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STIMULATORY EFFECT OF GONADOTROPINS ON THE SYNTHESIS OF ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE AND PROGESTERONE BY SUSPENSIONS OF RAT OVARIAN INTERSTITIAL CELLS

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Summary

A cell suspension was prepared from immature rat ovaries by treatment with trypsin and collagenase. The isolated cells were capable of converting [8-^{14}C]adenine to cyclic [^{14}C]AMP and the rate of this conversion was stimulated in vitro by luteinizing hormone and human chorionic gonadotropine, but not by prolactin, norepinephrine, dopamine or albumin. The accumulation of progesterone was also measured in these cells by radioimmunoassay. In vitro addition of luteinizing hormone stimulated the accumulation of radioimmunoassayable progesterone. The conversion of [8-^{14}C]adenine to cyclic [^{14}C]AMP showed a rapid increase during the first 30 min of the incubation period when luteinizing hormone was added to the incubation medium. Progesterone accumulation in response to the same dose of luteinizing hormone showed a lag period for the first 30 min of incubation after which there was an increase up to 2 h. The luteinizing hormone-induced progesterone accumulation was sensitive to puromycin, but there was no effect on the luteinizing hormone-induced increase in cyclic [^{14}C]AMP formation from [8-^{14}C]-adenine. Actinomycin D also inhibited the luteinizing hormone-induced progesterone accumulation, with no effect on cyclic AMP formation. The results suggest that the luteinizing hormone-induced progesterone accumulation in rat ovarian interstitial cell suspension is preceded by an increased accumulation of cyclic AMP and that the accumulation of steroid under the influence of luteinizing hormone involve processes sensitive to puromycin and antinomycin D.

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Introduction

Luteinizing hormone and human chorionic gonadotropin have been shown to activate adenylate cyclase in bovine corpus luteum [1,2], in the interstitial tissue from pseudopregnant rabbit [3] and in prepubertal rat ovary [4]. An increase in the cyclic AMP level in the ovarian tissue in response to these hormones has also been reported [5—9]. One of the major difficulties that one encounters when studying the effect of gonadotropic hormones in the target tissues is that the hormonal response to steroid conversion is observed only when an intact tissue is employed. The present experiments were aimed at studying the mode of regulation of gonadotropic hormones on the synthesis of cyclic AMP and progesterone using isolated cells prepared from immature rat ovary by treatment with trypsin and collagenase. This communication describes the temporal relationship between cyclic AMP and progesterone synthesis under the influence of luteinizing hormone and the effects of inhibitors of RNA and protein synthesis.

Materials and Methods

Chemicals

[8-^{14}C] Adenine (40—60 Ci/mol) was obtained from New England Nuclear Corporation and cyclic [^{3}H] AMP (28 Ci/mol) was purchased from Schwarz Mann Chemical Company. Cyclic AMP and cyclic nucleotide phosphodiesterase, and Dowex 50w were obtained from Sigma Chemical Company. Eagle's minimum essential medium was purchased from Grand Island Biochemical Company, Detroit. Collagenase CLS and Lima bean trypsin inhibitor were a product of Worthington Biochemical Corporation and methyl cellulose U.S.P. was obtained from Fisher Scientific Company. Trypsin 1 : 3000 was a product of General Biochemicals. Luteinizing hormone (NIH-LH-S16), follicle-stimulating hormone (NIH-FSH-P1), and prolactin were generously donated by the Endocrine Society Study Section of the hormone distribution program, National Institutes of Arthritis and Metabolic diseases. The α and β subunits of luteinizing hormone were kindly donated by Dr John G. Pierce, The University of California, Los Angeles. Actinomycin D and puromycin dihydrochloride were purchased from Sigma Chemical Company. Reagents for radioimmunoassay were kindly provided by Dr A.R. Midgley, Jr of this University.

Preparation of ovarian cell suspension

The procedure for the isolation of the cell suspension was adapted from that described by Liu and Gorski [10]. Holtzman rats (22—25 days old) were used in these experiments. After decapitation, ovaries were removed, and placed in ice cold Krebs-Ringer bicarbonate buffer (pH 7.4), containing 0.2% glucose and 0.1% methyl cellulose (Buffer A). The tissue was chopped into small pieces and transferred to a 25-ml Erlenmeyer flask and an aqueous solution of 1.5% trypsin (pH 7.6, adjusted with 1 M NaOH) was added to this and incubated at 37°C for 15 min in an atmosphere of 95% O_2 and 5% CO_2 with constant shaking. At the end of the incubation, the suspension was thoroughly mixed with a Pasteur pipet by upward and downward suction and centrifuged

at 600 × *g* for 2 min at room temperature. The pellet was washed twice with Buffer A and then suspended in the same solution containing 0.5% collagenase and incubated at 37° C for 50 min in an atmosphere of 95% O_2 and 5% CO_2, with mixing the contents approx. 20 times during the incubation period. At the end of the incubation, the suspension was again mixed and then centrifuged at 600 × *g* for 2 min at room temperature. The pellet was washed successively with 10 vol. of Buffer A containing 0.02% and 0.002% of Lima bean trypsin inhibitor and twice with Buffer B (Eagle's minimum essential medium containing 0.1% methyl cellulose and 0.0001% Lima bean trypsin inhibitor). The final pellet was resuspended in Buffer B and filtered through 2 layers of cheesecloth. The viability of the cells was ascertained by the inability of the cells to take up nigrosin when exposed to the dye. On average, the viability of the cells was above 90% and the yield was between $4-6 \cdot 10^5$ cells per mg ovarian tissue.

