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Regulation of the Cholesterol Ester Cycle and Progesterone Synthesis by Juvenile Hormone in MA-10 Leydig Tumor Cells

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We had previously reported that juvenile hormone III (JH III) and the JH analogue 2-(4-phenoxy phenoxy)-ethoxytetrahydropyran exert inhibitory effects on progesterone synthesis by blocking cAMP production in hCG-stimulated MA-10 Leydig tumor cells. In the present study, the effects of JH analogue upon the biosynthetic pathway of progesterone synthesis have been examined. Our results demonstrated that JH analogue inhibited progesterone production even in the presence of 20-hydroxycholesterol or 25-hydroxycholesterol. Furthermore, although JH analogue inhibited pregnenolone production in hCG-stimulated MA-10 cells the activity of the 3β-hydroxysteroid dehydrogenase (3β-HSD) was unaffected. These data suggest that JH analogue might inhibit the steroidogenic pathway in Leydig tumor cells by inhibiting the activity of the cholesterol side chain cleavage (CSCC) enzymatic complex. The JH analogue was also evaluated for inhibitory actions on cholesterol availability. An important effect of this compound was the interference with the cellular process of plasma membrane cholesterol internalization. Moreover, JH analogue inhibited not only the use of cholesterol ester for steroid biosynthesis under Bt2cAMP stimulation, but also the cholesterol ester hydrolase (CEH) activity in MA-10 Leydig tumor cells.

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INTRODUCTION

Previous studies have shown that during acute hCG-stimulation of MA-10 Leydig tumor cells, juvenile hormone (JH) analogue inhibits steroid production [1]. The mechanism whereby JH analogue causes this effect seems to be at the level of the production of the second messenger, cAMP. In addition, JH analogue has been shown to have a marked inhibitory steroidogenic effect in dibutyryl cyclic-AMP-stimulated MA-10 cells. Thus, it appears that JH analogue is capable of interfering with an essential step in the regulation of steroid production. The MA-10 Leydig tumor cell line has been used as a model system for investigating the regulation of steroidogenesis by JH analogue. Although these cells synthesize progesterone rather than testosterone as the major steroid hormone, MA-10 cells share many features with normal Leydig cells. MA-10 Leydig tumor cells use stores of free cholesterol and cholesteryl esters as substrates for steroid hormone synthesis [2–6]. These stores are the most important source of substrate for maintaining short-term (0–4 h) steroidogenesis [2], and can be correlated with the output of progesterone by these cells [3]. The free cholesterol used for steroidogenesis comes predominantly from the plasma membrane [6, 7]. The loss of cholesterol from the cell disrupts the balance of opposing enzymes: cholesterol esterase vs acyl-CoA:cholesterol acyltransferase. Cholesterol efflux from the cell deprives the cholesterol acyltransferase of substrate and results in a net cholesterol ester hydrolysis. Incubation of these cells with trophic hormones or cAMP analogues leads to rapid and profound cholesterol ester depletion [2]. Incubation of MA-10 cells with low-density lipoprotein (LDL) increases cellular cholesterol esterification [2] and cholesterol ester stores [3, 4]. Taking into account that the biosynthesis of JH involves reactions common to mammalian isoprene metabolism, the aim of this study was to determine whether JH affects the cholesterol ester cycle and/or cholesterol availability, as well as to elucidate by which mechanism JH analogue inhibits the biosynthetic pathway of steroid production.

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MATERIALS AND METHODS

Chemicals

Purified hCG was obtained from the National Institute of Health (batch CR 127). Progesterone, pregnenolone, cyanoketone, 20-hydroxycholesterol, 25-hydroxycholesterol, aminogluthetimide, cholesterol, cholesteryl linoleate, bovine serum albumin (BSA, RIA grade), and Bt_2cAMP were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The JH analogue [2-(4-phenoxy phenoxy)-ethoxytetrahydropyran] was synthesized as described by Gros *et al.* [8].

[1,2,6,7-^{3}H]progesterone (80.2 Ci/mmol), [7-^{3}H]-pregnenolone, [1,2,6,7-^{3}H]cholesterol (71.40 Ci/mmol), [26-^{14}C]cholesterol (54 Ci/mmol), and [26-^{14}C]cholesteryl oleate (54 Ci/mmol) were obtained from Du Pont New England Nuclear (Boston, MA, U.S.A.).

