Studies on the Role of Plasminogen Activator in Ovulation

IN VITRO RESPONSE OF GRANULOSA CELLS TO GONADOTROPINS, CYCLIC NUCLEOTIDES, AND PROSTAGLANDINS*

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SIDNEY STRICKLAND AND WILLIAM H. BEERS
From The Rockefeller University, New York, New York 10021

A quantitative method is described for measuring the amount of plasminogen activator produced by rat ovarian granulosa cells following exposure to hormones in vivo or in vitro. The results confirm the previously reported observation (Beers, W. H., Strickland, S., and Reich, E. (1975) Cell 6, (387-394) that granulosa cells in vivo produce increasing amounts of plasminogen activator as the time of ovulation approaches and that the enzyme is produced only by cells obtained from follicles destined to ovulate.

Inactive cells can be stimulated in vitro by gonadotropins to produce plasminogen activator. This response is time- and dose-dependent, and results in an increase of intracellular and extracellular enzyme. Studies of the specificity of this response indicate that preparations of follicle-stimulating hormone are much more effective than corresponding preparations of luteinizing hormone. The effect of other pituitary hormones is also presented.

Molecules other than gonadotropins are also capable of stimulating the cells to produce the enzyme. Prostaglandins E₁ and E₂ and analogues of cAMP effectively stimulated the cells to produce plasminogen activator, cGMP and its analogues and prostaglandins F₁α and F₂α were without effect as were the six steroids studied. The inactive compounds also did not inhibit the response of the cells to gonadotropins.

The granulosa cell plasminogen activator has been analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and has an apparent molecular weight of 75,000. By this and other criteria, the granulosa cell enzyme is similar to one of the species of plasminogen activators obtained from cultures of simian virus 40-transformed rat embryo fibroblasts.

In order for the ovum to escape from the graafian follicle at the time of ovulation, extensive degradation of the follicle wall is necessary. Although several theories have been proposed to explain this phenomenon, its biochemical basis is not firmly established. Schochet, in 1916, suggested that the process has an enzymatic rather than mechanical basis and gave evidence that a proteolytic enzyme is associated with preovulatory follicles (1). Other investigators have also proposed that changes in the amount of lytic enzymes might be involved in ovulation (2, 3).

Recently, a specific biochemical mechanism for ovulation has been proposed (4). According to this hypothesis, plasminogen activator is responsible for the disruption of the follicle. This proposal is based on the fact that rat ovarian granulosa cells produce plasminogen activator in a manner which is closely correlated with ovulation. The substrate for this enzyme, plasminogen, is present in follicular fluid, and the product of the reaction catalyzed by the action of plasminogen activator on plasminogen, plasmin, has been shown to weaken follicle wall strips in vitro (5). In addition, in vitro exposure of granulosa cell cultures to preparations of ovine luteinizing hormone and cAMP results in increased amounts of extracellular plasminogen activator.

Taken together, these findings suggest a role for plasminogen activator in ovulation. It was therefore of interest to examine the nature of the in vitro response of ovarian granulosa cells. Using a method which allows the response of the cells to be measured and quantitated easily, the effect of a number of polypeptide hormones, prostaglandins, cyclic nucleotides, and steroids has been investigated. These results indicate that preparations of follicle-stimulating hormone are the most effective of the polypeptide hormones tested. In addition, cAMP and prostaglandins E₁ and E₂ are also capable of stimulating the cells in vitro.

EXPERIMENTAL PROCEDURE

Materials—Rats of the Sprague-Dawley strain were obtained from Holtzman. Multi-well culture plates, containing 24 wells (16 mm diameter), were purchased from Linbro Chemical Co. and plastic Petri dishes were from Falcon. Medium 199, dog, chicken, and African green monkey sera were obtained from GIBCO; calf serum from Flow Laboratories; fetal bovine serum from Reheis; PMSG,¹ HCG, theo-

¹ The abbreviations used are: PMSG, pregnant mare serum gonadotropin; Bt₂cAMP, N', O'-dibutyryl CAMP; Bt₂cGMP, N', O'-dibutyryl cGMP; 8-Br-cGMP, 8-bromo cGMP, LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hor-
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Phyllium, cAMP, B₄₅cAMP, cGMP, B₄₅cGMP, DL-D-ribose, deoxymethasone, cytosine arabinoside, human serum albumin, indomethacin, and Triton X-100 from Sigma; Gestyl (PMSG, approximately 2500 IU/mg) and Pregnyl (HCG, approximately 2500 U.S. units/ml) from Organon; 17a- hydroxyprogesterone, 17β-estradiol, testosterone, and fibrinogen (77% clottable) from Calbiochem; Norit A-activated charcoal powder from Matheson, Coleman and Bell; ovalbumin, RNAase A, chymotrypsinogen, dextran T-70, and Sepharose 4B from Pharmacia; 20α-dihydroprogesterone from Steraloids; cycloheximide from ICN Pharmaceuticals; carrier-free Na⁺/²⁺ from New England Nuclear; sodium dodecyl sulfate, specially pure, from BDH Chemicals LTD; acrylamide, ammonium persulfate, N, N'-methylenebisacrylamide, and N,N,N',N'-tetramethylenediamine from Eastman Kodak Co.; trypsin from Nutritional Biochemicals; β-galactosidase from Brush; 3-indolylmethylsulfonfyl fluoride from Aldrich; and soybean trypsin inhibitor from Miles Saracov.