Incubation procedure

200 µl of the cell suspension ($1.8 \cdot 10^6-2.4 \cdot 10^6$ viable cells) were used in each incubation and duplicate determinations were performed in all experiments. The incubation procedure consisted of two stages; initial incubation with [8-^{14}C]adenine for usually 45 min to label the intracellular ATP pools in an atmosphere of 95% O_2 and 5% CO_2 and a second incubation in the presence of hormone or other test substances for different time intervals after replacing the supernatant with fresh buffer. The second incubation was performed in a final volume of 300 µl at 37° C in the presence of 5 mM theophylline.

Isolation and quantitation of nucleotides

(a) Isolation of the metabolites of [8-^{14}C]adenine. The incubations were terminated by inserting the incubation tubes in a boiling water bath for 3 min. One µmole of each ATP, ADP, 5'-AMP, adenosine, adenine and cyclic AMP were added to the contents of the incubation mixture. The contents of the tubes were then homogenized and were centrifuged at 5000 rev./min for 5 min in a Sorvall refrigerated centrifuge using an SS 34 rotor. Aliquots (200 µl) were then chromatographed on paper in 1 M ammonium acetate (pH 7.4)/ethanol (3 : 7) system using a descending technique at room temperature for 18 h. Authentic nucleotides were also chromatographed alongside unknowns. After drying the chromatogram, they were viewed under an ultraviolet lamp (Chroma-Vue) and the area corresponding to authentic, added nucleotides were marked and scanned for radioactivity using a Packard radiochromatogram Scanner (Model 7201).

(b) Quantitation of radioactive cyclic AMP. In those experiments in which the conversion of [8-^{14}C]adenine to cyclic [^{14}C]AMP was measured [11], the following procedure was employed. The incubation was stopped by inserting the incubation tube in a boiling water bath for 3 min and 1 µmol of cyclic AMP was added to the tube and homogenized in the same tube with a teflon pestle. The tube was then centrifuged in a Sorvall refrigerated centrifuge at 5000 rev./min for 5 min and 200 µl of the supernatant was then transferred to another tube. Approx. 5000 cpm cyclic [^{3}H]AMP were added and the volume was adjusted to 500 µl with water. Cyclic AMP was then separated from other nucleotides by the method of Krishna et al. [12] by chromatography on

Dowex-H^+ columns followed by precipitation with $BaSO_4$. The recovery of cyclic [^{14}C]AMP was corrected for losses on the basis of the recovered cyclic [^{3}H]AMP. By this procedure, the values obtained in duplicate determinations varied less than 10%. All incubations were, therefore, carried out in duplicate and the values represent the mean of duplicate determinations.

Characterization of cyclic [^{14}C]AMP was accomplished by subjecting it to hydrolysis by cyclic 3′,5′-phosphodiesterase [13] to yield 5′-AMP followed by paper chromatographic separation in 1 M ammonium acetate (pH 7.4)/ethanol (3 : 7) system.

(c) Assay of cyclic AMP. In those experiments in which the mass of cyclic AMP produced during the incubation were measured, the incubations were performed in the absence of [8-^{14}C]adenine. All other conditions were identical. Incubations were terminated by the addition of 1.0 ml of 5% trichloroacetic acid. The contents were then homogenized and centrifuged at 5000 rev./min for 5 min in a Sorvall centrifuge and extracted 5 times with 2 ml of acidified, anhydrous ether. The aqueous residue was evaporated under a stream of nitrogen and cyclic AMP was assayed by the competitive binding assay of Gilman [14].

(d) Radioimmunoassay of progesterone.

