Concerted Stimulation of Rat Granulosa Cell Deoxyribonucleic Acid Synthesis by Sex Steroids and Follicle-stimulating Hormone

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Although follicle-stimulating hormone (FSH) and estrogens are known to be the main physiological stimuli for the development of the ovarian follicle in mammals, their growth-promoting activity has not been clearly established “in vitro”. Furthermore, experimental evidence indicates that FSH and estradiol can independently inhibit granulosa cell proliferation. The present study was aimed at examining the effect of sex steroids in combination with FSH, on DNA synthesis in rat granulosa cells cultured in completely defined medium. Estradiol and FSH, when added separately, produced a significant inhibition of [3H] thymidine incorporation. In contrast, a combination of a low dose of FSH (20 ng/ml) with estradiol (100 ng/ml) produced a shift in the period of maximal DNA synthesis from 96 to 48 h after plating. Dose response studies showed that estradiol effects were produced at physiological intraovarian concentrations (1-100 ng/ml), whereas the effects of FSH were biphasic, with high doses (200 ng/ml) being inhibitory. A similar biphasic dose response curve was observed with increasing concentrations of a cAMP derivative in the presence of maximally effective doses of either an aromatizable steroid (androstenedione), insulin or insulin-like growth factor I. Non-aromatizable androgens (5α-dihydrotestosterone, 5α-androstane 3α-17β diol and androsterone) showed a potency comparable to that of estradiol. The effect of 5α-dihydrotestosterone was completely blocked by a specific antiandrogen (hydroxy-flutamide), indicating that it was mediated by the androgen receptor. The effects of estradiol and androgens were not additive. The interaction between estradiol and FSH was further amplified in the presence of a maximally effective dose of insulin. Data presented herein indicate that both estrogens and androgens are able to elicit a mitogenic response in purified granulosa cells, cultured in a completely defined medium, provided the cells are stimulated by a physiological dose of FSH. These results suggest that, during follicular development, the stimulus for granulosa cell proliferation is given by the concerted action of steroid and peptide hormones acting through different signalling pathways. © 1997 Elsevier Science Ltd
estrogens, when administered separately, can exert inhibitory effects on granulosa cell growth [4-7].

This apparent lack of direct stimulatory effects of FSH and estrogens, together with the demonstration of the mitogenic effects of several peptide growth factors on granulosa cell cultures [8], has led to the assumption that the growth-promoting actions of these hormones are mediated by paracrine or autocrine loops. On this basis most studies have focused on the interaction between FSH or estradiol and putative intraovarian growth regulators.

We have developed a rat granulosa cell culture system that enabled us to demonstrate that estradiol treatment sensitizes rat granulosa cells to the mitogenic stimulation of insulin and a theca/interstitial cell-derived growth factor [9]. In the same system we have shown that FSH exerts a cAMP-mediated co-mitogenic effect with insulin-like growth factor-I (IGF-I) [10]. In more recent studies, however, we have found that although FSH or estradiol had no effect when added separately, a combination of both hormones elicited a marked stimulation of DNA synthesis in cultures of purified granulosa cells [11].

The present study was aimed at characterizing the interaction between FSH and sex steroids in the regulation of rat granulosa cell DNA synthesis.

MATERIALS AND METHODS

Hormones and chemicals

Recombinant IGF-I and highly purified single component porcine insulin (26.2 U/mg) were generously provided by Chiron Corp. (Emeryville, CA, U.S.A.) and Eli Lilly Company (Indianapolis, IN, U.S.A.), respectively. Ovine FSH (NIADDK oFSH-17) was obtained from the National Hormone and Pituitary Program; [methyl-3H] thymidine (10 Ci/mmol) from American Radiolabeled Chemicals Inc. (St Louis, MO, U.S.A.). Dibutyryl cyclic AMP, DES and all the steroids were from Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals were reagent grade from standard commercial sources.

