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Cessation of Steroidogenesis in Leydig Cell Tumors After Removal of Luteinizing Hormone and Adenosine Cyclic 3′,5′-Monophosphate*

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SUMMARY

Luteinizing hormone (LH), but not follicle-stimulating hormone or prolactin, was shown to enhance steroid synthesis of Leydig tumor cells *in vitro*. Adenosine cyclic 3′,5′-monophosphate (cAMP) duplicated the effect of LH. Removal of LH from the medium within 1 hour of incubation by washing the cells had no effect on the rate of steroid synthesis previously stimulated by LH. Under these conditions, addition of LH antiserum was required to reduce steroid synthesis. In contrast, removal of cAMP by washing the tumor cells caused a rapid termination of the previously induced steroidogenesis. Cycloheximide reduced the steroid synthesis initiated by LH. These results suggest that (*a*) steroidogenesis may be controlled by short lived factors (proteins), (*b*) LH may be required continually to elevate cAMP levels to maintain steroid synthesis at stimulated rates, and (*c*) that LH is probably bound to the tumor cells.

Current concepts regarding the mechanism of luteinizing hormone action suggest that LH[1] stimulates the accumulation of adenosine cyclic 3′,5′-monophosphate, which in turn promotes steroidogenesis (2, 3). The action of cAMP is not well understood but apparently involves the production of an intermediary factor (protein) with a very high turnover rate which is responsible for the steroidogenic effect (4, 5). The basis for the latter idea stems from the observation that inhibitors of protein synthesis also inhibit the cAMP-induced stimulation of steroidogenesis without affecting the LH stimulation of cAMP accumulation (2). Experiments were designed to test this hypothesis by measuring steroidogenesis before and after the stimulatory effects of LH or cAMP were no longer present due to removal of these substances from the medium.

Previous studies have shown that steroidogenesis by Leydig cell tumors *in vitro* was enhanced within 15 min after addition of LH (6). The specificity of this effect has now been tested by incubation of tumor cells with prolactin, follicle-stimulating hormone, and antisera to LH. Results of experiments designed to examine whether cycloheximide, an inhibitor of protein synthesis, will inhibit steroidogenesis are also included in this report.

MATERIALS AND METHODS

Tumor Preparation—Leydig cell tumors, grown in C57Bl/6J mice were prepared for incubation as described earlier (6). Minced tumors were forced through nylon marquisette or dacron ninon with a Teflon pestle. The cell clumps were sedimented from the supernatant and used in the incubation. The clump size was less than 0.5 mm in diameter.

Incubation Procedure—Cell clumps were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 5.5 mM glucose (7) with LH (NIH-LH-S-16) at 37° in a shaking water bath. Incubation was carried out in 50-ml round bottom centrifuge tubes to facilitate centrifugation of the tissue. After 15 min of incubation, the 1.5 ml of incubation medium was diluted to 30 ml with additional Krebs-Ringer bicarbonate buffer and the cells sedimented by centrifuging at 150 × *g* in the cold. The supernatant was discarded and the cells again suspended in 30 ml of buffer, centrifuged, and the second supernatant discarded. The two washings and centrifugations required approximately 15 to 20 min. The washing procedure produced an approximate dilution of 400-fold in LH concentration. These levels of LH have been shown earlier (6) not to stimulate steroidogenesis.

The pellet of cells obtained after the second wash was suspended in 1.5 ml of the Krebs-Ringer buffer and incubated in the presence of diverse additives for varying periods (see tables for additives). Steroids synthesized by these cells were analyzed by methods described previously (6), namely, chloroform-methanol extraction, thin layer chromatography, and gas chromatography. The thin layer system was restricted to one dimension in hexane-diethyl ether-acetic acid (60:40:3). In experiments in which cAMP was used to stimulate steroidogenesis, the procedure was identical, with the exception that prior incubation was

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[1] The abbreviations used are: LH, luteinizing hormone; cAMP, adenosine cyclic 3′,5′-monophosphate; FSH, follicle-stimulating hormone; 20α-dihydroprogesterone, 20α-hydroxypregn-4-en-3-one.

continued for periods up to 1 hour and that no LH antiserum was added during the incubation of previously washed cells.