(a) Sample preparation: 10 000 cpm of [1,2-^{3}H]progesterone (45 Ci/mmol) were added to 200 µl of sample to account for losses during the extraction procedure. The tubes were thoroughly mixed using a Vortex mixer and then incubated for 1 h in a water bath at 40° C. Tubes were removed from the water bath, mixed and then placed at 4° C for 18—24 h. 10 ml of glass-distilled petroleum ether were added to each sample tube and the tubes were shaken vigorously for 1 min. The aqueous layer was frozen by placing the tube in a solid CO_2 and acetone mixture and the solvent layer was decanted into another test tube. The extraction procedure was repeated two more times. The petroleum ether extracts were washed with 1 ml of distilled water and the extracts were again decanted to another test tube using the above freezing technique. The extracts were dried under nitrogen. After drying, the sides of the tube were rinsed with 1 ml of distilled chloroform twice and the chloroform was transferred to a glass tube (75 × 12 mm internal diameter) with a disposable Pasteur pipet. The chloroform was dried under N_2 and 1 ml of phosphate-buffered saline containing 1% gelatin was added to dry residue. The tube was shaken vigorously and allowed to stand overnight at 4° C.

(2) Assay procedure: Samples were assayed in duplicate, at dose levels of 250 µl and 50 µl. Samples were aliquoted into assay tubes (75 × 12 mm internal diameter) with Hamilton syringes. The volume in each assay tube was made up to 500 µl with 0.1% gel-phosphate-buffered saline. A standard curve using unlabeled progesterone in 11 dose levels ranging in concentration from 10 pg/ml to 10 ng/ml was set up and run along with the samples. 100 µl of ^{125}I-labeled progesterone-11-tryrosylmethylester* (30 000 cpm) were added to each assay tube. The reaction was started by adding 200 µl of antibody formed against progesterone-11-bovine serum albumin** (1 : 2000 v/v) and the

* 11 α-hydroxypregn-4-ene-3,20–dione-hemisuccinate: tyrosine methylester

** 11 α-hydroxypregn-4-ene-3,20-dione-hemisuccinate: bovine serum albumin. The antibody was raised by the procedure of Midgley, Jr, A.R., Niswender, G.D., Gay, V.L. and Reichert, L.R. (1971) Rec. Prog. Horm. Res. 27, 235—301

solutions were mixed throughly and incubated at 4° C for 18—24 h. At the end of this incubation, 200 μl of anti-rabbit γ-globulin were added to each tube and mixed and then incubated at 4° C for 4—5 h. Following this incubation, 3 ml of cold phosphate-buffered saline were added to the tubes and they were centrifuged at 4° C for 30 min at 2000 rev./min. The supernatant was decanted and the amount of bound labeled progesterone in the precipitate was determined using a (Nuclear-Chicago) counter. Results were obtained with the use of a computer program which uses a logit-log transformation to obtain a linear inhibition curve [15]. Sample concentrations obtained from the computer analysis were corrected for procedural losses. The percentage recovery of [1,2-^{3}H] progesterone for each sample was determined by adding 100 μl of the reconstituted sample extracts to a scintillation vial containing 10 ml of a scintillation fluid (1 l of toluene and 4 g of omnifluor) and counted in a Beckman LS-230 liquid scintillation counter. The characteristics of the antibody have been recently described by Niswender [16]. The values obtained from duplicate determinations were essentially identical and all the incubations were performed in duplicate. The values are expressed as the mean of duplicate determinations.

Results

Incorporation of [8-^{14}C] adenine into cyclic [^{14}C] AMP and other metabolites by the ovarian cell suspension

In order to establish the optimal conditions for prelabeling the cells with [8-^{14}C] adenine to ensure the accumulation of intracellular ATP pools, the time course for the accumulation of the products was determined after incubation of the ovarian cell suspension with [8-^{14}C] adenine. The cells were incubated in the presence of [8-^{14}C] adenine and were subjected to paper chromatographic separation of the products as described under Materials and Methods. The profile of the radioactive metabolites formed at 0, 10, 20, 30, 45, 60, 90 and 180 min are shown in Fig. 1. With increase in the incubation period, increased accumulation of [^{14}C] ATP was observed up to 180 min. Although this chromatographic system did not separate 5′-AMP, adenosine, inosine and adenine, these metabolites were well separated from ATP and ADP. In those experiments in which cyclic [^{14}C] AMP was measured, the nucleotides were chromatographed on Dowex columns (see Materials and Methods). There was a significant amount of [^{14}C] ATP accumulation when the incubations were performed for longer periods, up to 3 h. Thus, in those experiments in which the effects of various test substances on cyclic [^{14}C] AMP formation was studied, the substrate [^{14}C] ATP was not limiting. The areas corresponding to ATP and ADP were eluted [17] and the radioactivity was determined by liquid scintillation spectrometry. The data as shown in Fig. 1 indicate that accumulation of total radioactivity in ATP and ADP fractions increased progressively with incubation period. The predominant component in the combined fractions was ATP, as can be seen from the radiochromatogram scan.