Ovine LH (Batch S-19), FSH (S-11), TSH (S-8), PRL (S-12), GH (S-11), and rat LH (B-1) and FSH (B-1) were provided by the National Institute of Arthritis, Metabolic and Digestive Diseases. Prostaglandins E₂, F₂α, and P₄ were the gift of Dr. J. D. Johnson, Upjohn Chemical Co. and cytoskeleton was the gift of Dr. M. I. Sherman. Ro 20-1724 was donated by Hoffman-LaRoche Inc. and actinomycin D by Merck Chemical Co.

All other materials were of reagent grade.

Preparation of 125I-Fibron-coated Multi-well Plates—The preparation of 125I-fibron-coated wells was similar to that described by Unkless et al. (6). Fibrinogen (77% clottable) was precipitated with ammonium sulfate (7), resuspended in 0.6 M NaCl, and then precipitated with 7% ethanol in the presence of lysine (8). The precipitate was allowed to form overnight at 4°C, resuspended in 0.6 M NaCl and the ethanol precipitation was repeated. The final precipitate was dissolved in a volume of NaCl/PO₄, sufficient to bring the protein concentration to 10 mg/ml.

The purified fibrinogen was radioiodinated at 0°C by the method of Helman et al. (9), and the specific radioactivity of the iodinated protein was approximately 300,000 cpm/μg of protein. This represents the incorporation of 1 to 2 atoms of 125I per molecule of fibrinogen. Purified unlabeled fibrinogen (10 mg/ml in NaCl/PO₄) was diluted in a solution containing 1 part NaCl/PO₄, to 11 parts H₂O to a concentration of 0.19 mg/ml. Sufficient 125I-fibrinogen was then added so that the final solution contained approximately 400,000 cpm/ml. To each well of the multi-well plate, 0.25 ml of the above solution was added. The plates were then dried at 37°C for 3 days. During this process, the tops of the plates were elevated to facilitate drying. The plates were stored at room temperature.

Prior to use each well was incubated for 2 h with Medium 199 supplemented with 5% plasminogen-depleted fetal bovine serum at 37°C in a 5% CO₂ atmosphere. This treatment results in the conversion of fibrinogen to fibrin. Following this procedure, each well was washed twice with Medium 199, and used within 24 h. At this point each well contained 50,000 to 70,000 cpm which could be solubilized by trypsin.

Preparation of Rat Granulosa Cell Cultures and Analysis for Plasminogen Activator Production—Female rats, 26 days old, were injected subcutaneously with 5 IU of PMSG in 0.1 ml of 0.9% NaCl. For all of the studies involving in vitro exposure of the cultures to hormones, the animals were decapitated 48 h after injection with PMSG and the cells were collected from graffian follicles. Approximately 10⁵ cells per ovary could be obtained by this procedure. For studies involving in vitro stimulation, LH (100 μg), FSH (100 μg), or HCG (25 IU) was injected subcutaneously in 0.1 ml of 0.9% NaCl 48 h after the injection of PMSG. Identical results were obtained with each of these gonadotropin preparations. Cells were prepared at desired times from appropriate follicles following the second injection.

Granulosa cells were prepared and cultured as previously described (4). Briefly, the contents of individual follicles were expressed into Medium 199 and the cells were collected and cultured at 37°C in a 5% CO₂ atmosphere. Plasminogen activator production by cultured granulosa cells was measured by platting the cells in 125I-fibron-coated wells (6). The fibrin had no effect on the maintenance of the cells in culture. The cells were plated in 0.5 ml of Medium 199 containing 10% fetal bovine serum and 100 μg/ml of soybean trypsin inhibitor or Medium 199 containing 10% acid-treated fetal bovine serum. Aliquots of the medium were removed at various times and solubilized ³⁵S was counted in a Packard Auto Gamma spectrometer.

All assays were performed in duplicate. Control incubations with hormones but without cells were always included and the amount of radioactivity released (1 to 2%) was subtracted from the experimental value. The results are presented as the fraction solubilized of the total available substrate. Total digestible fibrin was determined by exposing representative wells to a solution of 0.25% trypsin. To control for variations in cell preparations and the fibrin-coated wells, each experiment included cells stimulated by a standard solution containing 10% activity of FSH.

