, whereas 8-Br-cAMP had no effect on granulosa cell function. Both 8-Br-cAMP and cholera caused granulosa cells to form multilayered aggregates of epithelial-shaped cells, similar to those induced by FSH. The gonadotropin-releasing hormone analog, [d-Ala²]des-Gly⁰-GnRH N-ethylamide, markedly inhibited the morphological and biochemical changes induced by FSH and choleracon, concomitant with a reduction in cAMP production. The analog also prevented the actions of 8-Br-cAMP on granulosa cell function, indicating that its inhibitory effect occurs at a step beyond cAMP biosynthesis. These results demonstrate that endogenous or exogenous cAMP can produce the functional and structural features of granulosa cell maturation, and indicate that cAMP mediates the hormonal induction of phenotypic expression in the ovarian follicular cell.

cAMP has been shown to induce metaplastic growth in mammary glands (4), to transform embryonal carcinoma cells into parietal endoderm (5), to inhibit cellular proliferation (6, 7), to increase the number of insulin receptors in cultured lymphocytes (8) and melanocyte-stimulating hormone receptors in melanoma cells (9), and to mediate steroidogenesis in the adrenal, testis, and ovary (10–13). These effects of cAMP appear to be expressed through stimulation of protein kinase and the subsequent phosphorylation of intracellular proteins involved in gene expression, protein biosynthesis, and enzyme activation (14, 15).

Ovarian follicular tissue provides a model of cellular development from the undifferentiated to the mature state. Prior to pituitary gonadotropin stimulation, ovaries from immature rats consist predominantly of primitive granulosa cells surrounding intact ova (16). Treatment with FSH induces biochemical and morphological alterations including cyclic nucleotide accumulation and steroidogenesis, LH and prolactin receptor formation, organization of microtubules and microfilaments, microvillus formation, and follicular antrum development (17–23). Many of these changes occur both in vivo and in vitro, although the absence of serum or the presence of growth factors are crucial for the in vitro response to FSH (18, 24). The role of cAMP in the phenotypic expression and organization of ovarian tissue has not been explored previously, although its role in acute hormonal regulation is well defined. Thus, elevations in intracellular cAMP mediate steroidogenesis (25), stimulate protein kinase activity (26), and prevent dedifferentiation of ovarian cells in culture (22, 27). We have recently described the ability of FSH to stimulate cAMP accumulation (17) and to induce morphological alterations in granulosa cells isolated from hypophysectomized diethylstilbestrol-implanted rats (28). The present study investigates the regulatory role of cAMP and agents elevating cAMP levels on granulosa cell differentiation and suggests that cAMP modulates both the acute and long term aspects of hormone-dependent ovarian development.

MATERIALS AND METHODS

Twenty-five-day-old female, hypophysectomized rats were obtained from Harlan Sprague-Dawley Laboratories (Madison, WI). Silastic capsules (10 mm) containing diethylstilbestrol were implanted subcutaneously at the time of hypophysectomy in order to stimulate granulosa cell proliferation (29). Five days after surgery and diethylstilbestrol treatment, ovaries were excised and granulosa cells were isolated as previously described (17). Cell viability, as determined by trypan blue exclusion, was normally between 60 and 70%. Approxi-
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approximately 1-2 x 10^6 viable granulosa cells were added to 35-mm plastic tissue culture dishes in a total volume of 1.0 ml of McCoy's 5A medium (modified, without serum) supplemented with 10 mM Heps, pH 7.4, 4 mM L-glutamine, 100 units/ml of penicillin, and 100 µg/ml of streptomycin sulfate. Ovine FSH (NIH-FSH-S13), 8-Br-cAMP and other agents affecting cAMP production were added in 10-µl volumes at the beginning of culture. The GnRH analog, [α-Ala^2,Des-Gly^10, Nal^4]** GnRH N-ethylamide was obtained from Peninsula Laboratories, San Carlos, CA. Cells were cultured at 37°C in a humidified 95% air, 5% CO2 environment. Media were removed after 48 h of culture, heated at 100°C for 10 min, and stored at -20°C for radioimmunoassay of extracellular cAMP (17). Extracellular accumulation of cAMP at 48 h of culture is approximately 5-fold greater than intracellular levels (17). Progesterone levels were determined by radioimmunoassay on separate aliquots of media (17).