Effects of gonadotropins on cyclic [^{14}C] AMP synthesis from [8-^{14}C] adenine

The effect of varying concentrations of N.I.H.-luteinizing hormone-S16 on

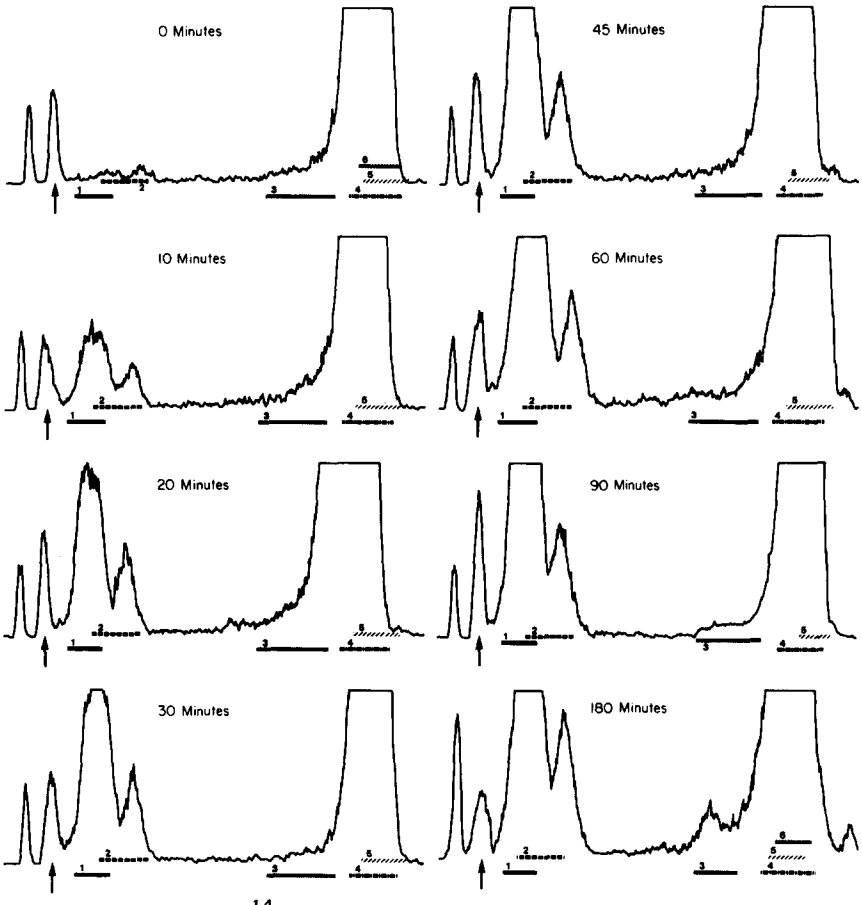

Fig. 1. Incorporation of [^{14}C]adenine into nucleotides by the ovarian cell suspension. The numbers on the radiochromatogram scan refer to: 1, ATP; 2, ADP; 3, cyclic AMP; 4, 5'-AMP; 5, adenosine; and 6, adenine. The chromatograms were scanned at a scale of 3000-C at a speed of 0.75 cm/min. The radiochromatogram shows the incorporation of [8-^{14}C]adenine into ATP, ADP, cyclic AMP, adenosine, inosine and 5'-AMP. 200 μl of the cell suspension ($2.4 \cdot 10^6$ cells) were preincubated at different intervals of time (0—180 min) at 37°C in an atmosphere of 95% O_2 : 5% CO_2 with 2 μCi of [8-^{14}C]adenine and then subjected to paper chromatography as described under Materials and Methods.

the conversion of [8-^{14}C]adenine to cyclic [^{14}C]AMP is shown in Fig. 3. In this experiment the cells were labelled with [8-^{14}C]adenine for 45 min and then the hormone was added and incubated for 30 min. Cyclic [^{14}C]AMP was separated from other metabolites of adenine by chromatography on Dowex columns (Materials and Methods) and the identity was further established by treatment with cyclic nucleotide phosphodiesterase [13], Materials and Methods).

The stimulatory effect was observed even at 0.5 ng of luteinizing hormone and reached a plateau of 20 ng. Human chorionic gonadotropin also stimulated cyclic [^{14}C]AMP formation at comparable concentrations. The dose-response curve for human chorionic gonadotropin is shown in Fig. 4. The formation of cyclic [^{14}C]AMP increased with the increase of human chorionic gonadotropin

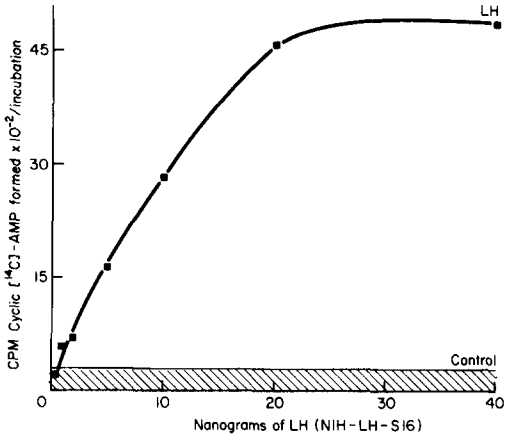

Fig. 2. Effect of increasing concentrations of luteinizing hormone (N.I.H.-luteinizing hormone-S16) (LH) on cyclic [^{14}C]AMP synthesis. 200 μl of ovarian cell suspension (3.1 · 10^6 cells) was prelabelled with [^{14}C]adenine and the second incubation was carried out in the presence or absence of the hormone for 30 min. Hatched lines at the bottom indicate cyclic [^{14}C]AMP formed in control incubations.

concentration up to 40 ng. The stimulatory effect of gonadotropins on the formation of cyclic [^{14}C]AMP from [8-^{14}C]adenine was also confirmed by measuring the amount of cyclic AMP formed de novo by the competitive protein binding assay of Gilman [14].

