A Cell Culture Assay for Follicle-stimulating Hormone*

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Cultured rat ovarian granulosa cells respond to follicle-stimulating hormone (FSH) by synthesizing and secreting plasminogen activator. The specificity of the response for FSH prompted us to explore the use of this system as an in vitro bioassay for FSH. The release of FSH by pituitary cell cultures has been examined by this method, as have been preparations of FSH by preparing pituitary cell cultures has been examined by this method, as have been preparations of FSH by synthesizing and releasing hormone. Furthermore, the method obviates the problem of metabolic clearance and is extremely sensitive: as little as 10^-12 mol (100 pIU) of FSH can be detected.

In a previous publication (1) we demonstrated that rat ovarian granulosa cells respond in vitro to gonadotropins by synthesizing and secreting plasminogen activator. These determinations were conducted with extremely small amounts of gonadotropin using a method of analysis that is simple and rapid. Further studies, reported below, have revealed that this response of the cells is specific for follicle-stimulating hormone.

The routine bioassay for FSH, the HCG augmentation assay of Steelman and Pohley (2), requires large amounts of gonadotropin and large numbers of rats. Furthermore, it is time consuming and suffers from the lack of precision generally associated with bioassay. Because it appeared that the in vitro granulosa system would provide a rapid method for the detection of FSH which is more sensitive than the Steelman-Pohley assay by a factor of 10^1 to 10^5, we have performed the studies described in this report. These experiments further characterize the cell system and illustrate its usefulness as an in vitro bioassay for FSH.

EXPERIMENTAL PROCEDURES

Materials—Rats of the Sprague-Dawley strain were obtained from the Holtzman Co. Multiwell culture plates, containing 24 wells (16 mm diameter), were purchased from Linbro Chemical Co. Penicillin, streptomycin, Medium 199, and Leibovitz's L-15 were obtained from Gibco; fetal bovine serum from Reheis Chemical Co.; pregnant mare serum gonadotropin, HCG, and bovine serum albumin from Sigma; trypsin from Nutritional Biochemicals; fibrinogen (77% clottable) from Calbiochem; Sepharose 4B from Pharmacia; LRH from Beckman Instruments, Inc.; and soybean trypsin inhibitor from Miles Seravac.

Ovine FSH (S-12), rat FSH (I-3) and LH (I-4), and antisera to the rat gonadotropins (FSHS-7 and LHS-4) were provided by the National Institute of Arthritis, Metabolism and Digestive Diseases.

All other materials were of reagent grade.

Preparation of Rat Granulosa Cell Cultures and Analysis of Plasminogen Activation Production—The methods employed for the preparation of cells and materials for plasminogen activator analysis have been described elsewhere (1). The routine procedure was as follows: female rats, 26 days old, were injected subcutaneously with 5 IU of pregnant mare serum gonadotropin. After 48 h the animals were decapitated, the ovaries removed, and granulosa cells harvested by puncturing follicles and expressing their contents into Medium 199 or L-15 (L-15 is a phosphate-buffered medium that maintains a pH of 7.2 to 7.4 in air). The cells were then plated in culture wells (90,000 cells/well) that had been previously coated with 125I-fibrinogen. The culture medium (0.5 ml/well) consisted of either Medium 199 or L-15 containing 100 units/ml of penicillin and 100 μg/ml of streptomycin and supplemented with 10% plasminogen-depleted fetal bovine serum. After 3 h, during which time the granulosa cells attach to the dish and the fibrinogen is converted to fibrin, the medium was removed and replaced with 0.5 ml of fresh medium with or without gonadotropin. Under routine conditions the culture cell suspensions were exposed to hormone for 4 h, the medium was again removed and, after washing the cultures twice with serum-free medium, replaced with 0.5 ml of medium containing 10% acrid acid-treated fetal bovine serum. The cultures were then incubated overnight. Aliquots of the culture fluid were then collected and analyzed for soluble 125I-peptides in a Packard Auto Gamma spectrometer. All of the above procedures were conducted at 37°C. Cultures containing Medium 199 were incubated in the presence of an atmosphere of 5% CO2, 95% air. Cultures containing L-15 were incubated in air. Equivalent results were obtained with either medium.

All assays were performed in duplicate. The difference between duplicates was routinely less than 10%. Thus all values are given as the mean of the two determinations and are presented as the fraction of the total available substrate solubilized. Total digestable fibrin was determined by exposing wells to 0.25% trypsin in 0.1 M Tris, pH 8.1. To control for variations in cell preparations and the condition of the radioactive substrate, each plate included cells which had been maximally stimulated (10 μg/ml of NIH-FSH-S-12).