Preparation of Serum-free Conditioned Medium from Granulosa Cell Cultures—In contrast to the in vitro studies, rats for these experiments were treated with a different hormone regimen which results in superovulation: 26-day-old female rats were injected subcutaneously with 50 IU of PMSG, and 48 h later the animals received 25 IU of HCG via the same route. After an additional 8 h, the cells were harvested from prevolutionary follicles and plated at a density of 1 × 10⁶ cells per 35-mm Petri dish in Medium 199 supplemented with 10% plasminogen-depleted fetal bovine serum. Following a 4-h incubation at 37°C the cultures were washed twice and incubated overnight in 1 ml of Medium 199. The conditioned medium was centrifuged at 250 × g for 10 min to remove cells and debris and the supernatant stored at 20°C.

Conditioned medium from cultures of SV40-transformed rat embryo fibroblasts was prepared in a similar manner (5). Preparation of Polyacrylamide Gel Electrophoresis of Conditioned Medium—Granulosa and SV40 rat embryo fibroblast conditioned media (2 ml) were dialyzed overnight at 4°C against two changes of 0.1% SDS (1 liter). The resulting solutions were lyophilized and the solids redissolved in 100 μl of 0.0625 M Tris/HCl, pH 6.8, containing 10% glycerol and 0.1% bromphenol blue. Samples (50 μl) were applied to an SDS-polyacrylamide slab gel (0.15 × 13 × 10 cm, 11% acrylamide) prepared as described by Laemmli (13) using a stacking gel of 4% acrylamide. Proteins of known molecular weight, dissolved in the same buffer containing 2.5% SDS, were run as molecular weight markers on the same gel. The samples were not reduced or boiled, and the electrophoresis was conducted at a constant current of 7 mA for 16 h at room temperature.

The gel was sliced longitudinally between the individual sample lanes. The lanes containing the molecular weight markers were stained for protein with 0.2% Coomassie blue, 50% methanol, 1% acetic acid.
in water for 2 h at room temperature and destained with 30% methanol, 7% acetic acid in water. The length of the gel and the distance of migration of each standard from the origin was recorded.

The lanes containing conditioned media were measured and then cut into 1.1-mm slices. To assay for plasminogen activator each slice was placed in a 121-fibrin-coated culture well containing 1 ml of 0.1 M Tris/HCl, pH 8.1, supplemented with 2.5% of either native or plasminogen-depleted African green monkey serum. The assay was allowed to proceed for 4 h at 37°. The position of active fractions was compared to the position of the standard proteins after correcting for differences in the lengths of the individual lanes.

Preparation and Analysis of Granulosa Cell Lysates—Cells were plated on 60 mm Petri dishes at a density of 6 × 10⁶ cells per dish in Medium 199 supplemented with 10% plasminogen-depleted fetal bovine serum and various concentrations of LH and incubated at 37° in a 5% CO₂ atmosphere for 8 h. The cultures were washed twice with NaCl/PO₄, and the cells were removed from the dish by scraping with a Teflon policeman. The cells were collected by centrifugation, washed twice with NaCl/PO₄, and lysed by the addition of 0.2 ml of 0.1% Triton X-100. The lysates were assayed on 121-fibrin-coated plates in 0.25 ml of 0.1 M Tris/HCl, pH 8.1, supplemented with 2.5% African green monkey serum as the source of plasminogen.

Protein Determination—Protein was determined by the method of Lowry et al. (14) using bovine serum albumin as the standard.

RESULTS

Characteristics of Fibrinolysis Assay

It has been demonstrated that granulosa cells harvested from preovulatory follicles produce elevated amounts of plasminogen activator (4). The method of analysis used in those studies was a fibrin/agar overlay technique which allowed direct visualization of fibrinolysis by individual cells. For further studies of this phenomenon, a more quantitative and convenient assay was desirable. This was achieved by culturing granulosa cells on 121-fibrin and measuring the solubilization of the radioactive substrate. To establish the validity of this reaction as an assay for plasminogen activator production by granulosa cells, the dependence of the reaction on time, cell number, and plasminogen was investigated.

