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Effects of Juvenile Hormone on Mammalian Steroidogenesis

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The effects of juvenile hormone-III (JH-III) and the JH analogue 2-(4-phenoxyphenoxy)-ethoxytetrahydropiran on testicular steroidogenesis were studied. By using cultured MA-10 Leydig tumor cells as a model, these compounds were found to be potent inhibitors of LH/hCG steroidogenic action in a dose-dependent manner. Scatchard plot analysis of the binding data indicated that the JH analogue did not significantly alter the affinity nor the number of hCG binding sites, as well as GTP binding to plasma membranes. JH analogue inhibited the stimulatory action of both cholera toxin and forskolin on cAMP production and the concomitant steroidogenic response. JH analogue inhibited $(Bu)_2$cAMP-stimulated progesterone synthesis, indicating that a process downstream to the adenylyl cyclase in the steroidogenic pathway is also affected.

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INTRODUCTION

Juvenile hormone (JH) regulates a variety of processes during both insect development and reproduction. Their biosynthesis takes place in endocrine glands called corpora allata during precisely determined periods of insect development. Together with the steroid molting hormone 20-hydroxyecdysone, JH controls the transition from one developmental stage to the next [1].

Their continuous presence during the last larval instar for example, inhibits metamorphosis and leads to death of the insect without its becoming an adult [2]. By analogy with natural JHs, a great variety of terpenoid JH analogues have been synthesized [3]. A class of JH analogues with nonisoprenic structure, such as 2-(4-phenoxyphenoxy) ethoxytetrahydropiran (one of the most effective insect growth regulators), has been produced.

In contrast to its morphogenic role during development prior to adult eclosion, in the adult stage JH is considered the major gonadotrophic hormone. Taking into account that JH biosynthesis begins with reactions common to mammalian isoprene metabolism to form the 15 carbon farnesyl pyrophosphate, a cholesterol, ubiquinones, farnesyl proteins, juvenile hormones precursor; and that JH regulates the steroidogenic competence of endocrine glands in insects [4], studies to determine whether JH has an effect on steroidogenesis in mammals, in particular in Leydig cells, have been initiated.

MA-10 cells are a clonal strain of cultured Leydig tumor cells that produce progesterone rather than testosterone as the major steroid hormone, when stimulated by LH/hCG or by cAMP analogues [5, 6]. These cells share many common features with normal Leydig cells. The advantages offered by continuous cell lines and the availability of MA-10 cells, provided a valuable tool for the study of the possible effects of JH on the steroidogenic pathway.

MATERIALS AND METHODS

Chemicals

Purified hCG was obtained from the National Institute of Health (batch CR 127). Progesterone,

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cAMP, 3-isobutyl-1-methyl-xantina (IBMX), cholera toxin (CT), forskolin, bovine serum albumin (BSA, RIA grade), Juvenile Hormone-III (JH-III), and Bt_2cAMP were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The JH analogue [2-(4-phenoxyphenoxy)-ethoxytetrahydropiran] was synthesized as described by Gros *et al.* [3].

[1,2,6,7-^{3}H]progesterone (80.2 Ci/mmol), [α-^{32}P]ATP (50–200 cpm/pmol), guanosine 5′-[γ-[^{35}S]thio] triphosphate ([^{35}S]GTP[S]) (1500 Ci/mmol) and [^{125}I]cyclic adenosine 3′,5′phosphoric acid 2′-*O*-succinyl (iodotyrosine methyl ester (150 Ci/mmol) were obtained from Du Pont New England Nuclear (Boston, MA). Culture supplies and Waymouth's MB 752/1 medium were purchased from Gibco, and cell culture plasticware was from Corning or Falcon. Antisera to cAMP was obtained from Chemicon, while antisera to progesterone was a gift of Dr Leonardo E. Bussmann (IBYME). All other materials were of reagent grade.