Granulosa cell preparation and culture

Ovaries were obtained from 24 25-day-old female Sprague-Dawley rats after 3 days of DES treatment (subcutaneous silastic implants containing 5 mg diethylstilbestrol; DES). Granulosa cells were prepared and cultured as previously described [9]. Briefly, the ovaries were punctured with a 30-gauge needle and incubated in DMEM (4.5 g glucose/l):F12 (Gibco BRL, Gaithersburg, MD, U.S.A.), EDTA (6.8 mM), HEPES (10 mM) (15 min at 37°C) and then washed twice and incubated in DMEM:F12, sucrose (0.5 M), HEPES (10 mM) (5 min at 37°C). After incubation the medium was diluted with two volumes of DMEM:F12, HEPES (10 mM) and then the ovaries were allowed to sediment. Granulosa cells were obtained by pressing the ovaries within two pieces of nylon mesh (Nytex 50) and were purified by density gradient centrifugation as described by Magoffin and Erickson [12]. The cell suspension was layered over a 40% Percoll solution in saline and centrifuged at 400g for 20 min. The granulosa cell layer was aspirated from the top of the Percoll solution and resuspended in DMEM:F12 (1:1), sodium bicarbonate (2.2 g/l, pH 7.4). For the separation of the granulosa cell subpopulations, the cell suspension was layered on a continuous Percoll gradient (densities 1.014-1.054 g/ml) and centrifuged for 30 min at 400g. Cell viability, determined after 5 min incubation with trypan blue, ranged from 35 to 45%.

Cells were seeded on plastic 96 multi-wells (Nunc, Roskilde, Denmark) precoated with rat tail collagen [9]. The initial plating density was 3.0 x 10^5 viable cells per cm². Cells were maintained at 37°C with 5% CO₂. After 2 h media were changed to remove non-attached cells, and were replaced by fresh medium containing the different factors to be tested.

DNA synthesis measurements

For the time course experiment (Fig. 1) cells were incubated with tritiated thymidine (4 μCi/ml) during 12 h consecutive periods. In all other experiments, incubations with tritiated thymidine were carried out for 24 h starting 24 h after plating. Cells were harvested in glass hollow fibres (Whatman GFC), with a multi-well cell harvester (Nunc). Excess ³H-thymidine was washed with six volumes of distilled water followed by one volume of ethanol. Filters were allowed to dry and transferred to counting vials. Radioactivity was measured in a scintillation counter (efficiency 50%). Under these conditions thymidine incorporation reflects the rates of DNA synthesis and this measurement has previously been validated by the following criteria: correlation with cell numbers [9]; labelling index [10] and suppression by a DNA synthesis inhibitor [13].

Labelling index

To determine the percentage of cells synthesizing DNA, granulosa cells were cultured in 35 mm plastic dishes coated with collagen. Twenty-four hours after plating duplicates were radioactively labelled by exposure to ³H-thymidine (3 μCi/ml) for 24 h. Incubation was stopped and the cells were fixed by washing them with phosphate-buffered saline (PBS) (0 4°C) twice, with 5% trichloroacetic acid (0 4°C), and with ethanol twice. Dishes were then processed for autoradiography. The percentage of radioactively labelled cells was determined after counting at least 500 cells/dish [10].
FSH and Sex Steroid Effects on Granulosa Cell DNA Synthesis

Fig. 1. Effects of FSH and estradiol on DNA synthesis in cultured rat granulosa cells. Rat granulosa cells were isolated from immature DES-treated rats and purified by centrifugation on Percoll. Cells (1 × 10⁶) were cultured on collagen coated 96-well multi-wells in serum-free medium (DMEM: Hams's F12) in the absence (upper panel) or presence (lower panel) of estradiol (100 ng/ml) and increasing concentrations of FSH. [³H]-Thymidine incorporation was performed on consecutive 12 h periods. Each time-point corresponds to the harvest of one set of cultures and the addition of thymidine to the next. Results are means ± SEM of triplicate determinations in a single experiment that was repeated two additional times. Asterisk indicates significant difference (P < 0.05, Scheffé's multiple range test) when compared with the respective control cultures.