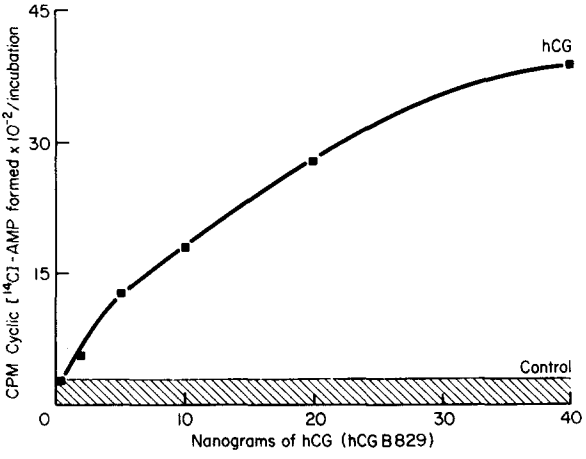

Fig. 3. The effect of increasing concentrations of human chorionic gonadotropin (hCG) on cyclic [^{14}C]-AMP synthesis. The cell suspension (2.05 · 10^6 cells) was prelabelled with [^{14}C]adenine and the second incubation was carried out in the presence or absence of the hormone for 30 min.

Specificity

Other pituitary hormones such as ACTH (adrenocorticotropic hormone) and prolactin were ineffective at concentrations tested (Table I). Bovine serum albumin did not have any stimulatory effect suggesting the specificity of gonadotropin to stimulate cyclic [^{14}C]AMP production. The effects of norepinephrine and dopamine were tested in this system, as these adrenergic drugs

TABLE I

EFFECT OF VARIOUS HORMONES AND OTHER SUBSTANCES ON CYCLIC [^{14}C] AMP SYNTHESIS BY OVARIAN CELL SUSPENSION

Cell suspension ($2.0 \cdot 10^6$ cell) was preincubated for 45 min with [8-^{14}C] adenine. The second incubation in the presence and absence of test substances was carried out for 30 min in a final volume of 300 µl.

Substance added	Amount added	Cyclic [^{14}C] AMP formed (X 10^{-2} cpm)
		4.4
Adrenocorticotropic hormone (Organon)	1 unit	3.4
	2 units	4.5
Bovine serum albumin	5.0 µg	4.2
	10.0 µg	4.6
N.I.H.-follicle-stimulating hormone-P1	5.0 µg	16.6
	10.0 µg	23.5
Prolactin	5.0 µg	4.6
	10.0 µg	4.7
Human chorionic gonadotropin	5.0 ng	11.6
	10.0 ng	18.0
N.I.H.-luteinizing hormone-S16	5.0 ng	7.2
	10.0 ng	28.5
Luteinizing hormone α subunit	5.0 µg	5.7
	1.0 µg	6.8
Luteinizing hormone β subunit	5.0 µg	17.8
	1.0 µg	26.0

are known to activate adenylate cyclase in neural tissues [18]. These agents did not stimulate cyclic [^{14}C] AMP formation in the ovarian cell suspension. Both the α and β subunits of luteinizing hormone were also tested to determine their ability to stimulate cyclic [^{14}C] AMP formation from [^{14}C] adenine at a relatively high concentration. Both subunits stimulated cyclic AMP formation with β subunit more effectively than the α subunit. Whether this stimulatory effect is due to the β subunit alone or is a result of contamination with the intact hormone is not known at present. N.I H.-follicle-stimulating hormone-P1 also stimulated the formation of cyclic [^{14}C] AMP from [^{14}C] adenine at higher concentrations, but at concentrations comparable to that of luteinizing hormone which gave a stimulatory response, follicle-stimulating hormone was ineffective.