Cell culture

Culture conditions for the MA-10 cells were as described previously [5, 6]. For experiments, cells were plated at a density of 7×10^4 cells/well in 24 × 16 mm wells and grown for 3 days in medium supplemented with 15% horse serum. Cells were then washed and incubated in assay medium (Waymouth's 752/1 with 20 mM HEPES, 1.2 g/l $NaHCO_3$, and 1% BSA, pH = 7.4). HCG, $(Bu)_2cAMP$ and cholera toxin were added in NaCl/HEPES, while forskolin was dissolved in 95% ethanol.

JH-III and JH analogue were dissolved in ethanol and added in 1/100th the volume of medium in the well 30 min before steroidogenic stimulation. The addition of phosphodiesterase inhibitors is known to enhance the accumulation of cAMP, so the experiments were carried out by incubating cells with 0.1 mM IBMX. Cells were incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

Recovered medium was boiled for 5 min in a water bath to inactivate the phosphodiesterase and, after centrifugation, the supernatants were stored at −20°C for cAMP and progesterone determination.

Membrane preparation

The following method was used to obtain a membrane pellet. Confluent MA-10 cells from a 75 cm^2 Falcon flask were trypsinized, resuspended in 10 ml of Waymouth's medium with 0.1% BSA and centrifuged for 10 min at 800 *g*. The cell pellets were then resuspended in 2 ml of 1 mM $NaHCO_3$, 1 mM EDTA and 1 mM EtSH. Cells were ruptured by ultrasonic oscillation. The homogenate was centrifuged for 10 min at 7000 *g* and the supernatant solution ultracentrifuged for 60 min at 45 K. The pellet was then used as a membrane preparation.

[^{125}I]hCG binding study

The membrane suspensions were incubated overnight at room temperature with [^{125}I]hCG (specific activity 50 μCi/μg). These incubations were performed in PBS–0.2% BSA buffer in a final volume of 0.5 ml. Nonspecific binding was assessed in the presence of excess hCG. The incubation was stopped by the addition of 2 ml of ice-cold PBS–0.2% BSA buffer containing 1 mg/ml cellite followed by centrifugation for 10 min at 2000 *g*. The supernatant fraction was removed by aspiration and the radioactivity of the pellets counted in an automatic gamma counter.

Steady state binding was analyzed by Scatchard analysis [7] to determine the maximum binding capacity and the equilibrium dissociation constant (K_d) of the hCG binding sites.

[^{35}S]GTP[S] binding assay

Specific binding of [^{35}S]GTP[S] to total membranes was assayed using a modification of the method described by Northup [8]. Samples containing 30–50 μg of membrane protein were incubated for 30 min with 30 nCi of [^{35}S]GTP[S] (specific activity 1500 Ci/mmol) in the presence or absence of unlabeled GTP[S] in 200 μl of 20 mM Tris–HCl (pH 8.0), 1 mM EDTA and 1 mM DTT at 30°C.

Bound [^{35}S]GTP[S] was measured by a rapid filtration method through 0.45 μM HA Millipore membranes.

Adenylyl cyclase activity assay

Adenylyl cyclase activity was measured according to the method of Salomon [9]. MA-10 membranes were incubated in a solution containing 50 mM Tris–HCl buffer (pH 7.4), 0.2 mM IBMX, 2.5 mM $MgCl_2$ or $MnCl_2$, 0.5 mM [α-^{32}P]ATP (specific activity 50–200 cpm/pmol), 2 mM phosphoenol pyruvate and 20 μg of pyruvate kinase.

Incubations were initiated by the addition of 50 μl of membranes (50–70 μg) and were continued for 15 min at 30°C in a total volume of 100 μl. The reaction was stopped with a solution containing excess ATP and the synthezised [^{32}P]cAMP isolated and counted.

Radioimmunoassays

Progesterone and cAMP were assayed in unextracted cell culture media by RIA [10, 11].