Statistical analysis

Results are expressed as means ± SEM of triplicate cultures. Statistical comparisons of the results were made using one-way analysis of variance (ANOVA) and Scheffé’s test for multiple comparisons [14]. Fitting of the dose-response curves and calculation of the ED₅₀s were performed using a computer program based on a four-parameter logistic equation [15]. Experiments were carried out at least three times with similar results.

RESULTS

Figure 1 shows the time course of [³H]-thymidine incorporation in rat granulosa cells treated with different doses of FSH, in the absence or presence of estradiol. As can be seen, in the absence of estradiol or FSH, incorporation levels dropped from the comparatively high initial values observed after plating to a low baseline and then started to increase after 60 h in culture. The addition of increasing doses of FSH produced a progressive decrease in the overall incorporation during the 96 h culture period. At the highest dose (200 ng/ml) a slight increase in the rates of thymidine incorporation was observed between 48 and 60 h after plating. In cultures treated with estradiol alone, there were no evident changes in the time course, but maximal incorporation rates, from 60 to 96 h in culture, were diminished by approximately 60% (compare control curves in both panels). However, estradiol treatment produced a marked change in the responses to FSH. In the presence of this steroid, FSH, at a dose of 20 ng/ml, elicited a dramatic increase in the incorporation rates observed after 24 h. This effect, albeit less pronounced, was also observed with the highest dose (200 ng/ml). In all subsequent experiments, thymidine incorporation was therefore performed from 24 to 48 h after plating.

The observed changes in thymidine incorporation were correlated with the labelling index of the granulosa cell cultures. Treatment with a combination of FSH (2 ng/ml) and estradiol (100 ng/ml) resulted in a three-fold increase in the percentage of labelled nuclei when compared with non-treated cells (1.8 ± 0.4 vs...
Fig. 3. Effects of non-aromatizable androgens on DNA synthesis in cultured rat granulosa cells. Rat granulosa cells were obtained and cultured as indicated in Fig. 1 in the absence or presence of FSH (20 ng/ml) and the indicated concentrations of dihydrotestosterone (DHT), 5α-androstan-3α, 17β-diol (3α-Diol) or androsterone. [3H]-Thymidine incorporation was carried out for 24 h, starting 24 h after plating. Results are means ± SEM of triplicate determinations in a single experiment that was repeated two additional times. No significant differences were observed in the magnitude of the stimulations elicited by 10 or 100 ng/ml of the three steroids. Values with no common superscripts are significantly different (P < 0.05, Scheffe's multiple comparison test).

0.5 ± 0.1, P < 0.05). No significant effects were observed when FSH or estradiol were added alone (0.6 ± 0.2 and 0.2 ± 0.1, respectively).

To test the specificity of the estrogen effect we compared different steroids in their ability to interact with an optimal dose of FSH in the stimulation of DNA synthesis. Aromatizable androgens such as androstenedione and testosterone were found to be as potent as estradiol in the stimulation of thymidine incorporation (Fig. 2). Similarly, non-aromatizable 5α-reduced androgens, such as 5α-dihydrotestosterone (DHT), 5α-androstan-3α, 17β-diol (3α-Diol) and androsterone, were also able to synergize with FSH (Fig. 3). Comparison of the dose-response curves for estradiol and dihydrotestosterone (Fig. 4) showed no significant differences in the ED₅₀s for both steroids (10 ± 5 ng/ml).

Neither the 5β isomer of dihydrotestosterone (5β-androstan, 17β-ol,3-one, 5β-DHT; Fig. 5) nor the 17β epimer of testosterone (data not shown) showed a significant interaction with FSH. The synthetic steroid medroxyprogesterone acetate (MPA), known to act as progestagen, androgen and glucocorticoid [16, 17], showed a potency comparable with that of dihydrotestosterone (Fig. 5). Under the same conditions, progesterone was completely ineffective (Fig. 5). Furthermore, the addition of progesterone (100 ng/ml) did not alter the responses elicited by FSH in combination with either estradiol or dihydrotestosterone (data not shown).