Eastman Kodak silica thin layer chromatography plates (TLC) were from Fisher Scientific Co. Culture supplies and Waymouth's MB 752/1 medium were purchased from Gibco, cell culture plasticware was from Corning or Falcon.

Cell culture

Culture conditions for the MA-10 cells were as described previously [9, 10]. For experiments in which steroid production was measured, 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 and Bt_2cAMP were added in NaCl/HEPES. For experiments in which cholesteryl ester mass was measured, cells were plated at a density of 2.5×10^5 cells/well in 6 well-plates, and loaded overnight with 50 μM LDL. JH analogue was dissolved in ethanol and added in 1/100th the volume of medium in the well, 30 min before steroidogenic stimulation. Cells were incubated 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, supernatants were stored at −20°C for pregnenolone and progesterone determination.

Lipid methods

After incubation, cells were scraped and extracted with 4 ml of chloroform:methanol (2:1) as described previously [11]. The dish extracts were evaporated to dryness under N_2, and mass and radioactivity determinations of free cholesterol and cholesteryl esters were performed after separation by TLC using Kodak plastic-baked TLC plates developed in heptane:ethyl ether:acetic acid (85:12:2). Radioactivity was quantified by cutting out the areas of the plate corresponding to free and esterified cholesterol and counting in scintillation fluid. Cholesterol and cholesteryl ester masses were quantified after eluting the steroids from the TLC using an assay kit supplied by Boehringer Mannheim. Assay sensitivity and intra- and inter-assay coefficients of variations were as follows: cholesterol, 1 μg/assay, 5 and 14%; cholesteryl esters, 2 μg/assay, 4 and 10%, respectively. All values were corrected according to the recovery of an internal standard.

Labeling of plasma membrane with cholesterol

The procedure for plasma membrane labelling has been described previously [11]. Briefly, assay medium (2 ml) containing 1 μCi of [^{3}H]cholesterol (71.40 Ci/mmol; in ethanol solution) was added to each dish of cells. After 2 h at 37°C dishes were washed with 2 × 2 ml of assay medium. Each dish received 2 ml of assay medium containing the indicated additions and was returned to the incubator at 37°C until harvesting.

Plasma membrane cholesterol internalization was determined by measuring the quantity of free [^{3}H]-cholesterol incorporated to intracellular cholesteryl esters [11].

Cholesterol ester hydrolase activity

Cholesterol ester hydrolase activity was measured in intact cells according to the method described by Freeman [12]. After 4 h of incubation at 37°C, the medium was removed from the dishes, cells were scraped and extracted in chloroform–methanol (2:1), and cholesteryl esters were separated from the other lipids as described above. Cholesteryl esters were eluted from the appropriate section of the silica TLC plates and quantified. The percentage of cholesterol ester hydrolase activity was calculated as the difference (in cholesteryl ester concentrations) between untreated control cells at time zero (C_0) and treated cells after 4 h incubation. 100% activity was considered as the difference between control (0 h) cells and Bt_2cAMP-ACAT inhibitor 58-035-stimulated cells. All other percentages were estimated taking into account this difference.

Incorporation of radioactivity into extracellular steroids

In order to determine the incorporation of radioactivity into extracellular steroids (mainly progesterone in the case of these cells), 1 ml aliquots of the medium were extracted with 10 ml of ethyl ether. The upper phase was transferred to scintillation vials, evaporated to dryness and counted [2].

Radioimmunoassays

Pregnenolone and progesterone were quantified directly from aliquots of medium recovered from control and treated cell by RIA using specific antibodies, as previously described [13, 14]. Assay sensitivity and intra- and inter-assay coefficients of variation were

as follows: progesterone, 12.5 pg/100 μl, 4 and 10%; and pregnenolone, 12.5 pg/100 μl, 10 and 18%, respectively. Data were expressed as picograms of steroid per nanogram of DNA.

Other methods

Protein content was determined by the method of Lowry *et al.* [15] using bovine serum albumin as standard. The DNA content of cell cultures was quantified by fluorometry using a solution of Hoechst dye 33258 (Sigma, St Louis, MO) with a sensitivity of 10 ng [16]. Cellular viability was assessed using Trypan blue stain.