Intracellular cAMP assay

The cells were scraped into 2 ml of ethanol and disrupted on ice by ultrasonic oscillation. Samples were then placed in 1.5 ml Eppendorf tubes and allowed to dry in a Speedvac, followed by resuspension in 250 mM acetate buffer. Precipitated proteins were removed by centrifugation at 800 *g*. The cAMP content of the supernatant was determined by RIA.

Statistical analysis

Data were analyzed by analysis of variance. Significant differences among treatment means were determined using Tukey test. A P value less than 0.05 was considered statistically significant.

Other methods

Intracellular progesterone was determined following the extraction method described in the intracellular cAMP assay, by resuspending the dried samples in the progesterone assay buffer.

Protein content was determined by the method of Lowry *et al.*[12] using BSA as standard. The DNA content of cell cultures was quantified by fluorometry using a Hoescht dye 33258 (Sigma, St Louis, MO) solution, sensitivity of 10 ng per 20 μl solution [13]. Cellular viability was assessed using Trypan blue stain.

RESULTS

Effects of JH III and 2-(4-phenoxyphenoxy)-ethoxyte-trahydropiran (JH analogue) on MA-10 Leydig tumor cells progesterone synthesis

To determine the possibility of an effect of JH-III on progesterone secretion, MA-10 Leydig tumor cells were cultured for 30 min in the presence of increasing doses of JH-III (30 ng/ml–30 μg/ml) followed by a 4 h incubation with a maximal stimulating dose of hCG (5 ng/ml).

The presence of JH-III resulted in a dose-dependent inhibition of hCG-induced progesterone production [Fig. 1(A)]. This effect was characterized by a half-maximal inhibitory dose (IC_{50}) of 5×10^{-5} M (15 μg/ml). At the highest inhibitor concentrations tested (10^{-4} M), progesterone synthesis was inhibited by over 90%.

As shown in Fig. 1(A), under similar assay conditions the JH analogue was more potent than the JH-III. The JH analogue effect was characterized by an IC_{50} of 3×10^{-6} M (0.9 μg/ml). The inhibitory effect of the analogue on hCG-stimulated progesterone formation could not be accounted for by a decrease in Leydig cell viability.

Effects of JH-III and JH analogue on cAMP synthesis

In Leydig cells, the activation of the LH/hCG transduction pathway results in an increase in the second messenger cAMP.

JH-III, as well as its analogue, inhibited hCG-induced cAMP synthesis in a dose-dependent manner with IC_{50}s of 5×10^{-5} and 10^{-5} M, respectively [Fig. 1(B)]. This result suggests different sensitivity of cAMP and progesterone to inhibition by the JH analogue.

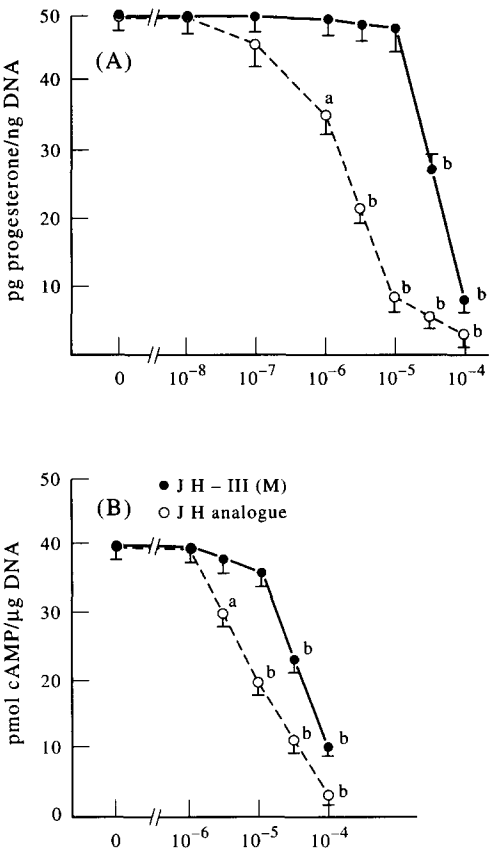

Fig. 1. Effects of increasing doses of JH-III and JH analogue on MA-10 tumor Leydig cells. MA-10 cells were stimulated for 30 min with increasing concentrations (30 ng–30 μg/ml) of juvenile hormone III (●) or JH analogue (○), followed by 4 h of a maximal dose of hCG (5 ng/ml). Extracellular progesterone (A) and cAMP (B) secretions were determined by RIA. The results of a representative experiment are shown. Each point represent the mean (±SE) of triplicate wells. $^{a}P < 0.01$ and $^{b}P < 0.001$ when compared to control.