To verify that the effects of dihydrotestosterone were mediated by the androgen receptor, we tested the effect of the pure antiandrogen hydroxyflutamide. As can be seen in Fig. 6, this antagonist did not modify the effect of estradiol but completely suppressed the effect of dihydrotestosterone.

When estradiol and dihydrotestosterone were added at maximally effective doses (100 ng/ml) in the presence of 20 ng/ml of FSH their effects were not additive (FSH: 1227 ± 244; FSH + estradiol: 9269 ± 383; FSH + dihydrotestosterone: 9593 ± 973 and FSH + estradiol + dihydrotestosterone: 10652 ± 420 cpm/well, n = 3).

To test whether the interaction between FSH and sex steroids could be mediated through increases in cAMP levels, we studied the effect of dibutyryl cAMP, (Bu)₂cAMP, in the presence of androstenedione. Increasing doses of (Bu)₂cAMP, in combination with this steroid, produced a biphasic effect on DNA synthesis (Fig. 7). An increase in thymidine incorporation was observed in the range 0.10 to 0.5 mM, whereas concentrations higher than 1 mM of the cAMP derivative produced a complete inhibition...
Fig. 5. Effects of 5β-dihydrotestosterone, medroxyprogesterone, and progesterone on DNA synthesis in cultured rat granulosa cells. Rat granulosa cells were obtained and cultured as indicated in Fig. 1 in the absence or presence of FSH (20 ng/ml) and the indicated concentrations of 5α-dihydrotestosterone (DHT), 5β-dihydrotestosterone (5β-DHT), medroxyprogesterone acetate (MPA) or progesterone (P). [3H]-Thymidine incorporation was carried out for 24 h, starting 24 h after plating. Results are means ± SEM of triplicate determinations in a single experiment that was repeated two additional times. No significant differences were observed in the magnitude of the stimulations elicited by 10 or 100 ng/ml of either DHT or MPA. Values obtained in the presence of 5β-DHT or P were not different from controls without steroids. Values with no common superscripts are significantly different (P < 0.05, Scheffé’s multiple comparison test).

of DNA synthesis. In the absence of androstenedione, (Bu)2cAMP (0.2–0.4 mM) stimulated thymidine incorporation (P < 0.05), and this effect was reversed at higher concentrations.

As shown in Fig. 8, a striking parallelism was observed in the dose-response curves for FSH obtained in the presence of androstenedione, insulin or insulin-like-growth factor-I (IGF-I). The main difference found was that, in contrast with insulin or IGF-I, androstenedione had no effect in the absence of FSH or in the presence of highest doses of the gonadotropin.

Although the maximal incorporation rates, observed in the presence of an optimal dose of FSH, were similar for either steroids (androstenedione or estradiol) or insulin/IGF-I the three types of stimuli produced supra-additive effects. Table 1 shows the results obtained in cultures treated with different combinations of FSH, estradiol and insulin. Insulin, when added alone or in combination with estradiol produced a slight stimulation of thymidine incorporation (in the experiment shown in Table 1 these differences do not appear to be statistically significant because of the high stringency posed by the multiple comparison test).

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Fig. 6. The effects of dihydrotestosterone were blocked by a pure antiandrogen. Rat granulosa cells were obtained and cultured as indicated in Fig. 1 in the absence or presence of hydroxyflutamide (1 μg/ml) and the indicated combinations of FSH (20 ng/ml), dihydrotestosterone (DHT, 10 ng/ml) and estradiol (E, 10 ng/ml). [3H]-Thymidine incorporation was carried out for 24 h, starting 24 h after plating. Results are means ± SEM of triplicate determinations in a single experiment that was repeated two additional times. An asterisk indicates significant difference (P < 0.05) when compared with values obtained in the absence of the antiandrogen.