As seen in Fig. 1, the extent of fibrinolysis was directly proportional to the number of cells plated. The reaction was also linear with time, whether the cells were stimulated in vivo (Fig. 2) or in vitro (Fig. 3). Finally, no fibrinolytic activity was observed when plasminogen-depleted serum was used in the assay (Table I). This result was not due to deleterious effects of the modified serum on the cells, since the addition of purified bovine plasminogen to the depleted serum fully reconstituted the activity. These characteristics establish that this method is

![Graph](image_url1)

**Fig. 1.** Dependence of the extent of fibrinolysis on number of granulosa cells. Animals received PMSG and then HCG as described under "Experimental Procedure." Cells were collected from preovulatory follicles 0 h after the HCG injection and the indicated number of cells was plated per well in 0.5 ml of Medium 199 supplemented with 10% plasminogen-depleted fetal bovine serum. After 15 h at 37°, the cultures were washed twice with Medium 199 and then 0.5 ml of Medium 199 supplemented with 10% acid-treated fetal bovine serum was added to each well. Aliquots were taken 1 h later and analyzed for soluble radioactive material.

![Graph](image_url2)

**Fig. 2.** Kinetics of the fibrinolysis assay using cells exposed to gonadotropins in vivo and correlation of the response with ovulation. Animals received PMSG for 48 h and then LH for 0, 4, or 8 h. Two different classes of cells were collected from animals after 8 h exposure to LH: one class was from preovulatory follicles (pink, protuberant, and within 1 to 2 h of ovulation) and the other from non-preovulatory follicles (well developed graafian follicles, but not destined to ovulate). Granulosa cells were plated at a density of 10⁶ per well in 1 ml of Medium 199 containing 10% fetal bovine serum and 100 μg of soybean trypsin inhibitor. After 16 h in culture, each well was washed twice with Medium 199 and then 0.5 ml of Medium 199 supplemented with 10% acid-treated fetal bovine serum was added. At the indicated times, aliquots were removed and counted: •, 0 h LH; A, 4 h LH; x, 8 h LH, non-preovulatory follicles; O, 8 h LH, preovulatory follicles. Identical results were obtained if FSH or HCG was administered in place of LH.

![Graph](image_url3)

**Fig. 3.** Kinetics of the assay using cells stimulated in vitro. Animals received PMSG for 48 h and granulosa cells were prepared as described under "Experimental Procedure." The cells were plated at a density of 5 × 10⁶ cells per well in Medium 199 containing 10% plasminogen-depleted fetal bovine serum. After 3 h, the culture medium was removed and replaced with the same medium with or without 10 μg/ml of LH. Four hours later the cultures were washed twice with Medium 199 and 0.5 ml of Medium 199 supplemented with 10% acid-treated fetal bovine serum was added to each well (0 h). At the indicated times, aliquots were removed and counted.
suitable for measuring plasminogen activator production by granulosa cells in culture.

Correlation of Plasminogen Activator Production with Ovulation

The fibrinolysis assay using ¹²⁵I-fibrin as the substrate has been used to corroborate the previously reported finding that granulosa cell plasminogen activator levels and ovulation are closely correlated (4). Fig. 2 shows that cells collected from rats exposed to a gonadotropin regimen which results in ovulation, produce increased amounts of enzyme as the time of ovulation approaches.

While this experiment suggests that a correlation exists between the level of plasminogen activator and the time of ovulation, it was difficult to determine whether the response was correlated with this process or simply with the hormone regimen to which the animals were exposed. However, at the time of ovulation only a small fraction of the well developed graafian follicles present (~5%) will actually rupture. These follicles, designated as preovulatory, are easily recognized on the basis of their appearance and cells can be harvested from them selectively. Cells from the remaining follicles (non-preovulatory) can likewise be collected and cultured. Since the cells from both types of follicles exist in a common endocrine environment (in terms of the exogenously administered hormones) it is possible to test for a functional correlation in addition to the temporal one described above. The data in Fig. 4 show that at a time immediately prior to the time of ovulation high levels of plasminogen activator are associated with cells obtained from preovulatory follicles only. Since the cells from preovulatory and non-preovulatory follicles were obtained from the same ovary, it must be concluded that the observed correlation is with ovulation and not with the exposure of the cells to a particular regimen of gonadotropins.

Further evidence that the production of plasminogen activator by granulosa cells is closely correlated with ovulation has been derived from the following facts. First, in normally cycling rats, only cells obtained from preovulatory follicles late on the night of proestrus, i.e. shortly before ovulation, were active (data not shown), and second, regardless of which hormone regimen was used to induce formation of preovulatory follicles (see “Experimental Procedure”), cells from these follicles invariably produced high levels of enzyme. These results are in good agreement with those which have been obtained using the fibrin/agar overlay technique (4).

In Vitro Response of Granulosa Cells

Granulosa cells collected from rats 48 h after PMSG injection produce barely detectable amounts of plasminogen activator (Fig. 2). However, if these cells are exposed in vitro to preparations of gonadotropins, the amount of extracellular enzyme increases markedly. Cells that have been stimulated in vitro can also be assayed by the ¹²⁵I-fibrin method with essentially linear kinetics (Fig. 3). The reliability and convenience of this assay has allowed a detailed investigation of the characteristics and specificity of the in vitro response.