Effects of JH analogue on hCG dose–response curves. Time course of the inhibitory effect of JH analogue on hCG-stimulated progesterone production

The addition of JH analogue (10^{-4} M) to the culture medium of MA-10 Leydig tumor cells during 4 h incubations, resulted in a consistent and significant ($P < 0.001$) inhibition (up to 80%) of the gonadotropin effect for all hCG doses tested (0.3–10 ng/ml), suggesting that the magnitude of the JH analogue effect is independent of the hCG concentration [Fig. 2(A)]. Time course studies of the inhibitory effect of JH analogue (10^{-4} M) on the accumulation of progesterone in the incubation medium also indicated a strong inhibition [Fig. 2(B)].

Intracellular progesterone was also measured to determine if the inhibitory effect was related to a change in the intracellular level. We found no change between control and JH treated cells in intracellular progesterone (Data not shown).

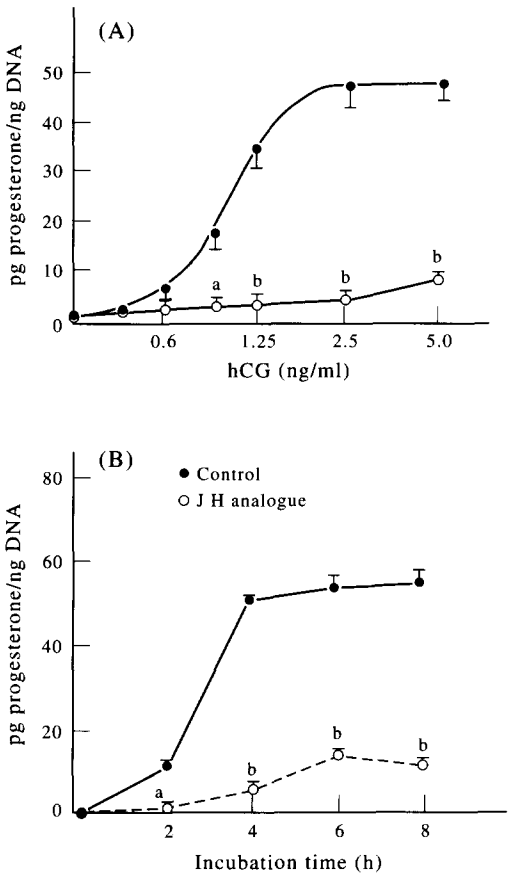

Fig. 2. Effects of increasing doses of hCG on time course experiments. (A) Effect of JH analogue (10^{-4} M) on progesterone accumulation in cells treated with increasing concentrations of hCG. MA-10 cells were preincubated for 30 min with JH analogue and then stimulated with the indicated doses of hCG (0.3–20 ng/ml). The amount of progesterone accumulated in the medium was determined by RIA after 4 h of incubation at 37°C. (B) Time course of the inhibitory effect of JH on hCG-stimulated progesterone production. Cell cultures of MA-10 cells were maintained as described in Materials and Methods and treated with media containing 5 ng/ml hCG (control), or both hCG and JH analogue. Media were collected 2, 4, 6 and 8 h after treatment and progesterone levels measured. The results of a representative experiment are shown. Each point represents the mean (± SE) of triplicate wells. [a] indicates value is different from control with $P < 0.005$ and [b] with $P < 0.001$.

JH analogue effects on hCG binding

Since JH analogue inhibited hCG-stimulated progesterone synthesis and cAMP production, the effects of JH analogue on [^{125}I]hCG binding to MA-10 Leydig tumor cells were determined (Fig. 3).