Fig. 7. Effects of a cAMP analogue on DNA synthesis in rat granulosa cells. Rat granulosa cells were obtained and cultured as indicated in Fig. 1 in the absence (open symbols) or presence (closed symbols) of androstenedione (100 ng/ml) and the indicated concentrations of dibutyryl cAMP [(Bu)2cAMP]. [3H]-Thymidine incorporation was carried out for 24 h, starting 24 h after plating. Results are means ± SEM of triplicate determinations in a single experiment that was repeated two additional times. Values with no common superscripts are significantly different (P < 0.05, Scheffé’s multiple comparison test).
Estradiol 836 ± 40 a 2288 ± 169 a'b

Treatment Insulin
FSH + estradiol 9880 ± 1410 b'c 21093 _ 3384 d
FSH 852 ± 130 a 10578 ___ 592 c

...whether we could identify subpopulations with differ-

ting responses to FSH and steroid hormones in

Table 1. Effect of insulin on the interaction between FSH and
estradiol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3H-Thymidine incorporation (cpm/well)</th>
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<tbody>
<tr>
<td>Control</td>
<td>788 ± 27a</td>
</tr>
<tr>
<td>Estradiol</td>
<td>836 ± 40c</td>
</tr>
<tr>
<td>FSH</td>
<td>852 ± 130c</td>
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<tr>
<td>FSH + estradiol</td>
<td>9880 ± 1410d</td>
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...It has been reported that granulosa cell subpopu-
lations can be separated by density gradient centrifu-
gation [18, 19]. Therefore we sought to determine
whether we could identify subpopulations with differ-
tial responses to FSH and steroid hormones in

...test). The insulin effect was markedly amplified by
FSH. The interaction between FSH and estradiol was
still evident in the presence of insulin. Maximal
stimulations, observed when all the three hormones
were present, represented a 27-fold increase over
basal values.

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gation [18, 19]. Therefore we sought to determine
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...In spite of this apparent inhibitory effect, in the
presence of estradiol there was a dramatic change in
the effects of FSH, shown by the appearance of a
terms of DNA synthesis. Five fractions, corresponding
to densities ranging from 1.031 to 1.017 g/ml, were
isolated and cultured in the presence of FSH, andros-
tenedione or a combination of both. With the excep-
tion of a fraction that corresponded to the lowest
density, accounting for only 5% of the total cell num-
ers, that was not stimulated by a combination of
FSH plus androstenedione, all the other fractions showed a comparably responsiveness (data not shown).

...DISCUSSION

...Although estradiol and FSH are potent stimuli for
granulosa cell proliferation "in vivo" [1], the mito-
genic effects of these hormones had been difficult to
demonstrate in the culture systems commonly used
for the study of granulosa cell differentiation [3]. We
have developed a rat granulosa cell culture system
that allowed us to show stimulatory actions of estra-
coating of the culture dishes, high plating densities
and an adequate time for thymidine incorporation
measurement were found to be fundamental to
demonstrate these effects.

...Even under these optimized culture conditions,
only a small fraction of the cells are able to enter into
the S-phase [10]. This seems to be the consequence
not only of the low replicative potential of rat granu-
losa cells [9,20-22], but also of some peculiarities in
their cell cycle. In fact, Hirshfield et al. [23] noticed
that, in contrast with other rapidly growing tissues,
only approximately 10% of the granulosa cells were
found to be in the S-phase during follicular growth in
the adult cyclic rat. Similarly, van Weissenbruch et al.
[24] reported 2-16% of S-phase nuclei in antral folli-
cles obtained after PMSG treatment of immature
rats. This low percentage of growing cells has hin-
dered the study of rat granulosa cell proliferation "in vitro" by the usual approaches of measuring changes
in cell numbers and/or DNA content.

...Data presented here demonstrate that, in this sys-
tem, a combination of FSH and sex steroids at phys-
iological concentrations can exert a pronounced effect
on rat granulosa cell DNA synthesis.

...When purified rat granulosa cells are cultured with
increasing doses of FSH the overall effect on DNA
synthesis was inhibitory. Estradiol, as previously
reported [9], did not stimulate DNA synthesis on
purified cells. Furthermore, a slight inhibitory effect
was associated with the continuous presence of estra-
diol in the culture media. This effect would be con-
sistent with reports showing that prolonged estrogen
exposure inhibits granulosa cell growth [7,25].