For analysis of LH receptor sites, media were removed after 48 h of culture and cells were scraped from the dish with a rubber policeman. Cells were washed twice by centrifugation with 2-ml aliquots of phosphate-buffered saline, pH 7.4, supplemented with 0.1% bovine serum albumin, and resuspended in phosphate-buffered saline/bovine serum albumin at a concentration of 1-3 x 10^6 cells per tube in the presence of a saturating concentration of [125I]-hCG CR119 (4-5 ng, approximately 50,000 to 100,000 cpm/ng). Nonspecific binding was determined in the presence of an excess (10 µg) of unlabeled hCG. After incubation for 15 h at room temperature, membrane-bound radioactive hormone was measured after separation by centrifugation. Nonspecific counts were subtracted to give the specifically bound hormone and counts were converted to picograms of bound hCG/2 x 10^6 cells. Scatchard analysis of LH receptors was performed with increasing amounts of radiolabeled hCG added to a constant quantity of cells.

The prolactin receptor content of cells cultured for 48 h was evaluated by incubating an increasing number of cells (2 x 10^5-5 x 10^6 cells) with 1 x 10^6 cpm of [125I]-human growth hormone (70 µCi/µg) in a final volume of 300 µl. Binding of labeled hormone was linear with respect to cell number. Nonspecific binding of radiolabeled hormone was determined in the presence of an excess of ovine prolactin (100 µg). Bound and unbound hormone was separated as described above. Human growth hormone was used as the lactogenic ligand for assay of prolactin receptors as previously described (30).

For examination of cultured cells by light microscopy, sterile glass cover slips (9 x 9 mm) were introduced into 35-mm wells prior to cell plating. After culture for 48 h, media were removed and cells were fixed with 3% glutaraldehyde in Millonig's phosphate buffer, pH 7.4, and were visualized by phase contrast optics (Leitz ortholux light microscope) at x 400 magnification.

RESULTS AND DISCUSSION

**cAMP-mediated Increase in LH Receptor Formation—**

Granulosa cells from hypophysectomized diethylstilbestrol-implanted rats exhibit a marked increase in LH receptors during culture with FSH in the absence of serum (18). Recent observations in vivo (31) and in vitro (17) have indicated that doses of FSH which cause LH receptor induction also stimulate cAMP accumulation. Evidence that cAMP plays a direct role in the induction of LH receptors is shown in Fig. 1. 8-Br-cAMP, a cAMP derivative that is relatively resistant to degradation by phosphodiesterase (32), induced a dose-dependent increase in binding of radiolabeled hCG to membranes of granulosa cells cultured for 48 h in the presence of the nucleotide. Concentrations of 8-Br-cAMP as low as 3 nM doubled the levels of hCG bound by granulosa cells, while 10 nM 8-Br-cAMP increased hormone binding 6-fold higher than basal levels, to approximately 40% of the concentration induced by FSH. The rate of receptor formation induced by 8-Br-cAMP was not more rapid than that produced by FSH, since only minor changes in hCG binding were elicited by either FSH or 8-Br-cAMP during the first 24 h of culture (data not shown).

The higher levels of gonadotropin binding induced by FSH than by 8-Br-cAMP could be due to several factors. FSH may produce higher concentrations of intracellular cAMP than those attained by diffusion of exogenous 8-Br-cAMP across the plasma membrane. Also, the added 8-Br-cAMP could be progressively degraded during incubation, whereas the production of cAMP in FSH-stimulated granulosa cells has been shown to increase during the period of incubation (17). Although alterations in cellular proliferation and viability could affect receptor content, no changes in these parameters were observed after FSH or 8-Br-cAMP treatment. Analysis of hormone binding data showed that the LH receptor content was higher after FSH treatment than with 8-Br-cAMP (Fig. 2) and that the affinity constants were similar (2.9 x 10^-9 M^-1 for 8-Br-cAMP and 2.6 x 10^-9 M^-1 for FSH). The binding capacity of cells treated with FSH was double the amount obtained with 8-Br-cAMP, 1.2 fg/cell versus 0.6 fg/cell, or approximately 18,350 versus 9,000 receptor sites/cell.

Further investigation of the cAMP-mediated effect on LH receptor induction suggested that this response was specific for cAMP and agents elevating cAMP accumulation. Cholerae inhibits the GTPase activity associated with the guanyl nucleotide regulatory subunit of adenylate cyclase (33). Thus, cellular cAMP levels rise in the presence of cholerae due to

![Fig. 1. Induction of LH receptor formation by 8-Br-cAMP in cultured granulosa cells. FSH, 250 ng, or increasing concentrations of 8-Br-cAMP were added to granulosa cells immediately prior to incubation. Levels of LH receptors were measured after 48 h of culture. Each bar is the mean ± S.E. of duplicate tubes from at least 5 separate experiments.](image1)