Binding experiments were conducted for 24 h at room temperature using 150 μg plasma membrane protein per sample (obtained as previously described), from untreated or JH analogue-treated Leydig tumor cells, in the presence of increasing concentrations of [^{125}I]hCG. Non-specific binding was determined in parallel incubations containing 100-fold unlabeled hCG.

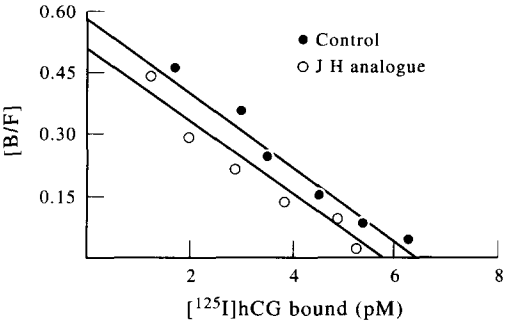

Fig. 3. Scatchard plot analysis of hCG binding. hCG/LH receptors in cultured MA-10 tumor Leydig cells. Leydig cells were incubated in the presence (○) or absence (●) of JH analogue and increasing concentrations of unlabelled hLH as described in Materials and Methods. Data were plotted as a Scatchard curve to calculate binding parameters.

Scatchard plot analysis [7] of the binding data indicated that JH analogue did not significantly alter the affinity (K_d 6 × 10^{-11} M) nor the number of hCG binding sites.

Effects of JH analogue on cholera toxin and forskolin-induced cAMP synthesis

Taking into account that the proteins of the LH/hCG signal transduction pathway in Leydig cells have an essential and specific role in the stimulation of steroidogenesis, the effects of JH analogue on cAMP synthesis in MA-10 Leydig tumor cells were investigated (Table 1). Results indicated that cAMP levels increased in the presence of both cholera toxin and forskolin (which stimulate adenylyl cyclase activity). Increasing doses of forskolin (1–100 μM) or cholera toxin (5–50 ng/ml) enhanced cAMP formation to the same extent as hCG. In the presence of JH

Table 1. *Inhibitory effect of JH analogue on cAMP production*

	pmol cAMP/μg DNA	
Treatment	− JH analogue	+ JH analogue
Control	0.10 ± 0.05	0.13 ± 0.06
Cholera toxin (ng/mg)		
5	1.11 ± 0.08	0.25 ± 0.01*
50	14.73 ± 2.16	3.87 ± 1.35*
Forskolin (μM)		
1	2.78 ± 0.06	0.67 ± 0.26*
10	22.20 ± 0.88	1.65 ± 0.14*
100	99.69 ± 5.43	5.95 ± 0.32*

MA-10 cells were incubated with or without JH analogue in the presence of cholera toxin (5–50 ng/ml) for forskolin (1–100 μM). The cAMP content of the extracellular medium was measured after 4 h of incubation. Each number is the average (± SEM) of nine replicates (three independent experiments, triplicate wells/experiment). In all cases, IBMX was present at a final concentration of 1 mM. *$P < 0.001$ when compared to − JH analogue.

analogue, cAMP production was reduced in all cases, suggesting that JH interfered with cAMP production and/or action.

Inhibition of the adenylyl cyclase catalyst by the JH analogue

Experiments were carried out on membranes obtained from MA-10 cells as described previously, including a 4 h pretreatment with the JH analogue ((B) *in vivo* assay), or on membranes with no pretreatment to which the JH analogue was added during the experiment ((C) *in vitro* assay). In the presence of both Mg^{2+} or Mg^{2+} + hCG, adenylyl cyclase activity was reduced by the addition of the JH analogue (Fig. 4).

Basal activity could be inhibited by about 60% in both types of assay. When Mg^{2+} was replaced by Mn^{2+} in the assay mixture, there was a marked increase in catalytic activity (data not shown).