Preparation of Pituitary Cultures and Conditioned Medium—Cells were prepared from the pituitaries of rats that were 26 to 30 days old by a method similar to one described by Hopkins and Farquhar (3). After decapitation, the anterior portion of the pituitary gland was removed from the skull and minced with a scalpel. When sufficient tissue had been collected (usually from 15 to 30 animals), it was incubated with stirring at 37°C for 2 h in Medium 199 containing 1 mg/ml of bovine serum albumin and 1 mg/ml of trypsin. Throughout this treatment the tissue was repeatedly passed through a 20-g hypodermic needle to facilitate dispersion of the cells.

After 2 h little if any undissociated tissue remained and the cells were collected by centrifugation and then washed six times by resuspension and centrifugation in Medium 199 containing antibiotics, 10% plasminogen-depleted fetal bovine serum, and 100 μg/ml of soybean trypsin inhibitor. After the final wash, the cells were plated in 0.5 ml of the above medium but without trypsin inhibitor at desired densities (usually > 0,000 cells/well) in untreated Linbro wells. After 48 h the medium was removed, the cultures washed twice with medium containing trypsin inhibitor, once with medium alone, and replaced with 0.25 ml of medium with or without LH. Conditioned medium was then harvested at desired times, usually after 2 to 3 h, for subsequent analysis in the granulosa cell cultures. For these determinations...
aliquots of the above samples were analyzed in the granulosa cell system in parallel with a series of FSH standards. The conditioned medium was routinely assayed in the absence of granulosa cells to detect trypsin which might have been carried through from the procedure used to disperse the pituitary cells.

Immunoprecipitation of Gonadotropins—Rat LH and FSH were iodinated with 125I as described by Liu et al. (4). The specific activity of the derivatized LH and FSH was 54 μCi/μg and 42 μCi/μg, respectively. The immunoprecipitations were performed using rabbit antirat LH and anti-rat FSH antisera. The antisera were diluted according to NIH instructions and stored frozen in Medium 199 containing 3% plasminogen-depleted fetal bovine serum.

Each precipitation reaction contained the following: 1) 0.1 ml of Medium 199 with 10% plasminogen-depleted fetal bovine serum containing 125I-LH, 125I-FSH, or authentic rat FSH; or 0.1 ml of pituitary cell-conditioned medium; 2) 20 μl of the appropriate antiserum or 20 μl of Medium 199 with 5% plasminogen-depleted fetal bovine serum. This mixture was incubated on ice for 2 h and then 20 μl of a 10% solution of heat-killed Staphylococcus aureus (5, 6) in Medium 199 was added to precipitate the antibodies. Control experiments demonstrated that the suspension of S. aureus had no effect on the gonadotropin solutions in the absence of antibody. After a further 30 min on ice, the mixtures were centrifuged at 4°C for 4 min in an Eppendorf centrifuge, model 5412. Supernatants to be tested for FSH activity were assayed using the granulosa cell system with the following modifications. After attachment, only 0.25 ml of medium was added to the cells, and 0.05 ml of the appropriate sample was added. In addition, aliquots were counted after 5 h rather than overnight. The amount of gonadotropin precipitated was determined by comparison to a standard dose-response curve of rat FSH performed simultaneously on the same granulosa cell preparation. The supernatants and pellets from the samples containing radiolabeled gonadotropin were counted.

RESULTS AND DISCUSSION

Specificity, Sensitivity, and Characteristics of in Vitro Response—When rat granulosa cells were exposed to highly purified preparations of rat gonadotropins, the response was specific for FSH. Fig. 1 shows that FSH was more potent than equivalently purified LH by a factor of approximately 10. As analyzed by the Steelman-Pohley assay, this LH preparation possesses an FSH activity <1/5000 that of the FSH preparation. Half-maximal response to FSH was achieved with ~3 ng/ml of the purified gonadotropin, which, assuming homogeneity (resulting in a conservative estimate), is equivalent to a concentration less than 10^-10 M. The determinations described in Fig. 1 were conducted with cultures that contained 0.5 ml of media; thus half-maximal stimulation was observed with 1.5 ng of gonadotropin. The sensitivity of the system can be further increased by a diminution of the volume of media in each culture well and by the inclusion of a cyclic nucleotide phosphodiesterase inhibitor, which augments the magnitude of the response, during exposure to gonadotropin. Under these conditions, the sensitivity of the assay can be increased 5- to 10-fold. In addition, hormone concentrations 10-fold lower than those required to reach half-maximal stimulation can be detected. These modifications have allowed us to measure quantities of rat FSH as small as 30 pg; less than 10^-17 mol.