Statistical analysis

Data were analysed by analysis of variance. Significant differences between mean treatment values were determined using the Tukey test. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of JH analogue on 25-hydroxycholesterol-stimulated steroidogenesis

Initial studies on the inhibition of progesterone production in MA-10 cells by the JH analogue showed that the inhibitory effect persisted in these cells despite Bt_2cAMP stimulation.

In order to investigate the possibility that insufficient delivery of cholesterol to mitochondria was the cause of the impaired steroidogenesis in JH-treated cells, the effect of 25-hydroxycholesterol (25-HC) on steroidogenic activity was determined. If the impairment were indeed due to a limitation in cholesterol availability, supplementation of the cells with 25-hydroxycholesterol should revert the inhibitory effect. However, the results shown in Fig. 1 clearly indicate that 25-hydroxycholesterol (50 μM) increased progesterone synthesis by these cells but did not reverse the inhibitory action of JH analogue (10^{-4} M) on steroid production. A similar pattern was observed when MA-10 cells were incubated with 20-hydroxycholesterol in the presence of the JH analogue (10^{-4} M) (Fig. 1). Under similar assay conditions, the addition of hCG (5 ng/ml) to cells treated or not with 20- or 25-hydroxycholesterol increased progesterone synthesis, but the presence of the JH analogue also inhibited the steroid production. It is possible that the decrease in the amount of progesterone produced in response to 25-HC or 20-HC is a reflection of the decrease in cholesterol side chain cleavage (CSCC) and/or 3β-hydroxy-steroid-dehydrogenase (3β-HSD) activities, though we cannot exclude the possibility that JH analogue also affects additional steps in the metabolic pathway preceding cholesterol synthesis.

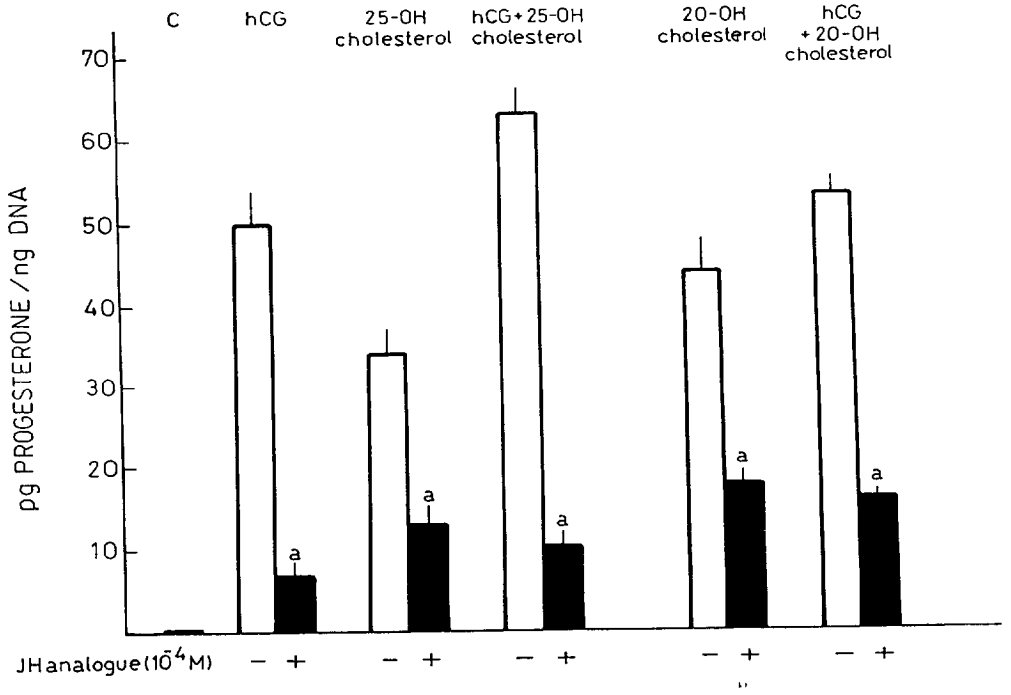

Fig. 1. Inhibition of steroidogenesis in JH analogue-treated cells. MA-10 cells were grown as described in Materials and Methods. At 0 h, wells were washed twice with 2 ml assay medium and placed in 2 ml of assay medium containing 25-OH-cholesterol (50 μM) or 20-OH-cholesterol (50 μM), with or without 5 ng/ml hCG, and, where indicated, in the presence of 10^{-4} M JH analogue. After 4 h of incubation, medium was removed for progesterone measurement. Each bar shows the average (±SE) of three independent experiments (triplicate wells/experiments). $^{a}P < 0.001$ vs corresponding value without JH analogue treatment.