![Fig. 2. Saturation binding analysis (inset) and Scatchard plot of 8-Br-cAMP and FSH-induced LH receptor formation in vitro. Granulosa cells were cultured with either 5 mM 8-Br-cAMP or 250 µg of FSH for 48 h and prepared for LH receptor assay. Approximately 2 x 10^6 cells were incubated with increasing amounts of radiolabeled hCG as indicated in the inset. Scatchard analysis was derived from the binding data shown in the inset. O—O, 8-Br-cAMP-treated cells; □—□, FSH-treated cells.)
the maintenance of the active catalytic state. Table I shows that cholera toxin induced a concentration-dependent elevation in LH receptor production after 48 h of granulosa cell incubation. Similarly, the increase in LH receptor production induced by cholera toxin was correlated with a parallel rise in cAMP accumulation in the extracellular medium. Prostaglandins also stimulate cAMP production by activating adenylyl cyclase (26) and prostaglandin Ei induced a slight increase in LH receptors and extracellular cAMP levels (Table I). It is noteworthy that FSH induced larger increments in both cAMP and LH receptor levels than those attained by cholera toxin or prostaglandin Ei. 8-Br-cAMP failed to induce LH receptor development at concentrations of 10 mM or 1 mM (Table I), and down to 50 µM (not shown), indicating that the effect was specific for the adenine cyclic nucleotide. Although dibutyryl cAMP did not elevate LH receptor levels, the butyrate ion was inhibitory to this process since 2 mM sodium butyrate inhibited the effects of FSH on LH receptor formation and cAMP accumulation. These findings may explain the inability of dibutyryl cAMP to cause induction of LH receptors in a previous report (29) and in the present study.

**CAMP-mediated Increase in Prolactin Receptor Formation**—Several reports have indicated that prolactin has a specific luteotropic effect in the rat ovary (16, 34), and FSH has been shown to induce prolactin receptor formation in cultured granulosa cells (19). As noted above for the LH receptor, we found that prolactin receptor formation was stimulated by 8-Br-cAMP. Table II shows that 5 mM 8-Br-cAMP stimulated a 7-fold increase in binding of radiolabeled hormone to cultured granulosa cells, an increase in lactogenic receptors similar to that induced by FSH. These findings indicate that the regulation of both LH and prolactin receptors by FSH is mediated by cAMP. This form of heterologous receptor regulation can be contrasted with that exerted by several steroid hormones (35), in which cytosolic receptor activation and nuclear translocation are responsible for regulating the expression of receptors for polypeptide hormones.

### Table I

**LH receptor induction by factors elevating cyclic AMP accumulation in cultured granulosa cells**

Granulosa cells were cultured for 48 h in McCoy’s 5A medium in the presence of the reagents indicated. Media were then removed and saved for determination of extracellular cAMP. Data are the mean ± S.E. of the number of determinations shown in parentheses. ND = not determined.

| Treatment          | Cyclic AMP μmol/2 × 10^5 cells | Bound hCG pg/2 × 10^5 cells |
|--------------------|-------------------------------|-----------------------------|
| Control            | 0.2 ± 0.03 (9)                | 10 ± 3 (9)                  |
| FSH, 250 ng        | 18.9 ± 2.1 (11)               | 198 ± 42 (7)                |
| 8-Bromo-cyclic AMP, 10 mM | ND                           | 64 ± 12 (6)                 |
| Choleragen 10 μg/ml | 0.6 ± 0.2 (6)                 | 10 ± 4 (4)                  |
| 1 μg/ml            | 3.0 ± 0.6 (14)                | 51 ± 12 (4)                 |
| 100 μg/ml          | 5.0 ± 0.5 (12)                | 69 ± 17 (5)                 |
| 10 ng/ml           | 8.3 ± 0.7 (12)                | 106 ± 27 (5)                |
| 1 μg/ml            | 7.7 ± 0.8 (12)                | 133 ± 36 (5)                |
| Prostaglandin E1   |                              |                             |
| 10 ng/ml           | 1.6 ± 0.4 (11)                | 14 ± 3 (3)                  |
| 1 μg/ml            | 3.9 ± 0.4 (14)                | 22 ± 3 (4)                  |
| 8-Bromo-cyclic GMP |                              |                             |
| 1 mM               | ND                            | 10 ± 3 (5)                  |
| 10 mM              | ND                            | 8 ± 3 (5)                   |
| Dibutyryl cyclic AMP |                             |                             |
| 1 mM               | ND                            | 12 ± 3 (4)                  |
| 10 mM              | ND                            | 9 ± 5 (4)                   |
| FSH + Na butyrate, 2 mM | 2.0 ± 0.3 (6)                | 9 ± 2 (3)                   |