Effect of Polypeptide Hormones—The effect of gonadotropins on the cells has been examined for its dependence on time, dose of hormone, and various aspects of cellular metabolism. An example of the time dependence of the response to several concentrations of ovine LH is shown in Fig. 4A. The cells respond rapidly to LH and a change in enzyme level can easily be detected at all three concentrations in 0.5 h, maximal stimulation being achieved in 8 to 10 h. The dose-dependent characteristics of the response in the presence and absence of the cyclic phosphodiesterase inhibitor, theophylline, are shown in Fig. 4B. In both cases, half-maximal stimulation was obtained with approximately 3 μg/ml of LH; however, the presence of theophylline potentiated the effect of LH, increasing the activity observed as much as 2-fold. This is consistent with the finding (see below) that cAMP is also capable of increasing the level of plasminogen activator in these cells and

![Graph](https://via.placeholder.com/150)

*Table I*

<table>
<thead>
<tr>
<th>Serum supplement in assay</th>
<th>% substrate solubilized</th>
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</thead>
<tbody>
<tr>
<td>Acid-treated fetal bovine serum</td>
<td>38.3</td>
</tr>
<tr>
<td>Acid-treated fetal bovine serum, plasminogen-depleted</td>
<td>0.2</td>
</tr>
<tr>
<td>Acid-treated fetal bovine serum, plasminogen-depleted plus 26 μg/ml bovine plasminogen</td>
<td>40.1</td>
</tr>
</tbody>
</table>

Fig. 4. Time and dose dependence of plasminogen activator production by granulosa cells in response to ovine LH in vitro. Cells were prepared and plated as in Fig. 3. A, cells were allowed to plate for 3 h, and then exposed to the indicated concentration of LH. At the times indicated, replicate cultures were washed and 0.5 ml of Medium 199 containing 10% acid-treated fetal bovine serum was added to each well. Aliquots were removed and counted after 18 h. B, cells were allowed to plate for 3 h and then exposed to the indicated concentration of LH with (●) or without (〇) 10⁻³ M theophylline for 10 h. The cultures were washed and 0.5 ml of Medium 199 containing 10% acid-treated fetal bovine serum was added. Aliquots were removed and counted after 9 h. Theophylline alone did not stimulate the cells.

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Table II
Plasminogen activator levels in lysates of granulosa cells stimulated in vitro by LH

Animals received PMSG for 48 h and granulosa cells were prepared. The cells were plated in the presence of the indicated amount of LH, cultured for 8 h, and lysates prepared as described under "Experimental Procedure." The lysates were assayed on 125I-fibrin-coated wells in 0.25 ml of 0.1 M Tris/HCl, pH 8.1, containing 2.5% African green monkey serum as a plasminogen source and 20 µg/ml of cellular protein. The assay was conducted for 2 h at 37°. The values are corrected for release of radioactivity by Triton (1%). Similar assays performed using plasminogen-depleted African green monkey serum showed no fibrinolytic activity above the Triton control.

<table>
<thead>
<tr>
<th>Additions to culture medium (per ml)</th>
<th>% substrate solubilized</th>
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<tbody>
<tr>
<td>None</td>
<td>2.0</td>
</tr>
<tr>
<td>0.1 µg LH</td>
<td>8.3</td>
</tr>
<tr>
<td>1.0 µg LH</td>
<td>15.5</td>
</tr>
<tr>
<td>10.0 µg LH</td>
<td>21.8</td>
</tr>
</tbody>
</table>

Table III
Effect of macromolecular synthesis inhibitors on the in vitro response of granulosa cells to LH

The experiment was performed as in Fig. 3. The inhibitors were added at the same time as LH. Cycloheximide and cytosine arabinoside were also included in the assay medium. Aliquots were counted after 14 h.

<table>
<thead>
<tr>
<th>Additions to culture medium (per ml)</th>
<th>% substrate solubilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>10 µg LH</td>
<td>61.7 ± 1.0</td>
</tr>
<tr>
<td>10 µg LH + 10 µg cytosine arabinoside</td>
<td>63.8 ± 5.4</td>
</tr>
<tr>
<td>10 µg LH + 1 µg actinomycin D</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>10 µg LH + 10 µg cycloheximide</td>
<td>10.1 ± 0.2</td>
</tr>
</tbody>
</table>

suggests that the effect of gonadotropins may be mediated by cyclic nucleotides.