Inhibition of pregnenolone production by JH analogue. Effects on 3β-hydroxy steroid dehydrogenase activity

Taking into account that JH analogue inhibited progesterone production even in the presence of 25-hydroxycholesterol, the effects of the analogue on pregnenolone (3β-hydroxy-5-pregnen-20-one) production and 3β-HSD activity were determined (Fig. 2). For this purpose, MA-10 Leydig cells were incubated per 4 h in the presence of inhibitors of pregnenolone metabolism (5 μM cyanoketone) at 37°C in 5% CO_2 air. Pregnenolone production was determined as described in Materials and Methods. Figure 2(A) shows the effect of cyanoketone on progesterone production, with almost total steroid production corresponding to pregnenolone. The addition of JH analogue (10^{-4} M) to the culture medium of MA-10 Leydig tumor cells during the 4 h incubation, resulted in a consistent and significant ($P < 0.001$) inhibition of the effect of the gonadotropin on pregnenolone production.

3β-HSD activity was determined by incubating intact cells in the presence of the direct substrate of the enzyme, pregnenolone [Fig. 2(B)]. The inhibition of progesterone production by JH analogue (10^{-4} M) was totally reverted in cells incubated with 10 μM pregnenolone, indicating that JH analogue did not affect the activity of the 3β-hydroxy-steroid dehydrogenase enzyme.

The inhibitor effect upon pregnenolone production could be related to an effect on the CSCC enzymatic complex, which converts cholesterol to pregnelone.

Effect of JH analogue on plasma membrane-bound [^{3}H]cholesterol

Plasma membrane cholesterol acts as a major source of substrate for steroid hormone synthesis [6].