For the studies described here, however, these refinements have proved unnecessary.

The time course of the response to rat FSH was similar to that reported previously (1) for ovine gonadotropin preparations. However, maximum levels of activity were attained after approximately 4 h of exposure to the rat hormone (Fig. 2), in contrast to approximately 10 h for ovine FSH.

Analysis of FSH Secretion by Pituitary Cell Cultures—Several investigators have demonstrated that primary cultures of rat pituitary cells respond to gonadotropin-releasing hormone by secreting LH and FSH into the growth medium (7-9). Measurement of the secretion product is generally achieved by radioimmunoassay since this method possesses the requisite sensitivity. However, these measurements suffer from 1) the inherent drawbacks of competition assays, and 2) the fact that antigenic, not biological activity, is monitored (10). The use of the granulosa cell system overcomes these problems and the analysis of pituitary cell-conditioned medium demonstrates its facility as an in vitro bioassay under conditions that are experimentally realistic.

All of the experiments with pituitary cultures were performed in the following manner. Cells were prepared from the pituitaries of immature rats as described under "Experimental Procedures" and plated at the indicated densities. After 48 h the growth medium was removed and replaced with medium with or without LRH. Aliquots of the pituitary culture-conditioned medium were taken at desired times and added to granulosa cell cultures to determine the quantity of FSH present. Under these conditions, LRH itself had no effect on the granulosa cells. The time course of FSH secretion, as
monitored by the granulosa cell assay, in response to LRH is shown in Fig. 3. The cells secreted this gonadotropin during the first 1 to 2 h following exposure to LRH. This is similar to the response pattern seen when LH secretion is monitored (9). To provide further evidence that FSH was the hormone being measured, immunoprecipitations with specific antisera to FSH and LH were performed (Table I). These experiments demonstrated that antiserum to FSH was capable of decreasing the activity of the pituitary cell-conditioned medium by approximately 90%. This percentage was similar to the amount of radiolabeled FSH that could be precipitated with this antiserum, and also to the reduction in activity seen when unmodified FSH was treated in the same manner. Antiserum to LH, which was capable of precipitating 90% of a radiolabeled preparation of LH, had no effect on the activity. These results indicate that the active substance in the pituitary harvest fluid is FSH.

The magnitude of the response of the pituitary cell cultures is dependent on the number of pituitary cells (Fig. 4) and the concentration of LRH in the medium (Fig. 5). At densities less than 1 x 10^6 cells plated/well, we have had difficulty obtaining uniformly responsive cultures and consequently have conducted most determinations with at least 2 x 10^6 cells/well. Half-maximal response occurs at 1 x 10^-9 M LRH, a value which is in good agreement with the findings of other investigators using different techniques for the secretion of both FSH and LH (7, 9).

These results indicate that plasminogen activator production by granulosa cells can serve as a very sensitive assay for biologically active FSH. In situations where crude biological samples are analyzed, care must be taken to determine that none of the low molecular weight effectors of the response are present in the samples. For example, A, B, and E prostanoids and cAMP are known to stimulate the cells. All of these can be removed from specimens by gel filtration; however, only the prostanoids pose a serious problem under most experimental conditions. cAMP stimulation of the cells is measurable only at concentrations of approximately 10^-9 M or greater (11). The response to catecholamines also occurs at nonphysiological concentrations (10^-10^-6 M), is minimal in the absence of cyclic nucleotide phosphodiesterase inhibitors, and is blocked by β-adrenergic antagonists such as propranolol (12). Other potential, but as yet unknown, agonists of the granulosa cells can be detected from the results obtained from immunoprecipitation studies such as those described in Table I. In experimental situations where purified, or partially purified, gonadotropins are analyzed for activity, these precautions are not necessary.

Comparison of in Vitro FSH Activity Measurements with Those Obtained by Bioassay—The response of granulosa cells to FSH provides accurate relative measures of FSH activity. In addition, we have examined the extent to which the in vitro bioassay results correlate with values obtained by the Steelman-Pohley method. To conduct these comparisons, 15 samples of gonadotropin, prepared from 5 species, were provided by Dr. Leo E. Reichert. These specimens, which had been previously bioassayed, were made available as numbered unknowns and activity comparisons were made only after all specimens had been analyzed in the granulosa cell system. The results of this study are shown in Table II. All specimens were initially compared to the ovine preparation NIH-FSH-S12 as a reference standard. When the three ovine unknowns were compared to it, the granulosa cell assay and bioassay results were in good agreement. When the activities of preparations from other species were compared, relative values within each group were similar, but the absolute amount of activity determined by the granulosa cell assay was consistently lower than that determined by the Steelman-Pohley method if ovine FSH was used as a reference standard.