### Table II

**Prolactin receptor induction by 8-bromo-cyclic AMP and FSH in cultured granulosa cells**

Granulosa cells were cultured for 48 h in the presence of each reagent in McCoy’s 5A medium and were then assayed for prolactin receptor content. Numbers are the mean ± S.E. from 3 experiments.

| Treatment          | Bound human growth hormone µg/1 × 10^5 cells |
|--------------------|---------------------------------------------|
| Control            | 14 ± 7                                       |
| FSH 106, 250 ng    | 106 ± 34                                    |
| 8-Bromo-cyclic AMP |                                             |
| 1 mM               | 37 ± 12                                     |
| 5 mM               | 98 ± 36                                     |

### Table III

**Cyclic AMP-mediated progesterone biosynthesis and its inhibition by GnRHa in cultured granulosa cells**

Granulosa cells were cultured with the reagents indicated in McCoy’s 5A medium in the presence of or absence of GnRHa. After 48 h, media were removed and saved for determination of extracellular progesterone by radioimmunoassay. Data are the mean ± S.E. of the number of determinations shown in parentheses.

| Treatment          | GnRHa (10^8 M) | Nanograms progesterone/2 × 10^5 cells |
|--------------------|----------------|--------------------------------------|
| Control            | –              | 0.5 ± 0.1 (7)                        |
| FSH, 250 ng        | –              | 88.8 ± 6.5 (9)                       |
| +                  | 8.6 ± 0.6 (9)  |
| 8-Bromo-cyclic AMP |                |
| 5 mM               | –              | 58.0 ± 8.3 (9)                       |
| +                  | 47.9 ± 4.9 (9) |
| 10 mM              | –              | 145.4 ± 18.8 (6)                     |
| +                  | 73.7 ± 8.9 (6) |
| Choleragen         |                |
| 10 ng/ml           | –              | 26.2 ± 3.1 (12)                      |
| +                  | 14.8 ± 1.4 (12) |
| 1 μg/ml            | –              | 24.1 ± 3.3 (9)                       |
| +                  | 14.6 ± 1.1 (9) |

**inhibition of cAMP-mediated Granulosa Cell Function by a GnRH Agonist—GnRH and its potent agonists inhibit several aspects of granulosa cell function in the rat ovary. These include cAMP accumulation, steroidogenesis, LH receptor induction, and morphological differentiation (17, 36–39). The recent detection of specific GnRHa binding sites on ovarian membranes indicates that GnRH action is initiated by receptor-dependent mechanisms (40). Since the preceding
FIG. 4. Regulation of granulosa cell morphology by cAMP. Granulosa cells were cultured with no hormone, 250 ng of FSH, FSH plus $10^{-7}$ M GnRHa, 5 mM 8-Br-cAMP, 10 ng/ml of choleragen, or $10^{-7}$ M GnRHa alone. After 48 h, cells were fixed with 3% buffered glutaraldehyde and visualized by phase contrast optics at $\times 400$ magnification. a, in the absence of any hormone, granulosa cells formed monolayers and assumed a fibroblastic appearance. b, in the presence of FSH, cells were tightly packed with an epithelial shape and grew in multilayered aggregates. c, in the presence of FSH plus GnRHa, aggregation was markedly reduced. d, cells treated with 8-Br-cAMP were aggregated and had a round shape. Cell projections also interconnected cell clusters (arrows, compare to b). e, addition of choleragen caused aggregation and epithelial cellular shape (compare to b and d); f, incubation with GnRHa alone produced a monolayer of cells that were less flattened than untreated cells (a), with occasional aggregates of damaged cells. The bar represents 50 $\mu$m.

studies indicated that cAMP mediates many of the actions of FSH in granulosa cells, we examined the ability of GnRH to inhibit cAMP-induced LH receptor formation. As shown in Fig. 3, the GnRH agonist, GnRHa, at a concentration previously shown to inhibit cAMP accumulation (17), prevented the actions of 8-Br-cAMP, choleragen, and FSH on the induction of LH receptor synthesis. Levels of LH receptors were reduced by 95% with GnRHa treatment. GnRHa also lowered cAMP production in FSH-treated cells by 80% ($18.9 \pm 2.1$ pmol/2 x $10^6$ cells to $4.2 \pm 0.5$, $n = 11$), while similarly reducing choleragen stimulation of cAMP by 60% ($8.3 \pm 0.7$ pmol/2 x $10^6$ cells to $3.5 \pm 0.3$, $n = 12$). Since GnRHa also prevented the actions of 8-Br-cAMP, the GnRHa inhibition of LH receptor induction appears to be at a step beyond cyclic
nucleotide biosynthesis. Although it has been suggested that GnRH may affect cAMP accumulation and steroidogenesis by uncoupling the adenylate cyclase system (37, 41), other data indicate that GnRH does not acutely inhibit FSH-stimulated adenylate cyclase activity in membranes of luteal cells (42) or granulosa cells. Also, inhibitors of phosphodiesterase, the major degradatory enzyme(s) of cyclic nucleotides, prevent the GnRH inhibition of cAMP accumulation after FSH stimulation, suggesting that GnRH acts predominantly on catabolism rather than synthesis of the cyclic nucleotide (43). These data indicate that the inhibitory effects of GnRH on granulosa cell differentiation are through modulation of cAMP levels and emphasize the regulatory role of cAMP in ovarian development.