In experiments designed to determine whether FSH or prolactin (NIH-prolactin-S-9) stimulated steroidogenesis, the incubation procedure was identical with that described above. No "washing" studies were performed after stimulation with these gonadotropins. The highly purified FSH preparation used here was kindly donated by Dr. Harold Papkoff of the Hormone Research Laboratory, San Francisco, California, who estimated its potency to be 25 times that of FSH (NIH-FSH-S-1).[2]

Cycloheximide was dissolved in warm 0.9% NaCl solution and used in a concentration of 300 μg per ml in each incubation. A uniformly labeled ^{14}C-amino acid mixture (1.25 μCi per flask-52 mCi per mg atom of carbon (mixture B4) obtained from Amersham-Searle, Arlington Heights, Illinois) was used as a tracer in experiments in which protein synthesis was studied. The incubation procedure was identical with that used for steroid synthesis. At the end of the incubation, the medium and tissues were homogenized in 10 volumes of cold 10% trichloroacetic acid and the precipitate collected on Millipore filters (0.3 μm). After washing with 10 ml of 10% trichloroacetic acid, the filters were placed in scintillation vials with 10 ml of Triton X-100 scintillation fluid (Packard Instrument Company) and counted.

Preparation and Characterization of LH Antisera—The procedure for the preparation and characterization of LH antisera has been described earlier (8). This consisted essentially in the immunization of rabbits with LH (NIH-LH-S-16) over several months, collection of sera, and absorption for nonspecific antibodies. This preparation did not cross-react with prolactin and FSH as judged by immunochemical and biological end points (8).

RESULTS

Effect of LH, FSH, and Prolactin on Steroidogenesis by Leydig Tumor Cells—As can be seen from Table I, addition of LH to the tumor cells increased the synthesis of progesterone and testosterone from 0.28 μg per flask to 3.89 μg per flask, whereas addition of prolactin was without effect. FSH addition stimulated steroid synthesis marginally under conditions in which LH produced a marked rise in steroidogenesis. This stimulation by FSH, however, could be neutralized by LH antiserum, suggesting that the FSH preparation might be contaminated with small amounts of LH. This preparation had 0.034 unit of LH activity per mg of LH (NIH-LH-S-1).[2] It is possible that the slight inhibition seen after the addition of LH antiserum in the presence of FSH was due to removal of small amounts of endogenous mouse LH from the tissue. This suggestion is supported by the data in Table II. Addition of LH overcame the inhibitory effect of LH antiserum on steroidogenesis. Apparently, therefore, LH alone among the three gonadotropins acutely enhanced steroid production by tumor cells.

Effect of Removal of LH on Steroidogenesis—The results of attempts to inhibit steroidogenesis by washing LH from the cells are presented in Table II. Addition of LH to the incubation medium at concentrations above 0.1 μg per ml resulted in continued stimulation of steroid synthesis subsequent to washing the cells with buffer. In each experiment, the 100-fold smaller LH concentration failed to stimulate steroidogenesis. LH-induced steroidogenesis was significantly inhibited only when LH antiserum was added to the incubation medium. The inhibition of steroid synthesis by LH antiserum was overcome by reintroducing LH into the incubation, thus showing that the antiserum effect was caused specifically by neutralization of LH activity.

Steroid production after LH removal by addition of antiserum was studied as a function of time, and the results are shown in Fig. 1, *A* and *B*. As is evident in Fig. 1*A*, washing of the cells, followed by addition of normal rabbit serum, failed to inhibit

[2] Dr. Harold Papkoff, personal communication.

TABLE I

Effect of LH, prolactin, and FSH on steroidogenesis in Leydig tumor cells in vitro

Conditions of incubations were as described in the text. Values represent μg of steroid (testosterone plus progesterone) per incubation flask at the end of the 1-hour incubation period. All values are means ± standard deviations for duplicate flasks.

Hormone	Experiment I[a]	Experiment II[b]	Experiment III[b]
None	0.28 ± 0.093	0.88 ± 0.017	0.18 ± 0.011
LH	3.89 ± 0.285[c]	4.45 ± 0.669[c]	0.93 ± 0.082[c]
Prolactin	0.33 ± 0.025		
FSH		1.93 ± 0.131[c]	0.23 ± 0.018
FSH plus LH antiserum			0.11 ± 0.017[c]

[a] LH, 25 μg per ml; prolactin, 50 μg per ml.