The inhibition of the catalyst of the JH analogue was in agreement with previous findings in which cAMP production was diminished in hCG + JH analogue-treated cells.

Binding of GTP(S) to total membranes

In order to assess total GTP-binding proteins and any possible effects of the JH analogue on MA-10 cells, membranes were incubated with nonhydrolizable guanine nucleotide (^{35}S)GTP(S). The specificity of the sites for guanine triphosphates was established by incubations containing an excess of unlabeled GTP(S), which inhibited the binding of the tracer by more than 90%. The presence of the JH analogue did not reduce the binding of (^{35}S)GTP(S) to the membranes.

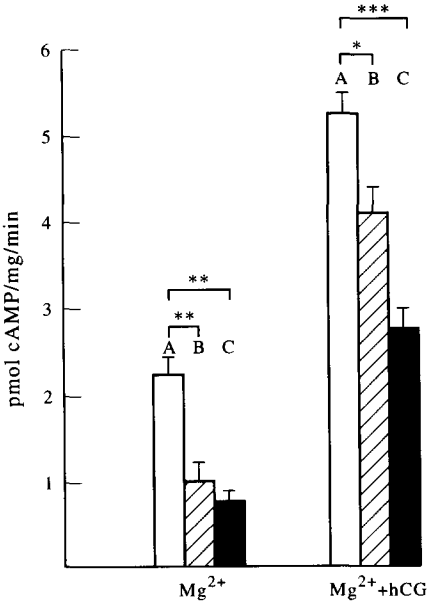

Fig. 4. Adenylyl cyclase activity. Experiments were carried out on membranes obtained from MA-10 cells as described in Materials and Methods, in the presence of both Mg^{2+} or Mg^{2+} + hCG. (A) control cells; (B) cells treated with the JH analogue for 4 h before the membrane isolation (*in vivo* assay); and (C) membranes incubated with JH analogue in the assay experiment (*in vitro* assay). The results of a representative experiment are shown. Each bar shows the mean (± SE) of triplicate determinations. *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.

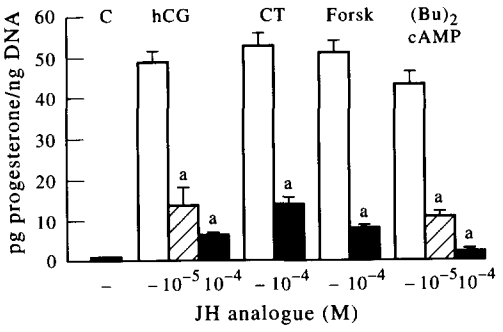

Fig. 5. Effects of juvenile hormone on progesterone synthesis. MA-10 cells were incubated in the presence of different effectors that stimulated progesterone synthesis with or without juvenile hormone analogue. Experiments were performed as described previously, with the addition of hCG (5 ng/ml), CT (500 ng/ml), forskolin (100 μM) and $(Bu)_2$cAMP (1 mM). Progesterone content of extracellular medium was determined by RIA. Each bar shows the average (± SE) of three independent experiments (triplicate wells/experiment). *$P < 0.001$ vs corresponding value without JH analogue treatment.

Effects of JH analogue on progesterone synthesis

The effects of JH analogue on progesterone production in MA-10 Leydig cells stimulated with different progesterone level-enhancing pharmacological agents (including cholera toxin and forskolin), were also studied. The results corresponding to the effects of a single concentration of JH analogue upon the activation of the steroid biosynthesis are shown in Fig. 5.

In the presence of JH analogue, the stimulation of steroidogenesis by 50 μM forskolin was abolished by 80%. In MA-10 cells incubated with 500 ng/ml cholera toxin, progesterone production was inhibited to the same extent.

Steroidogenesis could also be stimulated by addition of dibutyril cAMP. The inhibitory effects of 10^{-4} and 10^{-5} M JH analogue on progesterone synthesis were also observed in the presence of 1 mM $(Bu)_2$cAMP, indicating that JH analogue also affects MA-10 Leydig cell steroidogenesis at a biochemical step/s located downstream to cAMP formation.