...In spite of this apparent inhibitory effect, in the
presence of estradiol there was a dramatic change in
the effects of FSH, shown by the appearance of a
stimulatory effect of low doses of the gonadotropin. This effect seems to be specific for FSH because treatment of the cultures with increasing doses of hCG under the same conditions did not significantly alter thymidine incorporation (data not shown).

The synergistic action of FSH and estradiol on thymidine incorporation is consistent with data obtained by Reilly et al. [7], showing an increased responsiveness to FSH in vitro of granulosa cells that have been exposed to estrogens in vivo for a short period.

Changes in thymidine incorporation in the presence of FSH (20 ng/ml) and estradiol were consistent with an advancement in the entry into the S-phase. Assuming that the incorporation rates observed at the beginning of the culture reflect the completion of an S-phase initiated "in vivo", this would imply a shortening in the apparent doubling time from 84 to 48 h. This effect would be similar to that observed in intact mouse follicles maintained in culture, where the addition of a cAMP derivative induced a shortening of the doubling time of granulosa cells from 120 to 64 h [26].

Epstein-Almog and Orly [21] have previously reported that a sharp synchronous uptake of $^3$H-thymidine in rat granulosa cell cultures takes place between 4 and 6 days after plating. These authors, however, did not find a significant change between cultures treated with FSH or FSH plus androstenedione. Several differences in the experimental conditions, such as the use of a comparatively high (100 ng/ml) concentration of FSH and the supplements used for the culture medium, may account for these discrepancies.

On the other hand, McNatty and Sawers [27] had originally reported that the addition of a combination of FSH and estradiol to cultures of human granulosa cells from different follicular stages markedly stimulated the mitotic index. We have recently reported that in cultures of human granulosa-lutein cells, FSH and estradiol were unable to stimulate cell proliferation [11]. This effect seemed to be caused by the secretion of a growth-inhibitory factor by these highly differentiated cells.

The finding of a stimulatory action, comparable to that of estradiol for non-aromatizable androgens, was rather surprising because androgens are usually believed to exert a detrimental effect on follicular development. This assumption is mainly based on the association of high androgen/estrogen levels with atretic follicles [28]. However, the stimulatory effects on granulosa cells from preantral follicles and the induction of atresia may represent separate phenomena [29]. On the other hand, our data would be consistent with the report by Wang and Greenwald [30], showing that both estradiol and dihydrotestosterone can synergize with FSH in the stimulation of follicular DNA synthesis in hypophysectomized mice. Androgens, acting through specific receptors, have been shown to mimic some (but not all) of the estrogen effects on FSH-induced cell differentiation [3]. Therefore it seems possible that the synergism between either estrogens or androgens and FSH is another consequence of a pleiotropic action of the sex steroids on FSH-dependent granulosa cell functions. The present data constitute the first report showing that androstenedione and 3α-Diol, which have been shown to be the main steroids produced by theca/interstitial cells in the ovaries of immature rats [31, 32], may exert a marked stimulatory action on granulosa cell growth.

The stimulatory effect of medroxyprogesterone seemed to be caused by its androgenic action [16], because progesterone was completely inactive. This compound has also been shown to stimulate the proliferation of the androgen-sensitive mouse mammary carcinoma (Shinogi) cells in culture [33].

The low overall thymidine incorporation rates and labeling indexes [10] indicated that only a subpopulation of granulosa cells was undergoing DNA synthesis. Therefore we attempted to isolate this actively proliferating subpopulation by density gradient centrifugation. Granulosa cells separated according to their densities showed no major differences in their responsiveness to a combination of androstenedione and FSH, with the exception of a minor fraction (δ = 1.014) that was found to be non-responsive (data not shown). Previous studies by Kasson et al. [18] suggested the existence of subpopulations of granulosa cells isolated from hypophysectomized rats that could be isolated by density centrifugation. Although direct comparisons are not possible, because the cell densities of those subpopulations were not specified, it is to be noted that the minor fraction corresponding to the lowest density was found by these authors to be non-responsive to FSH.