[b] LH, 10 μg per ml; FSH, 10 μg per ml of Papkoff FSH (=250 μg of FSH (NIH)).

[c] Significantly different from control at $p < 0.05$ within the same experiment, as determined by the method of Dunnett (9).

TABLE II

Steroid production after incubation with LH and removal of latter by washing with buffer

See under "Materials and Methods" for description of the washing procedure. All values represent the mean ± standard deviation for three flasks.

Experiment	Prior incubation[a]	Incubation[b]	Steroid per flask[c]
	μg/ml		μg
1	1	NaCl solution	3.07 ± 0.276
	1	1 μg of LH per ml	3.68 ± 0.496
	1	LH antiserum[d]	1.88 ± 0.266[e]
	1	LH antiserum + LH[f]	4.02 ± 0.187
	0.01	0.01 μg of LH per ml	0.37 ± 0.076[e]
2	0.5	NaCl solution	0.82 ± 0.10
	0.5	0.5 μg of LH per ml	0.69 ± 0.07
	0.5	LH antiserum[d]	0.42 ± 0.09[e]
	0.5	LH antiserum + LH[f]	0.80 ± 0.17
	0.005	0.005 μg of LH per ml	0.09 ± 0.01[e]
	0.5	Not incubated	0.10 ± 0.02[e]

[a] Prior incubation period with LH was 15 min, after which all cells were washed.

[b] Incubation time, 1 hour.

[c] Tumors used in the first experiment synthesized testosterone as the major steroid, whereas those in the second experiment made progesterone primarily. Consequently, the steroids measured in Experiments 1 and 2 were testosterone and progesterone, respectively.

[d] LH antiserum neutralized 5 μg of LH.

[e] Significantly different from the control at $p < 0.05$, as determined by the method of Dunnett (9).

[f] LH added again at 10 μg per flask.