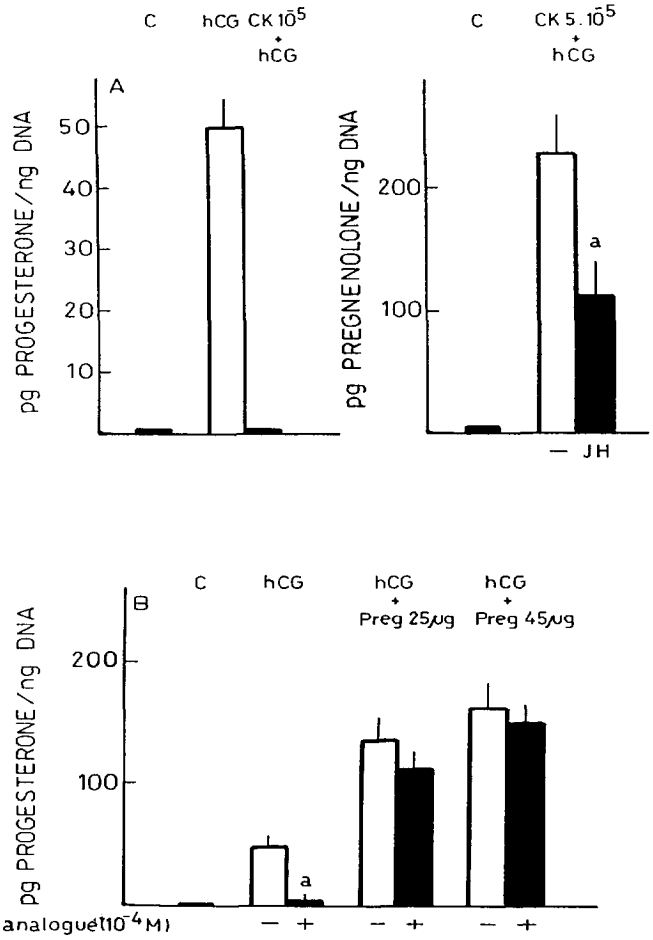

Fig. 2. Inhibition of pregnenolone production by JH analogue. Effects on 3β-hydroxy steroid dehydrogenase activity. MA-10 cells were grown as described in Materials and Methods. (A) hCG-stimulated cells contained 5×10^{-5} M cyanoketone to prevent further pregnenolone metabolism. After 4 h at 37°C, the medium was removed for pregnenolone and progesterone determination. (B) hCG (5 ng/ml)-stimulated MA-10 cells were treated with 25 or 45 μg of pregnenolone in the presence or absence of 10^{-4} M JH analogue. The presence of pregnenolone allowed the determination of the 3β-HSD activity by quantitating the progesterone production. This steroid was determined by RIA in unextracted medium after a 4 h incubatiron at 37°C. Each bar shows the mean (±SE) of triplicate determinations. $^aP < 0.001$.

Membrane cholesterol is constitutively internalized and esterified. Internalization can be accelerated by stimulating steroid hormone production in cells [6].

Since plasma membrane cholesterol is quantitatively important for maintaining steroidogenesis [3, 4, 6], interference with the normal cellular process of recycling plasma membrane would be expected to significantly inhibit steroidogenesis. For these experiments [^{3}H]cholesterol was employed as a plasma membrane marker. Figure 3(A and B) show the distribution of free and esterified [^{3}H]cholesterol radioactivity in control cells after 2 h of radiolabel incorporation (C_0), and the corresponding radioactivity after 4 h of incubation with different treatments. Control cells internalized a significant portion (approx. 25%) of membrane [^{3}H]cholesterol and converted it to [^{3}H]cholesteryl ester after 4 h of incubation (C_4). Bt_2cAMP stimulation of cells caused a loss of both free [^{3}H]cholesterol and intracellular [^{3}H]cholesteryl esters, with a corresponding increase in the radioactivity released into the medium [Fig. 3(C)].