DISCUSSION

The purpose of the present study was to investigate the effects of juvenile hormone on the gonadotropin

action of Leydig cells. We know JH is the major gonadotropic hormone in insects and shares its precursor Farnesyl pyrophosphate with mammals. The present findings demonstrate that in cultured MA-10 Leydig tumor cells, JH analogue is a potent inhibitor of progesterone production under (acute) gonadotropin stimulation. JH analogue inhibited hCG-stimulated progesterone secretion by up to 90% with an IC_{50} of 3×10^{-6} M after 4 h of incubation, at which time a maximal progesterone production can be found in this kind of cells.

JH analogue showed a more potent inhibitory effect than JH-III in this culture system. The inhibitory effect is independent of the hCG concentration, persists for at least 8 h and was not related to Leydig cell damage, since JH analogue had no significant effect on Leydig cell viability.

Efforts were directed toward identifying the critical sites of action of JH analogue in steroidogenic inhibition. A possible site of action is located in the signal transduction pathway between the binding of hCT to its receptor and the formation of cAMP [14].

It is well accepted that LH/hCG stimulates steroidogenesis as well as other gonadal functions, through the cAMP second messenger system [15]. This system is undoubtedly of great importance in the stimulation of steroid production by testicular Leydig cells.

JH analogue appears to modulate the function of the Leydig cell membrane, as shown by its capacity to modulate cAMP formation.

Since cAMP formation is due to the activity of the adenylyl cyclase, an enzyme regulated by G proteins [16, 17], it is tempting to speculate that these elements may be targets for the regulatory action of JH analogue in Leydig cells. The results obtained indicate that the JH analogue inhibits steroidogenesis at least by reducing cAMP formation.

Further proof that JH analogue is acting on a signal transduction pathway used by gonadotropins can be found in the experiment shown in Fig. 1(B). This contention is strengthened by the observation that JH analogue was capable of further inhibiting forskolin and cholera toxin cAMP formation [Table 1].

The inhibitory effect could not be associated with a reduction in the number of LH/hCG receptors or a modification in the gonadotropin receptor affinity constant. These kinetic parameters were similar to those reported previously [5].

The evidence provided by the forskolin experiments indicated that the inhibitory effect might be due to a direct effect on the adenylyl cyclase catalyst, since forskolin acts directly upon adenylyl cyclase.

The inhibition of cAMP formation, and the consequent inactivation of the protein kinase cascade, would be expected to significantly limit steroid hormone synthesis by MA-10 cells. Neither the inhibition of cAMP formation nor the effect on progesterone production were related to a change in the permeability of the plasma membrane. Experiments performed to determine extracellular and intracellular cAMP and progesterone showed that these inhibitions did not correspond to an increment in intracellular levels.

In summary, by using cultured MA-10 tumor Leydig cells as an experimental model, this study demonstrates that JH inhibits testicular hCG action mainly at the level/s of cAMP availability for steroid hormone biosynthesis activation. This is the first report demonstrating the action of juvenile hormone on mammalian steroidogenic cells.

Although the present studies emphasize the role of cAMP, the possibility that other sites could mediate the action of the JH analogue must be taken into account. In this context, although JH analogue decreases hCG-stimulated cAMP levels, it could be exerting its antigonadotropic effect mainly at a step/s located downstream to cAMP formation. This possibility is supported by the ability of JH analogue to inhibit progesterone secretion by Leydig cells by the same magnitude (up to 90%) both in cells incubated with RCG as well as those incubated with Bt2cAMP.

The studies presented here demonstrate that the JH analogue can regulate cellular steroidogenesis partly by action on the LH/hCG signal transduction pathway. It is important to note that these studies provide novel information on the mechanism of action of JH on steroidogenic MA-10 Leydig tumor cells. These investigations may also lead to research on a wide range of JH analogues with possible inhibitory influences on the mammalian endocrine system.

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