The above studies, and those reported elsewhere using a different method of analysis (4), demonstrate that in vitro exposure of granulosa cells to gonadotropins increases the extracellular level of plasminogen activator. Therefore, an analysis of the change in intracellular enzyme levels was also undertaken. The results shown in Table II demonstrate that the level of intracellular activity, like the extracellular, increases with increasing concentrations of LH. This result suggests that the cells respond to gonadotropins by increasing de novo synthesis of enzyme, a conclusion which is strengthened by an examination of the effect of inhibitors of RNA, DNA, and protein synthesis on the in vitro response (Table III). Actinomycin D and cycloheximide markedly inhibited the response whereas cytosine arabinoside did not, demonstrating the necessity of RNA and protein synthesis for the appearance of elevated levels of extracellular plasminogen activator. The dependence on macromolecule synthesis and the parallel increase in intracellular and extracellular activity indicates that the cells respond to gonadotropins by increasing enzyme synthesis and secretion.

Since the production of plasminogen activator is closely correlated with ovulation in vivo and can be effected in vitro by gonadotropins, an analysis of the specificity of the in vitro response was performed. It had been observed previously that LH, the presumptive ovulatory hormone, was not the most effective hormone in vitro (4). This observation has been explored in detail, and Fig. 5 shows the characteristics of the granulosa cell response to FSH. The time dependency of the effect of FSH is very similar to that of LH, and the level of activity observed is elevated by theophylline. FSH, however, is active at much lower concentrations than LH. Half-maximal stimulation occurs at 0.1 µg/ml with FSH as compared with 3 µg/ml with LH. Thus, on a weight basis, with the impurity of these preparations in mind (see "Discussion"), FSH is minimally 30-fold more active than LH.

To study the specificity of the in vitro response further, several other ovine pituitary hormones were tested for their ability to stimulate granulosa cells (Fig. 6A). Of all the hormones tested, FSH was by far the most effective. LH and TSH were equally active but at higher concentrations, whereas prolactin and growth hormone were inactive. In view of these results, PMSG and HCG, which have high FSH and LH activity, respectively, were also studied. As seen in Fig. 6B, the cells respond to PMSG at much lower concentrations than HCG, a result which is consistent with the specificity studies using ovine hormones.

While the above studies were of interest regarding the specificity of the rat granulosa cell response, their significance could not be judged until a similar study was conducted examining the response of the cells to homologous rat hormones. As seen in Fig. 7, rat FSH was more than 100 times more potent than rat LH, suggesting that the specificity observed previously has relevance to the physiological situation.

Effect of Cyclic Nucleotides and Cyclic Phosphodiesterase Inhibitors—In addition to gonadotropins, cAMP and Bt,cAMP are capable of stimulating granulosa cells in vitro to produce plasminogen activator. The dibutyryl derivative is capable of eliciting a response at lower concentrations than cAMP, perhaps due to the fact that cells are poorly permeable to cAMP. As seen in Fig. 8, the effect of Bt,cAMP was dose-dependent and was enhanced by theophylline. Other cyclic phosphodiesterase inhibitors, such as Ro 20-1724 and 3-isobutyl-1-methylxanthine also potentiated the response to Bt,cAMP. In contrast to these results obtained with cyclic adeno nucleotides, granulosa cells were not stimulated by cGMP or its derivatives Bt,cGMP and 8-Br-cGMP.

It has been well established that gonadotropins elevate ovarian cAMP levels (15) and it is often suggested that this nucleotide mediates the response to many polypeptide hormones. The fact that both gonadotropins and cAMP cause granulosa cells to produce the same enzyme is further evidence that they are linked in function. The finding that theophylline enhances the response to LH and FSH (Fig. 4B and 5B) also supports this view.

Effect of Prostaglandins—Prostaglandins have been shown to possess gonadotropin-like activities, such as increasing the synthesis of ovarian cAMP (15) and steroids (16). Furthermore, a role for prostaglandins has been suggested in ovulation (17, 18). Consequently, prostaglandins were tested for their ability to induce plasminogen activator production in granulosa cells. Prostaglandins E1 and E2 elicited a response at physiological concentrations, whereas prostaglandins F1α and F2α, were completely inactive (Fig. 9). The response to prostaglandins E1 and E2 was enhanced by theophylline, suggesting the possible intermediacy of cAMP in...
FIG. 5. Time and dose-dependence of plasminogen activator production by granulosa cells in response to ovine FSH in vitro. The experiment was performed as in Fig. 4, except that FSH was used in place of LH.

FIG. 6. In vitro response of granulosa cells to various polypeptide hormones. Cells were prepared and allowed to plate for 3 h as in Fig. 3. A, cells were exposed to hormones for 10 h, washed, and changed to Medium 199 containing 10% acetic acid-treated fetal bovine serum. Aliquots were counted after 6 h. B, cells were exposed to hormones for 4 h, washed, and treated as in A. Aliquots were counted after 15 h. Gestyl (PMSG) and pregnyl (HCG) were the hormone preparations used.

the effect. Although this is a feature shared with the response to gonadotropins, two lines of preliminary evidence suggest that prostaglandins and gonadotropins act independently, though possibly through the same final pathway, in their ability to stimulate the cells. First, indomethacin, an inhibitor of prostaglandin biosynthesis, does not inhibit the effect of gonadotropins. Second, prostaglandins will elevate the level of plasminogen activator produced by cells exposed to saturating levels of FSH.