Comparison of the time course for the stimulation by luteinizing hormone on the in vitro conversion of [8-^{14}C] adenine to cyclic [^{14}C] AMP and progesterone synthesis

The ovarian cell suspension was incubated in the presence or absence of luteinizing hormone and samples were removed at intervals shown in Fig. 4 and total progesterone produced was measured by radioimmunoassay. Accumulation of radioimmunoassayable progesterone was stimulated several fold in the presence of 100 ng of N.I.H.-luteinizing hormone-S16 as compared to the control. However, there was a lag period in progesterone production up to 30 min, after which the steroid production progressed almost linearly up to 120 min. After 2 h, the amount of progesterone accumulated started to decline rapidly. The control levels were not significantly altered. Cyclic [^{14}C] AMP

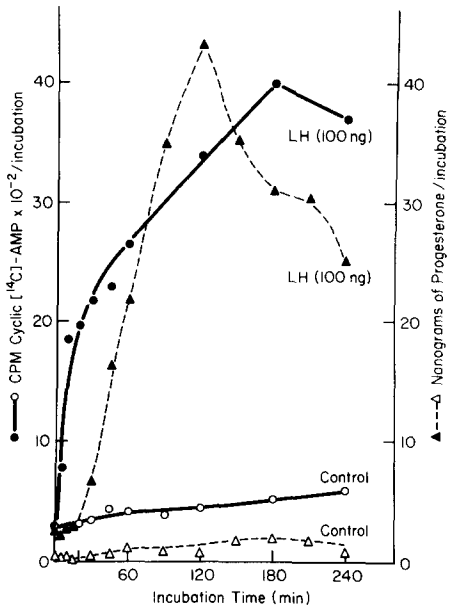

Fig. 4. Comparison of the time course of the in vitro conversion of [8-^{14}C]adenine to cyclic [^{14}C]AMP and progesterone synthesis under the influence of luteinizing hormone. When cyclic [^{14}C]AMP was measured, the cells were preincubated for 45 min with [8-^{14}C]-adenine and then 100 ng of (N.I.H.-luteinizing hormone-S16) was added. The incubations were then performed for the indicated time intervals. For the measurement of progesterone, incubation conditions were identical except that an equivalent amount (40 ng) of unlabelled adenine was included in each incubation and no labelled adenine was added.

production, on the other hand, showed a rapid increase during the early phase of the incubation period (30 min) in the presence of the hormone, while progesterone synthesis showed a lag period.

Role of RNA and protein synthesis on luteinizing hormone-induced synthesis of cyclic AMP and progesterone

To test whether luteinizing hormone-induced synthesis of cyclic AMP and progesterone involve protein and/or RNA synthesis, the following experiments were performed.

a. Effect of puromycin on cyclic AMP and progesterone synthesis. Cells were incubated in the presence and absence of 100 ng of luteinizing hormone (N.I.H.-luteinizing hormone-S16). After the incubation, progesterone was assayed at intervals as shown in Fig. 5. As expected, addition of luteinizing hormone caused a stimulation of progesterone synthesis. The cells were then preincubated in the presence of 10^{-5} M puromycin. After this preincubation, luteinizing hormone was added and the synthesis of progesterone was assayed by radioimmunoassay up to 1 h of incubation. The results showed that (Fig. 5) the synthesis of progesterone was completely inhibited by preincubation with puromycin. The effect of puromycin on progesterone synthesis was further tested by allowing the cells to synthesize progesterone for 30 min in the presence of luteinizing hormone after which time the inhibitor was added and the accumulated progesterone was then measured. The results shown in Fig. 6 indicate that puromycin inhibits luteinizing hormone-induced progesterone syn-