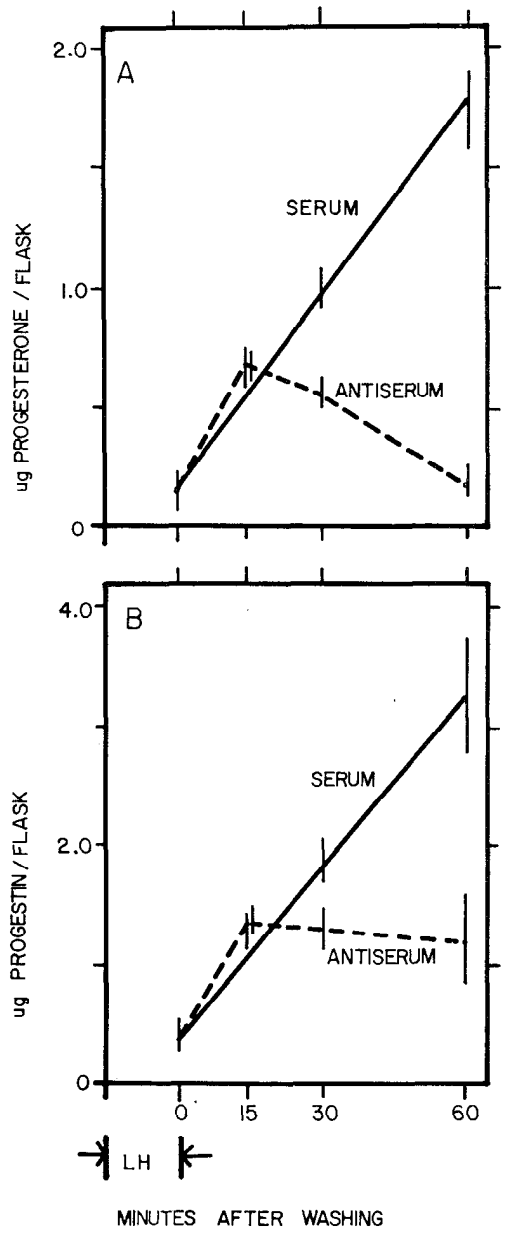

FIG. 1. Effect of removal of LH on steroid synthesis in the tumor cell preparation. Tumor cells were incubated with LH (0.5 μg per ml) for 15 min at which time the cells were washed. The cells were then incubated with either normal rabbit serum (*serum*) or LH antiserum (*antiserum*) for varying periods. The *upper graph* (*A*) represents progesterone synthesis while the *lower graph* (*B*) refers to the synthesis of progesterone plus 20α-dihydroprogesterone. See "Materials and Methods" for description of the washing procedure and steroid analysis. Each *point* represents the mean of two flasks with the *vertical bar* extending to the value of the standard deviation.

progesterone synthesis. On the other hand, treatment of the washed cells with LH antiserum inhibited progesterone synthesis during a subsequent 15 min incubation period. The production of progesterone and its 20α reduction product was also inhibited after 15 min in the presence of LH antiserum. The decline in progesterone concentration found after 15 min is due in part to its conversion to 20α-dihydroprogesterone.

Inasmuch as steroidogenesis proceeds at a nearly constant rate, despite washing of the cells (Fig. 1), the observation that antiserum did not immediately inhibit the LH effect suggested that antiserum must compete with a cellular binding site for LH. In all cases, enough LH antiserum was added to more than neutralize the total amount of LH included in the previous incubation.

TABLE III

Progesterone production after removal of adenosine cyclic 3′,5′-monophosphate by washing

See under "Materials and Methods" for description of the washing procedure. All values represent the mean ± standard deviation for three flasks.

Experiment	Prior incubation[a]	Incubation[b]	Progesterone per flask
			μg
1	0.02 M cAMP	NaCl solution	0.069 ± 0.010
	0.02 M cAMP	0.02 M cAMP	1.175 ± 0.041[c]
	0.02 M cAMP	Not incubated	0.462 ± 0.047[c]
	0.0002 M cAMP	0.0002 M cAMP	0.093 ± 0.023
2	0.02 M cAMP	NaCl solution	0.092 ± 0.009
	0.02 M cAMP	0.02 M cAMP	0.430 ± 0.010[c]
	0.02 M cAMP	Not incubated	0.133 ± 0.018[c]
	0.0002 M cAMP	0.0002 M cAMP	0.128 ± 0.008[c]

[a] Initial incubation was 1 hour, at which time all cells were washed.

[b] Incubation was 1 hour except where noted.

[c] Significantly different ($p < 0.05$) from the values obtained when NaCl solution alone was included in the incubation, as determined by the method of Dunnett (9).

Effect of Removal of Adenosine Cyclic 3′,5′-Monophosphate on Steroidogenesis—The effects of removing cAMP from the incubation medium are seen in Table III. Contrary to what was observed with LH, removal of cAMP by washing the cells terminated the stimulation of steroidogenesis. This indicated that the stimulus to steroidogenesis is labile and that continued presence of cAMP is necessary for maintenance of this stimulus. Closer inspection of the data revealed that after cAMP was removed, the amount of progesterone remaining in the incubation flask fell below that remaining after prior incubation alone. This decline in steroid content was not measurable as 20α-dihydroprogesterone even though progesterone and its 20α reduction product are the major ultraviolet-absorbing steroids produced during incubation.

Fig. 2, *A* and *B*, illustrates the production of steroids with time after removal of cAMP. Progesterone synthesis was affected well within 15 min after removal of cAMP, and the total of progesterone plus 20α-dihydroprogesterone fell to very low levels at 30 min of incubation. Again the data indicate that the presence of cAMP is required constantly to maintain steroidogenesis at a stimulated level. Further, the data suggest that the stimulus must be very labile as the effects of the washing procedure are seen shortly after the initiation of the incubation.

Effect of Cycloheximide on Steroidogenesis—Fig. 3, *A*, *B*, and *C* depicts steroid production after addition of cycloheximide to the incubation medium. The accumulation of testosterone, progesterone, and 20α-dihydroprogesterone is stimulated by LH. Addition of cycloheximide after 1 hour of incubation with LH abolished this stimulation, and the total amount of ultraviolet-absorbing steroids found after termination of the incubation was greatly reduced relative to that which was initially present as a result of 1 hour of LH stimulation. Cycloheximide also inhibited the incorporation of amino acids into protein (Table IV).