Effect of Steroids—Since ovarian tissue is the site of synthesis of several steroids, it was of interest to examine the effect of steroids on plasminogen activator production by granulosa cells. Of particular interest to these studies is the fact that progesterone has been implicated directly in amphibian (19) and indirectly in mammalian ovulation (3). Furthermore, the production of plasminogen activator by certain cells is inhibited at low concentrations of anti-inflammatory steroids (20, 21). However, none of the steroids stimulated the production of enzyme by inactive cells, nor did they affect the dose-response characteristics of the cells to gonadotropins. The following steroids were tested at concentrations up to $10^{-8}$ M: progesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 17β-estradiol, testosterone, and dexamethasone. Finally, cyanoketone, which blocks progesterone biosynthesis by inhibiting 3β-hydroxysteroid dehydrogenase, had no effect on the in vitro response to gonadotropins.

Other Compounds Which Were without Effect on Stimulated Cells—It has been shown in several experimental systems that cGMP exerts an action opposite to that produced by cAMP (22). As seen above, cGMP and structurally related molecules failed to stimulate inactive cells. These compounds were also tested for their ability to inhibit the response of the cells to gonadotropins and found to have no effect ($10^{-5}$ to $10^{-3}$ M). Prostaglandins F$_{16}$ and F$_{12}$ are also potential candidates as inhibitors; however at concentrations up to $10^{-8}$ M they did not influence the response of the cells to gonadotropins or prostaglandin E$_{1}$.

Characterization of Granulosa Cell Plasminogen Activator

The production of plasminogen activator is a property of many oncogenically transformed cells as well as some normal cells (see "Discussion"). Therefore, the characteristics of the
granulosa cell enzyme have been investigated to allow a comparison of its molecular weight and catalytic properties to other plasminogen activators.

Although only minute quantities of extracellular enzyme are produced by the most active granulosa cells, i.e., those obtained from preovulatory follicles, methods have been developed that enable the molecular weight of the enzyme to be determined (23). These methods take advantage of the fact that most plasminogen activators are not irreversibly inactivated by SDS (24). Thus, if serum-free conditioned medium from active cul-

Fig. 7. In vitro response of granulosa cells to rat LH and FSH. The experiment was performed as in Fig. 6A using rat hormones. The amount of rat LH available precluded analysis above 10 μg/ml. The magnitude of the response to 10 μg/ml of ovine FSH is shown by the bar.

Fig. 8. In vitro response of granulosa cells to Bt2cAMP (DBcAMP) and the effect of theophylline. Cells were prepared and allowed to plate for 3 h as in Fig. 3. The cells were exposed to Bt2cAMP alone (●) or Bt2cAMP and theophylline (○, □) as indicated in the figure for 4 h. The cultures were then washed and 0.5 ml of Medium 199 containing 10% acid-treated fetal bovine serum was added. Aliquots were counted after 15 h.

Fig. 9. In vitro response of granulosa cells to prostaglandins. The experiment was performed as in Fig. 8. The cells were exposed to prostaglandins E1, E2, F1α, or F2α at the indicated concentrations.

tures is fractionated by SDS-polyacrylamide gel electrophoresis and the gel sliced, the individual fractions can be assayed for plasminogen activator activity. The results of such an analysis are shown in Fig. 10A and demonstrate that the enzyme migrates with an apparent molecular weight of 75,000. In addition, a small amount of enzyme with molecular weight 45,000 was observed. Two species of plasminogen activator with molecular weights of 75,000 and 45,000 were also detected in conditioned medium obtained from SV40-transformed rat embryo fibroblasts (Fig. 10B).

One catalytic feature of most mammalian plasminogen activators is that they are unable to activate chicken plasminogen (25). As seen in Table IV, this is also true for the granulosa cell enzyme. Furthermore, the relative fibrinolytic activities generated by the enzymes from granulosa and transformed rat cells were equivalent when plasminogen from various species were used in the assay (Table IV). By these characteristics the granulosa cell plasminogen activator is similar to other mammalian activators.

**DISCUSSION**

In this paper we have presented evidence that granulosa cells can synthesize and secrete plasminogen activator and that this process is under hormonal control. In order to place this observation in a physiological context, it should be considered from three points of view: (a) the functional similarities between granulosa cells and other cell types that secrete this enzyme, (b) the potential function of this enzyme in ovulation, and (c) the possible relevance of the hormonal control of the synthesis and secretion of plasminogen activator to the endocrinology of ovulation.