The inability of the anti-FSH antiserum to quantitatively precipitate authentic FSH is not understood. The percentage of precipitation was not increased by longer incubation time with antiserum or with higher concentrations of antiserum. The residual activity could be due to slight modifications of FSH molecules (e.g. more or less glycosylation) which prohibit recognition by the antibodies but do not seriously affect biological activity. It seems reasonable to conclude, based on this result, that the residual activity in the pituitary cell harvest fluid is also due to nonprecipitable FSH and not other effectors of the response.
After 3 h the conditioned medium was removed and aliquots were assayed in the granulosa cell system as described in Fig. 3. O, +10^{-7} M LRH; ●, no addition. rFSH, rat FSH.

FIG. 4. Dependence of the extent of FSH secretion on the number of pituitary cells plated. Pituitary cells were prepared and plated at the indicated densities. After 48 h the cultures were washed and medium, with or without 10^{-7} M LRH, was added to replicate cultures. After 3 h the conditioned medium was removed and aliquots were assayed in the granulosa cell system as described in Fig. 3. O, +10^{-7} M LRH; ●, no addition. rFSH, rat FSH.

**TABLE II**

Comparison of biological activity of various FSH preparations as determined by granulosa cell in vitro assay and Steelman-Pohley bioassay

Preparations of gonadotropins were supplied as preweighed, numbered unknowns by Dr. Leo E. Reichert. The samples were solubilized in L-15 medium and were analyzed in the granulosa cell system at concentrations ranging from 0.001 to 10 µg/ml as described under “Experimental Procedures” and in a manner analogous to that described in Fig. 1. Parallel determinations were made with standard solutions of NIH-FSH-S-12. (S-1 and S-12 are similar preparations of ovine FSH; S-12 is 1.25 times more potent than S-1 by bioassay.) Half-maximal responses and relative potencies were then determined for each preparation and the results were compared to those obtained by bioassay. For both assays, potencies relative to an ovine standard (S-1 or S-12) are reported for all samples. In addition, potencies of the human preparations are reported relative to a human standard (LER 907).

<table>
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<th>Relative potency</th>
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<td>Granulosa cell in vitro assay versus</td>
<td>Steelman-Pohley bioassay* versus</td>
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<td></td>
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*Bioassays performed by L. E. Reichert.

### CONCLUSIONS

The stimulation of granulosa cell plasminogen activator production by FSH has been used as an in vitro bioassay for FSH. As with other in vitro bioassays, this method is limited available saving in time and materials.

**Analysis of Modified Gonadotropins—**Using previously available methodology, studies of the effect of chemical or enzymatic modification on the activity of FSH have been difficult if not impossible for the following reasons. First, the amount of material required to conduct bioassays is very large. Second, modifications which alter the metabolism of gonadotropins, e.g. by increasing the rate at which these substances are cleared from circulation, could cause enormous changes in their apparent biological activity, without concomitant changes in their effectiveness per se at the target organ (13, 14). Use of the granulosa cell assay obviates these difficulties. For example, this system has been used to analyze the effect of desialylation on the FSH activity of pregnant mare serum gonadotropin. These studies, which will be described elsewhere, were conducted using less than 150 ng of gonadotropin and demonstrated a parallel increase in the biological activity and receptor affinity of the modified gonadotropin.
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in its ability to predict biological activity in whole animals. However, this system has several features which make it attractive.

1. Among the gonadotropins it is specific for molecules that possess FSH activity.
2. It is very sensitive. Under routine conditions half-maximal activity is achieved with 1.5 ng (~6 mIU) of purified rat FSH/culture. If necessary the sensitivity can be increased further by a factor of 50 to 100.
3. The procedure is complete within 24 h and hundreds of samples can be processed simultaneously.
4. In contrast to radioimmunoassays and radioligand assays, a highly purified preparation of FSH is not required as a standard since the assay relies on the inherent specificity of the granulosa cell response.
5. It provides a quantitatively reliable method for assessing relative concentrations of FSH. If reference to previous bioassays is desired, a reference standard from the same species can be used to generate values which are directly comparable.
6. Activity determined by this method is not influenced by whole animal metabolism and thus provides a measure of the intrinsic activity of a preparation at a target cell. This consideration is particularly important in studies of the activity of structurally modified gonadotropins since certain manipulations can increase the metabolic clearance rate of a fully active hormone to the extent that it is difficult to demonstrate biological activity.

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