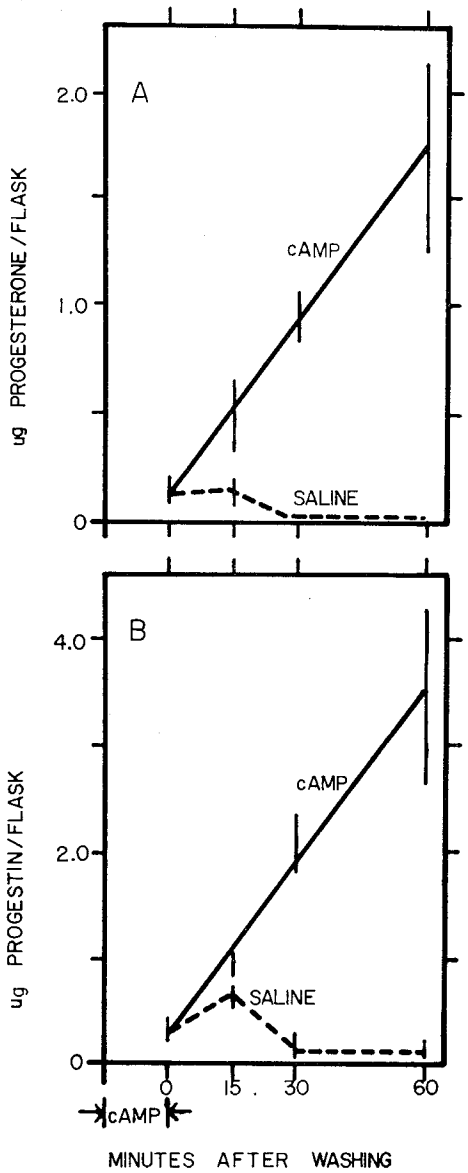

FIG. 2. Effect of removal of cAMP on steroid synthesis in the tumor cell preparation. Tumor cells were incubated for 15 min with cAMP (20 mM) and washed. Either 0.9% NaCl solution (*saline*) or cAMP (20 mM) in 0.9% NaCl solution was returned to the incubation flask for the time periods shown. The *upper graph* refers to progesterone synthesis while the *lower graph* depicts the synthesis of progesterone plus 20α-dihydroprogesterone. The level of steroids present immediately after washing was greater when cAMP rather than 0.9% NaCl solution was included in the prior incubation for 15 min (data not presented). Each *point* represents the mean of two values with the *vertical bars* extending to the value of the standard deviation.

Metabolism of Progesterone—[4-^{14}C]Progesterone (1 μCi) was included in the incubation flasks and its metabolism followed by autoradiography to determine whether it could be converted to steroids other than 20α-dihydroprogesterone or testosterone. The data (not presented) indicated that several metabolites other than 20α-dihydroprogesterone or testosterone were present after incubation. The decline in total steroids after removal of cAMP or addition of cycloheximide, therefore, may be related to the further metabolism of progesterone, 20α-dihydroprogesterone, and testosterone.