Bendell and Dorrington [34] have proposed that estrogen effects on rat granulosa cell proliferation are mediated by TGF-β. This factor, in turn, would synergize with FSH to stimulate DNA synthesis [22]. However, our preliminary data show that the synergism between estradiol and FSH is also evident in the presence of maximally effective doses of TGF-β [35]. Therefore, in our conditions the interaction between these two hormones does not seem to be mediated by an autocrine loop involving this growth factor. On the other hand, changes in the local production of IGF-I and/or its binding proteins would not be involved in the interaction between sex steroids and FSH, because it could also be observed in the presence of maximal doses of insulin.

The existence of an autocrine loop, involving a still unidentified growth factor, cannot be completely ruled out, because, as previously reported for the interaction between FSH and IGF-I [10], the synergism between FSH and sex steroids was only observed at high plating densities (data not shown).
we have recently reported that the stimulatory effect elicited by a combination of FSH, androgens and TGF-β can be markedly suppressed by the activin-binding protein follistatin [36]. These data, together with previous reports showing that activin is a potent stimulator of DNA synthesis in rat granulosa cells [37, 38], strongly suggest that this peptide is a key mediator of the growth-promoting actions of gonadotropin and steroid hormones in the ovarian follicle.

The actions of cAMP as a regulator of cell proliferation vary from inhibitory to stimulatory, depending on the cell type and experimental conditions [39]. The ovary, the adrenal and the thyroid are tissues under pituitary control by glandotrophic hormones that are thought to exert their trophic effects via stimulation of adenyl cyclase. However, at present it is unclear whether cAMP is fully responsible for the growth-promoting actions of these pituitary hormones. Data presented herein would further support the notion that FSH promotes granulosa cell growth through a cAMP-mediated process. In this regard, Peluso et al. [40] have shown that in perfused rat ovaries or isolated follicles, treatment with either FSH or a cAMP derivative increased DNA synthesis in granulosa cells. This effect was more pronounced in follicles with high estrogen levels. More recently, these authors have shown that although a cAMP derivative was unable to increase thymidine incorporation in isolated granulosa cells maintained in culture for 24 h, a cAMP antagonist blocked phorbol ester-induced mitosis [41].

The dose response curves for either FSH or a cAMP derivative, in the presence of androgens or estradiol were biphasic. Biphasic effects of cAMP were also reported for the stimulation of DNA synthesis in cultured human granulosa cells [42]. It is to be noted that data from Yong et al. [42], as well as our own [13], indicate that high concentrations of either FSH or cAMP, that had null or inhibitory effects on DNA synthesis, can maximally stimulate steroid biosynthesis. This would suggest a dissociation in the optimal cAMP levels required to induce growth and cell differentiation.

Regarding the mechanisms involved in the interaction between estradiol and FSH, increasing evidence is accumulating indicating a cross-talk between steroid hormone- and cAMP-signalling pathways [43, 44]. Therefore, it is unclear whether the interaction between estradiol and cAMP found in granulosa cells is peculiar to this cell type or if it corresponds to a more general mechanism by which estrogens activate cell division. In this regard, it is to be mentioned that an estrogen-specific mitogenic effect, in synergism with cAMP, has also been demonstrated even in non-classical target organs such as Schwann cells [45].

Although, as previously shown [10], insulin and IGF-I were able to interact with cAMP, giving dose response curves similar to those obtained with androgens or estrogens, a separate mechanism seems to be involved because the interaction between estradiol and FSH was further amplified in the presence of a maximally effective dose of insulin.

Our data would suggest that FSH, acting through a cAMP-mediated process, can directly stimulate granulosa cell DNA synthesis. This stimulatory effect is strongly dependent on the presence of sex steroids. The interplay between the tonic stimulation by the pituitary and the modulation by local regulators is likely to play a central role in the selection of the ovulatory follicles in mammals.

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