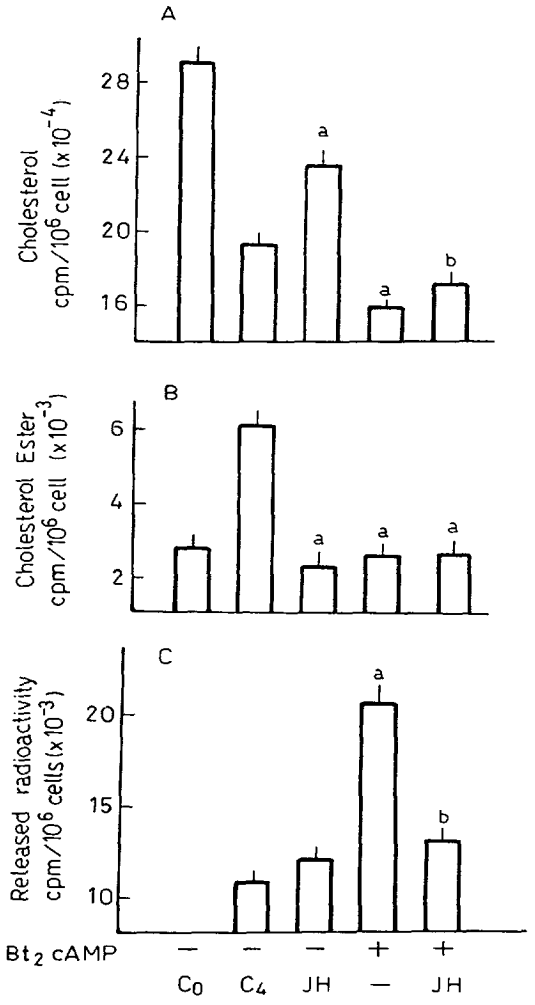

Fig. 3. Effect of JH analogue on plasma membrane-bound [^{3}H]cholesterol. MA-10 cells were grown and the cell surface labelled with 1 μCi of [^{3}H]cholesterol, as described in the Materials and Methods section. After surface labelling, dishes were washed twice with assay medium, scraped and harvested immediately (C_0 time zero controls), or incubated 4 h at 37°C with 2 ml assay medium containing either no additions (C_4), 10^{-4} M JH analogue (JH), 1 mM dibutyryl-cAMP, or both 10^{-4} M JH analogue (JH) and 1 mM dibutyryl-cAMP. After incubation, the medium was removed and saved for analysis of steroid radioactivity (lower panel), and wells were washed three times with 2 ml assay medium. Radioactivity in free cholesterol (upper panel) and esterified cholesterol (middle panel) was separated by TLC and quantitated as described in Materials and Methods. Data are expressed as the mean ± SD radioactivity per dish of two independent experiments (triplicate wells/experiments). $^aP < 0.001$ when compared to control (C_4); $^bP < 0.01$ when compared to control (C_4).

Treatment of cells with JH analogue (10^{-4} M) or JH analogue (10^{-4} M) and Bt_2cAMP (1 mM) had an inhibitory effect on plasma membrane cholesterol internalization and esterification, showing increased levels of free [^{3}H]cholesterol and basal levels of [^{3}H]cholesteryl ester. Figure 3(C) indicates that the general pattern of inhibition is similar to the inhibitory effect on steroidogenesis.

Effects of JH analogue on the mass of the esterified cholesterol pool

The MA-10 cells were loaded with cholesteryl ester by incubating overnight with 50 μg/ml LDL, so that the cholesteryl ester concentration would be high enough to be accurately measured.

The data presented in Table 1 show the effects of four treatments on the cellular cholesterol ester mass. Incubation of cells with Bt_2cAMP (1 mM) for 4 h resulted in cellular cholesterol ester concentrations decreasing substantially by an average of 50%. When these experiments were performed in the presence of JH analogue (10^{-4} M) or JH analogue (10^{-4} M) and

Table 1. Effects of JH analogue on the mass of esterified cholesterol

Additions		Cholesterol ester
Bt_2 cAMP	JH analogue	μg/10^6 cells
–	–	6.04 ± 0.50
–	+	6.24 ± 0.59
+	–	3.07 ± 0.20*
+	+	6.85 ± 0.38
+	AG	6.60 ± 0.40

Cells were incubated overnight with 50 μg of LDL cholesterol/ml. On the day of the experiment ($t = 0$), cells were washed three times with 2 ml warm assay medium and then placed in 2 ml assay medium containing either no additions, 10^{-4} M JH analogue, 1 mM Bt_2cAMP, 10^{-4} M JH analogue and 1 mM Bt_2cAMP, or 50 μM aminoglutethimide (AG) and 1 mM Bt_2cAMP. After 4 h at 37°C, the medium was removed, cells were scraped from the wells and the cellular mass of esterified cholesterol determined as described in Materials and Methods. Each value is the mean (±SE) of nine replicates (three independent experiments, triplicate wells/experiment). *$P < 0.001$.

Bt_2cAMP (1 mM), no changes in the cellular cholesterol ester mass were found. In addition, treatment of MA-10 cells with aminoglutethimide (50 μM), an inhibitor of the cholesterol side chain cleavage (CSCC) enzymatic complex [2], in combination with Bt_2cAMP (1 mM), did not alter the cellular cholesterol ester mass either. Thus, cAMP stimulation of MA-10 cells results in a net cholesterol ester hydrolysis only when cholesterol is utilized for steroid hormone synthesis.

Inhibition of cholesterol ester hydrolase by JH analogue

Cholesterol ester hydrolysis in intact cells can be measured by blocking cholesterol acyl-transferase (ACAT) with the compound 58-035 [17]. MA-10 cells were loaded with cholesteryl ester by overnight incubation with 50 μg/ml LDL. The data presented in Fig. 4(upper panel) show the effects of five treatments on the cellular mass of cholesterol ester. The compound 58-035 causes a loss in cholesterol esters of about 20% after 4 h incubation. The addition of both 58-035 and Bt_2cAMP to MA-10 cells resulted in a net cholesterol ester hydrolysis and cholesterol ester concentrations declined by 50%. Treatment of MA-10 cells with JH analogue (10^{-4} M) or JH analogue (10^{-4} M) and Bt_2cAMP (1 mM) in the presence of 58-035, does not alter the cellular mass of cholestol esters, indicating that JH analogue affects cholesterol ester hydrolase activity. The percentage of hydrolysis was determined as described in Materials and Methods. At the highest inhibitor concentration tested [Fig. 4(lower panel)], cholesterol hydrolase activity returned to basal levels. The variations in cellular ester concentrations resulting from preincubation with LDL, enhanced the variability of the treatment effects.