Since cAMP mediates steroidogenesis in granulosa cells (25), the effect of steroids on receptor formation and differentiation must also be considered. Indeed, it has been previously reported that progesterone secretion was correlated with microvilli formation in cultured granulosa cells (44). Further, other data suggest that LH receptors are located in microvillous regions of both luteal (23, 45) and granulosa (46) cell membranes. Table III shows that GnRH induced a 90% drop in progesterone accumulation after FSH treatment, as well as blocking LH receptor formation (Fig. 3). 8-Br-cAMP also stimulated progesterone production. However, GnRH blocked this steroidogenic activity by only 50% with 10 mm 8-Br-cAMP, but more markedly inhibited receptor induction by the nucleotide. Similarly, GnRH lowered progesterone production by approximately 40% in choleragen-treated cells, while substantially inhibiting LH receptor induction. Thus, progesterone formation does not appear to be directly related to receptor biosynthesis, since comparable levels of steroid production occurred in (8-Br-cAMP plus GnRH)-treated cells and those given FSH alone, despite the almost complete inhibition of receptor formation by GnRH after all treatments.

cAMP-mediated Morphological Differentiation of Granulosa Cells—The induction of in vitro cellular differentiation has been confirmed by the availability of simultaneous morphological and biochemical data. In the granulosa cell system, we have recently observed that FSH-induced differentiation in vitro closely resembles the in vivo process. Thus, cellular aggregation, formation of gap junctions and microvilli, and enlargement of mitochondria were stimulated by FSH in cultured granulosa cells (28). Fig. 4 presents the morphological alterations induced by the different agents that affect cAMP production in cultured granulosa cells. Whereas control cultures formed a typical monolayer of cells with a fibroblastic appearance (a), FSH-treated cells grew in multilayered aggregates of tightly packed epithelial-shaped cells (b). The cell clusters were interconnected by elongated extensions. Addition of GnRHs to FSH-treated cultures markedly impairs the morphological changes induced by FSH (c). Cells appeared less aggregated and more flattened than cells treated with FSH alone, although some clusters of more rounded cells showing regressive changes were still observed. 8-Br-cAMP also induced cellular aggregation (d). These cells were more round and less tightly packed than FSH-treated cells. In the presence of choleragen, cells again grew in multilayered aggregates (e). However, the cells were less rounded as compared to cultures treated with 8-Br-cAMP and thus more closely resembled FSH-treated cells. GnRHs alone also had an effect on granulosa cells (f), which were less flattened than in control cultures, but still formed a monolayer with essentially no aggregation. These experiments have demonstrated that treatment of cells with FSH, choleragen, and prostaglandins also modulates cellular aggregation and other morphological changes that are characteristic of the differentiated granulosa cell.

In conclusion, these data indicate that cAMP is responsible for many of the diverse actions of FSH in ovarian tissue. FSH elevates cAMP production, and this nucleotide mediates the phenotypic expression and differentiation of granulosa cells into luteal cells. Steroidogenesis, hormone receptor formation, and morphological development are enhanced by rises in cAMP, while prevention of cAMP accumulation inhibits cellular maturation. Whether the multiple actions of cAMP are initiated via phosphorylation of one or several intracellular proteins is presently unknown. While FSH, choleragen, and prostaglandins are known stimulators of cAMP production and have been shown to affect aromatase activity in granulosa cells (47), the present findings have emphasized a hitherto unrecognized ability of cAMP to initiate and maintain the extensive biochemical and morphological changes that occur during granulosa cell maturation. Also, these data provide unequivocal evidence in a mammalian cell system that cAMP regulates an entire differentiation process, similar to that produced by the trophic hormone in vivo. Further investigation of the mechanisms by which cAMP influences the maturing granulosa cell should provide useful insights into the hormonal control of cellular differentiation.

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