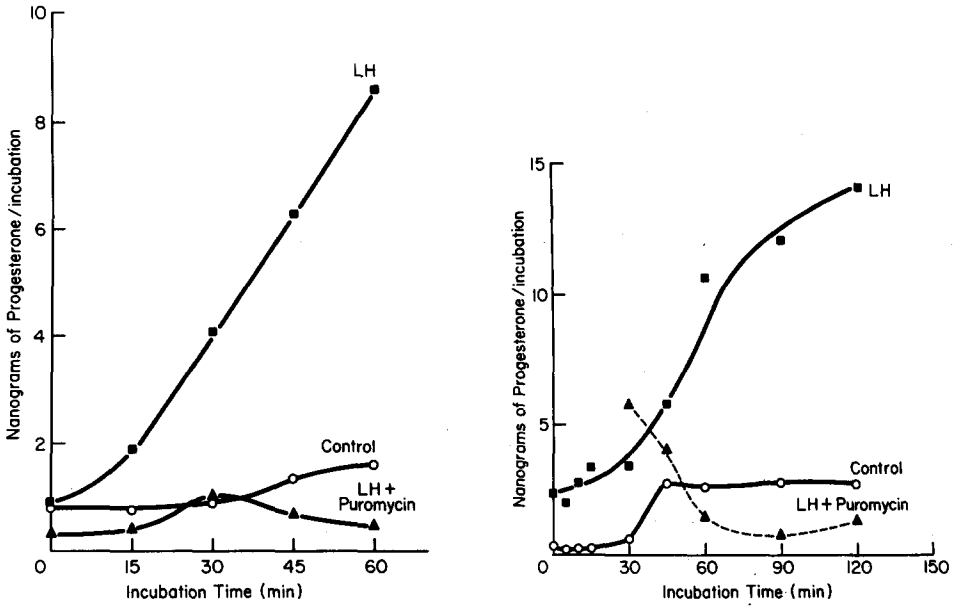

Fig. 5. Effect of puromycin on progesterone synthesis in ovarian cell suspension. The ovarian cell suspension ($1.03 \cdot 10^6$ cells) was preincubated for 45 min as described in the text. The second incubation was performed for 30 min with 100 ng of luteinizing hormone alone or in the presence of 100 ng of luteinizing hormone and 10^{-5} M puromycin. To the control tubes were added 10^{-5} M puromycin. After the second incubation progesterone was assayed by radioimmunoassay as described in the text. The control experiment represents incubation in the absence of puromycin and luteinizing hormone.

Fig. 6. The effect of puromycin on luteinizing hormone-induced progesterone synthesis by the ovarian cell suspension. After 45 min of preincubation, the cell suspension ($1.9 \cdot 10^6$ cells) was incubated in the presence or absence of 100 ng of luteinizing hormone (N.I.H.-luteinizing hormone-S16). In one set of experiments, puromycin (10^{-5} M) was added 30 min after the second incubation.

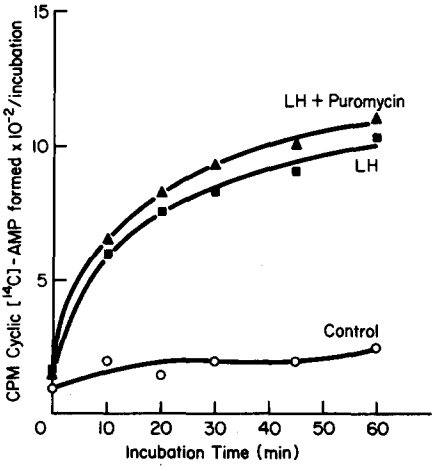

Fig. 7. Effect of puromycin on cyclic [^{14}C]AMP synthesis by the ovarian cell suspension. After preincubation of the ovarian cell suspension ($3.2 \cdot 10^6$ cells) for 45 min, 100 ng of luteinizing hormone was added to one set of incubation tubes and incubated for 30 min. To another set, 10^{-5} M puromycin was added in addition to 100 ng of luteinizing hormone. To the control tubes neither puromycin nor luteinizing hormone was added.

thesis despite allowing the reaction to proceed for 30 min. This indicates the extreme rapidity of the effect of puromycin on luteinizing hormone-induced progesterone synthesis.

The effect of puromycin was then tested on the conversion of [8-^{14}C]-adenine to cyclic [^{14}C]AMP. The data shown in Fig. 7 suggest that puromycin did not have an effect on adenylate cyclase activity under conditions in which progesterone synthesis was inhibited by the antibiotic. 3'-*N*-Glycylpuromycin amino nucleoside, an analogue of puromycin, had no inhibitory effect on progesterone synthesis.

b. Effect of actinomycin D on the synthesis of progesterone and cyclic AMP. The effect of an inhibitor of RNA synthesis was then tested on the in vitro synthesis of progesterone and cyclic AMP. The cells were preincubated with 10^{-5} M actinomycin D for 30 min and then luteinizing hormone was added to the incubation medium. Progesterone was measured at intervals of 15, 30 and 60 min after the addition of luteinizing hormone. A control experiment was performed under similar conditions with the exception that actinomycin D was not added. The results as shown in Fig. 8 indicate that there was approx. a 50% inhibition of progesterone synthesis caused by preincubation with actinomycin D. On the other hand, preincubation with actinomycin D under similar conditions did not inhibit the synthesis of cyclic [^{14}C]AMP from [8-^{14}C]-adenine suggesting that the antibiotic did not inhibit the adenylate cyclase activity (Fig. 9).