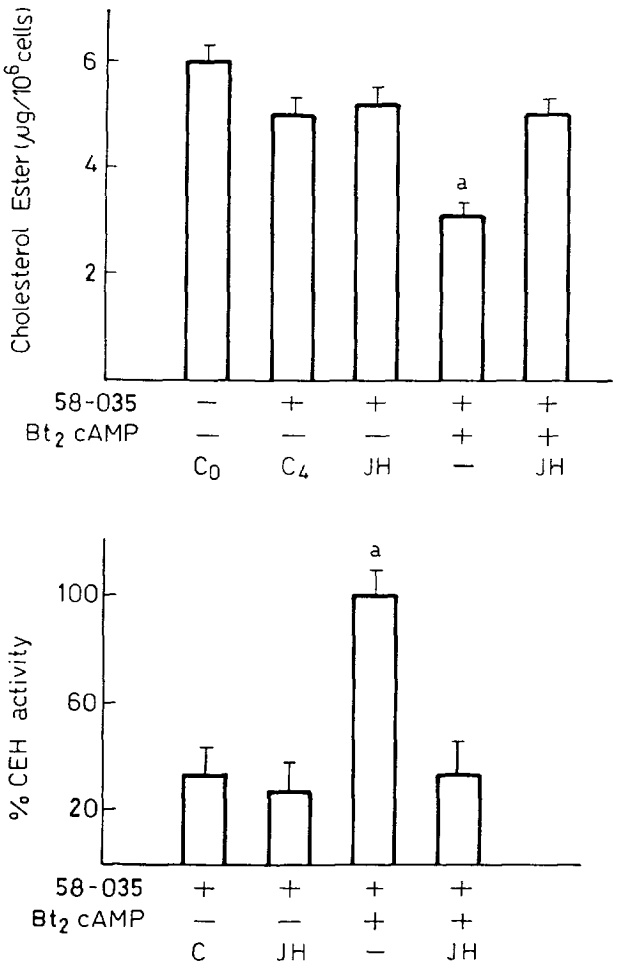

Fig. 4. Cholesterol ester hydrolase activity. Cells were incubated overnight with 50 μg of LDL of cholesterol/ml. Upper panel (time zero), wells were washed twice with 2 ml assay medium and then incubated in 2 ml of this medium containing the indicated additions. ACAT inhibitor 58-035 (2 μg/ml) was added to prevent ester resynthesis and, thus, allow measurement of the absolute rate of ester hydrolysis. After 4 h of incubation, medium was removed, cells scraped from the wells and esterified cholesterol determined as described in Materials and Methods. Lower panel, the percentage of cholesterol ester hydrolase activity was calculated by subtracting the cholesterol ester conent of control cells at time zero (C) from the cholesterol ester content of 58-035 (2 μg/ml), 58-035 (2 μg/ml) and 10^{-4} M JH analogue (JH), 58-035 (2 μg/ml) and 1 mM Bt_2cAMP, and 58-035 (2 μg/ml) and 10^{-4} M JH analogue (JH) and 1 mM Bt_2cAMP-treated cells after 4 h incubation. Experimental data were obtained as described in Materials and Methods. Each bar shows the average (±SE) of three independent experiments (triplicate wells/experiments). [a]Indicates value is different from control with $P < 0.001$.

DISCUSSION

We had previously reported that hCG-stimulated steroidogenesis in MA-10 mouse Leydig tumor cells could be inhibited by incubating the cells in the presence of JH. The inhibition of steroidogenesis was reported to be a result of the inhibition of cAMP formation by JH [1]. We also found that the inhibitory effect persisted by cells stimulated with cAMP analogues. Taking into account this previous finding, the purpose of the present study was to investigate the effects of JH analogue, 2-(4-phenoxyphenoxy)-etoxy tetrahydropyran, on the availability of cholesterol and upon some steps involved in the progesterone pathway.