The production of plasminogen activator is a property of many cell types such as oncogenically transformed cells (6, 26), activated macrophages (23), giant trophoblast cells, and stimulated granulosa cells (4). The fact that cells so dissimilar in morphology and origin secrete elevated levels of the same enzyme implies some similarity in function. Since plasminogen activator activity is expressed extracellularly, and since outside the cell the amount of proteolytic activity can be amplified

1 Since the plasmin molecules derived from these plasminogens are reported to have identical specific activities (22) this result should be a direct reflection of the efficiency of plasminogen activation by the two enzymes.

manyfold by the catalytic conversion of plasminogen to plasmin, it is reasonable to predict that the common function of these cells may involve their interaction with the external milieu. A case can be made that all the cell types mentioned share the capacity to disrupt connective tissue and plasminogen activator may provide the means for the disruption. For example, macrophages might require this enzyme in order to migrate from blood vessels (21), and trophoblasts to invade the uterine wall at the time of embryo implantation.

The production of plasminogen activator by granulosa cells is in accord with this concept. The maximum level of enzyme appears at a time coincident with the following events which occur in preovulatory follicles of the rat ovary. The ovum and its investments are dislodged from the granulosa cell layer and become free-floating in the follicular fluid (27). The granulosa cell layer is disrupted and in the region of the wall that is destined to rupture, the entire layer is removed (28). Finally, the connective tissue and thecal layers, which comprise the remainder of the follicle wall at the point of rupture, are degraded. The wall becomes thin, a stigma forms, ultimately tears, and the follicular contents, including the ovum, ooze out. These events imply degradation of connective and interstitial tissue, and could be explained by the action of intrafollicular proteases such as plasminogen activator and plasmin. This supposition is supported by the observation that plasmin is capable of weakening follicle wall tissue (5).

With every experimental approach used to date to examine the in vivo production of enzyme, elevated levels have been associated with granulosa cells from preovulatory follicles only. This suggests that the production of plasminogen activator by these cells can be used as a paradigm for the study of ovulation. Based on this concept and the results of our in vitro experiments, we propose the following scheme for the control and role of this enzyme in the ovulatory process.

1. An increase in gonadotropin or prostaglandin levels, or both, results in a corresponding increase of the intracellular CAMP level in the granulosa cells (15). The increased CAMP then stimulates the synthesis of plasminogen activator.

2. The enzyme is secreted into the follicular fluid.

3. Plasminogen, present in follicular fluid at serum levels (5), is converted to plasmin.

4. Plasmin initiates the sequence of events that culminates in rupture of the follicle.

Although we are aware that this scheme presents a simplified and idealized view of a complex process, it should provide the
basis for investigations into the specific biochemical events involved.

As seen from the results presented in this paper, the in vitro stimulation of granulosa cells allows a well defined analysis of the specificity of the response to gonadotropins. These studies show that FSH is much more effective than LH. In fact, since the LH preparation used is likely to contain traces of FSH, the possibility exists that the response to LH is due to contaminating FSH. The fact that TSH, which has no known function in the ovary, was as active as LH, also suggests that the stimulation by these two hormones might be due to the presence of FSH in the preparations. These three polypeptide hormones share a common subunit (29) and it is therefore difficult to obtain them in pure form. Prolactin and growth hormone, pituitary hormones that do not share this structural similarity, were not active.

Thus, FSH appears to be the hormone that controls plasminogen activator levels in granulosa cells. In light of the above model, this implies that FSH is ultimately responsible for the disruption of the follicle wall at the time of ovulation. This proposal runs counter to the classical view of ovulation in which LH is regarded as the ovulatory hormone. However, the experiments that form the foundation of this concept were performed on whole animals and suffer from the inherent limitations in scope and interpretation of such studies. In fact, a critical examination of the literature on this topic indicates that the identity of the ovulatory hormone is not established (30). A review of these data is not appropriate here, but it should be emphasized that in the rat, it has been reported that FSH alone can induce ovulation (31, 32).

It is known from experiments in a number of species that both LH and FSH reach peak levels in the blood at the same time prior to ovulation (33). Furthermore, granulosa cells have receptors for both hormones (34). On the basis of these observations, it seems likely that both FSH and LH are involved in ovulation, but that they are responsible for different biochemical events. Our results indicate that FSH controls the level of plasminogen activator. LH may be involved in other functions important at the time of ovulation, such as preparing the cells for luteinization if fertilization occurs.

Clearly, the endocrinology and biochemistry of ovulation are complex and not firmly established. Since the production of plasminogen activator by granulosa cells is the best defined enzymatic correlate of ovulation and is easily assayed, it should prove useful in analyzing this process.

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Studies on the role of plasminogen activator in ovulation. In vitro response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins.

S Strickland and W H Beers