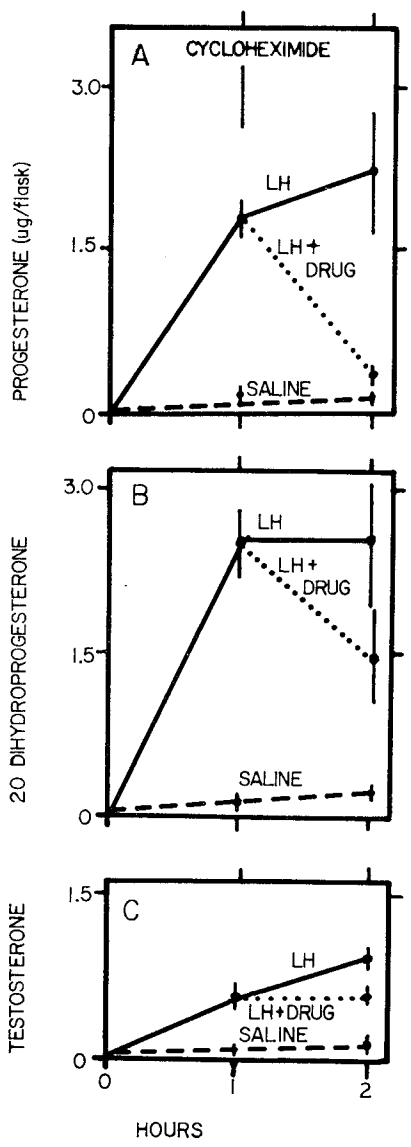

FIG. 3. Effect of cycloheximide on steroidogenesis previously initiated by LH. Cycloheximide was added to cells responding to LH after 1 hour of LH treatment. Figs. *A*, *B*, and *C* represent the amounts of progesterone, 20α-dihydroprogesterone, and testosterone, respectively, in micrograms per flask. All values represent the mean of three values with the *vertical bars* extending to the standard deviation. *Saline*, 0.9% NaCl solution.

TABLE IV

Incorporation of uniformly labeled amino acids into tumor cell proteins

See under "Materials and Methods" for description of the procedure. Values represent the mean ± standard deviation for duplicate flasks.

Treatment	Incorporation per flask
	cpm
Control	98,000 ± 3,200
LH	183,000 ± 41,000
Cycloheximide	27,000 ± 3,600
LH + cycloheximide	20,000 ± 600

DISCUSSION

The observation that LH, and not prolactin or FSH, stimulated steroidogenesis acutely is not unique to the tumor cells used in this study. Hall and Eik-Nes (10) have shown essentially the same result for normal testes. Studies utilizing LH antiserum show that the stimulatory effects of FSH may be due to enhancement of LH present either as contaminant in the FSH or bound to the tumor cells.

Throughout the course of these studies, a significant change in the type of steroid produced by the tumor cell became evident. The original tumors produced testosterone with lesser amounts of progesterone (6). More recently, the tumors which have been transplanted several times produced large amounts of progesterone and 20α-dihydroprogesterone and only small quantities of testosterone. Nonetheless, the change in the type of steroid synthesized has not influenced the responsiveness of the tumor to LH. The reason for maintaining continued responsiveness to LH may be that the point of stimulation by LH in the steroid pathway is between cholesterol and pregnenolone (11).

The results which have been presented show that cAMP increased steroidogenesis in Leydig cell tumors and that removal of this stimulus is followed by a rapid decline in the rate of steroidogenesis. Addition of cycloheximide to cells previously stimulated with LH also results in a rapid decline of steroidogenesis. Marsh *et al.* (2) have shown that inhibitors of protein synthesis do not inhibit steroidogenesis by inhibiting the increased accumulation of cAMP produced by LH. Marsh and Savard (3, 4) have shown that steroidogenesis induced by cAMP is inhibited by the addition of cycloheximide. It is apparent from these studies that cAMP is needed to produce high levels of an essential factor with a rapid rate of turnover that is involved in promoting steroidogenesis in the Leydig cells. Our data suggest that the requirement for cAMP is a continuous one.

The suggestion that LH binds to its target tissues is not a new one. Eshkol and Lunenfeld (12) and Espeland, Naftolin, and Paulson (13) have shown that human chorionic gonadotropin labeled with radioiodine is retained by ovarian tissues after *in vivo* administration. Similar findings have been reported for testicular binding of iodinated LH (14). The demonstration that repeated washing of the cells does not abolish the LH stimulation, even though unbound LH would have been diluted to a level well below that at which stimulation occurred, suggests that LH is being retained by the tissue. This suggestion appears more convincing when viewed with the data showing that steroidogenesis can be rapidly terminated when cAMP is removed. LH antiserum must be present to inhibit the LH effect. Two possible explanations become obvious for explanation of the antiserum effect. The antiserum may be acting by continually removing the LH as it slowly dissociates from the tissue LH receptor, until the tissue LH content falls below the level required to stimulate steroidogenesis. Alternatively, the antiserum might be removing LH immediately, although steroidogenesis continues for a short time, possibly due to the role of a labile intermediate acting between LH and cAMP. Studies demonstrating that LH is bound to the tumor cells are described in the accompanying paper.

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