Since progesterone production is a result of the activities of the CSCC and 3β-HSD enzymes in mitochondria [18] it is tempting to speculate that these elements may be targets for the regulatory action of the JH analogue in Leydig cells.

Our results demonstrated that steroid production was not inhibited by JH analogue when cells were treated with pregnenolone, indicating that JH analogue did not affect 3β-HSD activity. Subsequently, the use of JH analogue was found to result in an inhibitory effect in 25-hydroxycholesterol or 20-hydroxycholesterol-treated cells. In this context, the addition of the JH analogue to 20- or 25-hydroxycholesterol treated cells in the presence of hCG also resulted in a decrease in steroid production, indicating that neither the

hydroxylated compounds nor hCG could revert the inhibitory effect of the JH analogue. Both these hydroxylated compounds passed though the plasma membrane and entered the mitochondria to become substrates of the CSCC enzymes [19, 20]. These studies employed concentrations of the inhibitor that almost completely blocked steroidogenesis.

Moreover, with the use of pregnenolone metabolism inhibitors such as cyanoketone, we determined a decrease in pregnenolone production in stimulated cells treated with the JH analogue.

These results, together with the previous determination of 3β-HSD activity, led us to suggest that the inhibitory effect could be related to an effect on the CSCC enzymatic complex. In these studies, however, a direct estimation of CSCC activity was not performed.

The availability of cholesterol for steroidogenesis is regulated by several processes, including cellular uptake via lipoprotein receptors, release from esterified stores in lipid droplets by a cAMP-sensitive cholesterol esterase, transport to mitochondria, and translocation across the outer to the inner mitochondrial membrane [21].

The proportion of cell cholesterol in the plasma membrane, and the easy way in which radiolabeled cholesterol can be introduced selectively into this membrane, makes this molecule a strong candidate as a plasma membrane marker [22].

In non-stimulated cells, most cholesterol is transported to the plasma membrane where it remains indefinitely. Steroid hormone-synthesizing cells divert cholesterol, ordinarily destined for insertion into the membrane, towards the steroid biosynthetic pathway and cause plasma membrane cholesterol to become internalized [6]. We have found that JH analogue modified the level of [^{3}H]cholesterol used to radiolabel the plasma membrane, suggesting that the presence of the JH analogue affected the process described above. Blocking of free cholesterol usage would be expected to significantly limit steroid hormone synthesis by MA-10 cells.

Since the use of plasma membrane cholesterol and cholesteryl esters requires a functioning steroidogenic pathway, it seems likely that the inhibition of the pathway would account for the major effect of this compound on steroidogenesis. During the acute phase of steroidogenesis (0–4 h), cholesterol stores provide 73% of all substrate [5].

Cholesterol esters act as a readily available and rapidly mobilized source of cholesterol for steroidogenesis. Stimulation of MA-10 cells with trophic hormones or Bt_2cAMP, produces an increase in cholesterol ester hydrolase (CEH) and ACAT activities [5]. This leads to an increase in the cholesterol ester cycle turnover.

Thus, stimulated cells are able to convert cholesterol into steroid hormones; consequently, substrate for ester resynthesis is lost to steroidogenesis and results in a net ester hydrolysis. The presence of JH analogue in Bt_2cAMP-stimulated cells blocked this net ester hydrolysis effect. Control experiments were carried out using Bt_2cAMP in combination with aminogluthetimide, a CSCC inhibitor, with a similar inhibitory effect on net ester hydrolysis. The blocking of steroidogenesis using aminogluthetimide did not affect the increases in the activities of CEH and ACAT caused by Bt_2cAMP [5]. To determine whether the inhibitory effect observed in the presence of the JH analogue is similar to the aminogluthetimide effect, we determined the cholesterol ester hydrolase activity of intact cells. These experiments were carried out using the 58-035 ACAT inhibitor, which prevents cholesterol ester resynthesis [17], and CEH activity was determined as the percentage of cholesterol ester hydrolysed. The addition of both 58-035 and JH analogue to Bt_2cAMP-stimulated cells did not alter the cholesterol ester mass and decreased CE hydrolysis to basal levels, indicating that JH analogue effects CEH activity.

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