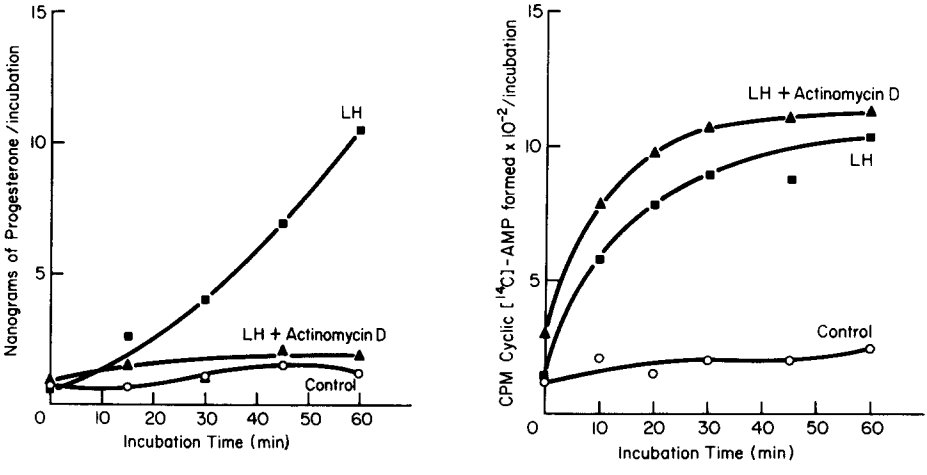

Fig. 8. Effects of actinomycin D on progesterone synthesis by the ovarian cell suspension. The ovarian cell suspension ($1.8 \cdot 10^6$ cells) was preincubated. For 45 min as described in the text. The second incubation was performed for 60 min with the additions as shown above. The final concentration of actinomycin D was 10^{-5} M.

Fig. 9. The effect of actinomycin D on luteinizing hormone-induced progesterone synthesis by the ovarian cell suspension. After 45 min of preincubation, the cell suspension ($2.2 \cdot 10^6$ cells) was incubated in the presence or absence of 100 ng of luteinizing hormone (NIH-luteinizing hormone-S16). In one set of experiments actinomycin D (10^{-5} M) was added 30 min after the second incubation.

Discussion

These studies present data on the successful isolation of an intact ovarian interstitial cell suspension from immature rates which responds to luteinizing hormone to increase the synthesis of cyclic [^{14}C]AMP from [8-^{14}C]adenine or when cyclic AMP synthesis was measured by the competitive protein binding assay of Gilman [14]. The system also responded to progesterone synthesis when the steroid was measured by radioimmunoassay. The cell suspension appears to be more suitable for the study of the mechanism of action of luteinizing hormone as compared to a slice or homogenate of the ovarian tissue. Although a stimulation of adenylate cyclase by luteinizing hormone can be demonstrated in a homogenate, the steroid interconversion is not stimulated in this preparation by the exogenously added gonadotropin. A cell suspension, therefore, appears to have an advantage in that it can support both the synthesis of cyclic AMP and progesterone at an increased rate when the tropic hormone is added in vitro.

Both luteinizing hormone and human chorionic gonadotropin are effective in stimulating cyclic AMP and progesterone synthesis. Follicle-stimulating hormone also caused an increase in cyclic AMP synthesis, but only at a higher concentration. As the follicle-stimulating hormone preparations were relatively impure, the stimulatory effect may be attributed to luteinizing hormone contamination. Similarly, both subunits of luteinizing hormone-stimulated adenylate cyclase activity, but the β subunit was far more effective than the α subunit. Whether the stimulatory effect of the β subunit is due to the contaminating luteinizing hormone activity or this is due to the inherent property of the molecule is not known. Catecholamines (dopamine and norepinephrine) at concentrations tested were not stimulatory. Similarly, other pituitary hormones such as ACTH and prolactin and albumin were not stimulatory. These experiments suggest that the cell suspension possesses a high degree of specificity which is not destroyed by treatment with collagenase and trypsin.

The present study also demonstrates the temporal relationship between cyclic [^{14}C]AMP formation from [8-^{14}C]adenine and progesterone synthesis under the influence of gonadotropic hormones. The stimulation of cyclic AMP synthesis appears to be the primary hormone-mediated cellular event in the ovarian cell suspension in response to gonadotropic hormone as an increase can be seen as early as 2.5 min after the addition of the hormone. Progesterone synthesis, on the other hand, seems to follow an initial lag period for about 30 min after which time the synthesis is increased with the incubation period. However, 2 h after incubation, the accumulation of progesterone declines. This decline may be due to the further metabolism of progesterone.

The synthesis of progesterone was inhibited by puromycin either when preincubated with the cell suspension or when added to the incubation medium after allowing the reaction to proceed for 30 min. This suggested that a labile protein may be involved in luteinizing hormone-mediated progesterone synthesis, an observation in keeping with earlier studies [19,20]. Puromycin did not inhibit cyclic [^{14}C]AMP formation from [8-^{14}C]adenine further suggesting that the puromycin sensitive step lies after cyclic AMP synthesis. That the synthesis of progesterone is also controlled by the transcriptional events are

documented by its sensitivity to actinomycin D, an inhibitor of DNA-dependent RNA synthesis. In this connection it is interesting to note a recent observation that in vitro addition of cyclic AMP to purified nuclei from bovine corpus luteum stimulated the incorporation of [^{3}H]UTP into RNA (Tack, K. and Menon, K.M.J. (1974) unpublished observations). Actinomycin D did not inhibit cyclic [^{14}C]AMP formation from [8-^{14}C]adenine. The present studies therefore strongly suggest that luteinizing hormone induced progesterone synthesis in the interstitial cells of the rat ovary involve initially the synthesis of cyclic AMP which then regulate steroidogenesis through processes sensitive to puromycin and actinomycin D.

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