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THYROID HORMONE ACTION IN PORCINE LUTEAL CELLS. EFFECT OF TRIIODOTHYRONINE ON MITOCHONDRIAL CYTOCHROME P450-SCC ACTIVITY

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In the present study hydroxylated cholesterol derivatives were used to monitor $P450_{scc}$ activity as an effect of triiodothyronine. Luteal cells isolated from mid-developing and mature corpora lutea were plated into 24-well plates by 18 h incubation with M199 supplemented with 5% of alf serum. After that time the plates were washed with fresh M199 and hydroxylated cholesterol derivatives (25-, 20-, and 22-hydroxylated cholesterol) were added to the control (not-T3-treated) and T3-treated cells. Two hours later, all cultures were terminated and the media were frozen until further progesterone analysis. Triiodotyronine added to the culture medium of cells isolated from mature corpora lutea increased both basal and hydroxylated cholesterol derivative-stimulated cells. Aminoglutethimide, a $P450_{scc}$ enzyme inhibitor, added to the culture medium in all doses used, had no effect on basal progesterone secretion while added to the T3-treated cells reduced progesterone production. This study strongly supports the hypothesis of a direct effect of thyroid hormone on mitochondrial cytochrome $P450_{scc}$ — catalysing side-chain cleavage of cholesterol enzyme in luteal cells.

K e y w o r d s: *thyroid hormone, corpus luteum, cytochrome $P450_{scc}$, progesterone production.*

INTRODUCTION

The effects of thyroid hormones on mitochondria and energy metabolism have been well documented (1—4). It is, however, commonly assumed that hormonal effects on mitochondria are secondary to a primary action on nuclear gene expression, generating regulatory signals channelled to the mitochondria.

A possible basis for direct thyroid hormone action on mitochondria was provided by findings of a thyroid hormone-binding component in mitochondrial membranes (5—7). The saturable receptor was found in the

inner mitochondrial membranes of rat liver, kidney, myocardium and skeletal muscle (8). Previous research from our laboratory (9) showed the direct effect of T3 on progesterone secretion by luteal cells. The effect of thyroid hormone on cyclic nucleotide release by ovarian cells was previously shown, suggesting the involvement of cAMP-dependent mechanism in the realization of T_3 action in ovarian cells (10). Surprisingly, there are no data concerning the presence of mitochondrial T3 receptors in the ovarian tissue. The activity of the mitochondrial cholesterol side chain cleavage cytochrome ($P450_{scc}$) has long been proposed as the rate-limiting step in the production of progesterone. It has been found that one cytochrome P-450 enzyme, is localized on the inner mitochondrial membrane (11). The study of Sugano $et\ al.$ (12) provide direct evidence that cholesterol is metabolized to pregnenolone by way of 22R-hydroxycholesterol and 20R, 22R-dihydrocholesterol by $P450_{scc}$.

The present experiment was therefore conducted to determine the effect of T3 on the activity of $P450_{scc}$ — by measuring the conversion of hydroxylated cholesterol derivatives to progesterone.

MATERIAL AND METHODS

Reagents

Triiodothyronine (10^{-9} M), 25-, 20-, 22-R-hydroxycholesterol (10 µg/ml) and aminoglutethimide (1, 10 or 20 µg/ml) were purchased from Sigma Chemical Co, St. Louis, MO, USA.

Medium M199, penicillin, trypsin, and calf serum were from the Laboratory of Vaccines, Lublin, Poland.

Animals

Porcine ovaries were collected in ice-cold physiological saline from a local abattoir immediately after slaughter and transported to the laboratory. The phase of the oestrous cycle was determined according to the established morphological criteria (13, 14). Ovarian tissues representing three different stages of the oestrus cycle were collected from: newly developed corpora lutea (CL1; 1—3 days after ovulation), mature corpora lutea (CL2; 7—10 days after ovulation), and regressing corpora lutea (CL3; 12—14 days after ovulation).

Cell culture

Dissected corpora lutea of each type were enzymatically dissociated according to the technique of Gregoraszczuk (15). The cells were suspended in medium M199 supplemented with 5% of calf serum at a concentration of 3.5×10^5 cells/ml medium.

Cells were grown in multiwell plates (Nunck) in a humidified atmosphere with 5% CO_2 in the air for 24 h. After 24 h incubation all cultures were terminated and the media were frozen until further steroid analysis. Cell viability using the trypan blue exclusion test was 85%.

Experimental procedure

Experiment 1. To show the dose response to T3 with progesterone secretion dispersed cells were incubated without (control) and with three doses (10^{-7} M, 10^{-9} M, and 10^{-11} M) of T3. After 24 h incubation, all cultures were terminated and the media were frozen until further progesterone analysis.

Experiment 2. This experiment required the use of hydroxylated cholesterol derivatives (25-, 20-, and 22-hydroxylated cholesterol) therefore a preliminary study evaluated different doses of those cholesterol derivatives on progesterone production. It was found that 10 μg/ml was a maximally stimulated dose for all cholesterol derivatives. This is in accordance with Wiltbank et al. (16). Cells were plated into 24-well plates by 18 h incubation with M199 supplemented with 5% of calf serum. After that time hydroxylated cholesterol derivatives (25-, 20-, and 22-hydroxylated cholesterol) was added to the control (not-T3-treated) and T3-treated cells. Six hours later, all cultures were terminated and the media were frozen until further progesterone analysis.

Experiment 3. Taking into consideration results obtained from Experiment 2,20-hydroxylated cholesterol was used in this experiment. Luteal cells were cultured for 18 h with M199 supplemented with 5% of calf serum and 20-hydroxylated cholesterol. After that time aminoglutethimide, the inhibitor of P450$_{scc}$ was added to the control (not-T3-treated) and T3-treated cells. Six hours later, all cultures were terminated and the media were frozen for further progesterone analysis.

Progesterone analysis

Progesterone was detected in the culture medium by a radioimmunoassay described elsewhere (13). A highly specific antibody raised in sheep against 11a-hydroxy-progesterone hemisuccinate coupled to bovine serum albumin was used. The cross-reaction with pregnenolone was 2.9%. All other tested steroids showed less than 0.1% cross-reaction. (1,2,6,7-^{3}H) progesterone (Radiochemical Centre, Amersham, England, sp. act. 80 Ci/mmol) was used as the tracer. The limit of sensitivity of the assay was 50 pg/ml. The coefficients of variation within and between assays were 15% and 2.5%, respectively.

All data points are expressed as means $\pm$ SEM derived from at least three different replications, each in triplicate, resulting in at least nine observations. Significant differences between steroid levels in control and treated cells were compared by analysis of variance and by using Duncan's new multiple range test.

RESULTS

The effect of exogenous triiodothyronine on progesterone secretion

All the applied doses of T3 stimulated progesterone secretion by cells isolated from early developing corpora lutea (CL1), in a doses 10^{-11} M and 10^{-9} M increased progesterone production by cells isolated from mature corpora lutea (CL2) and had no effect on progesterone production by cells isolated from regressing corpora lutea (CL3) (Fig. 1).
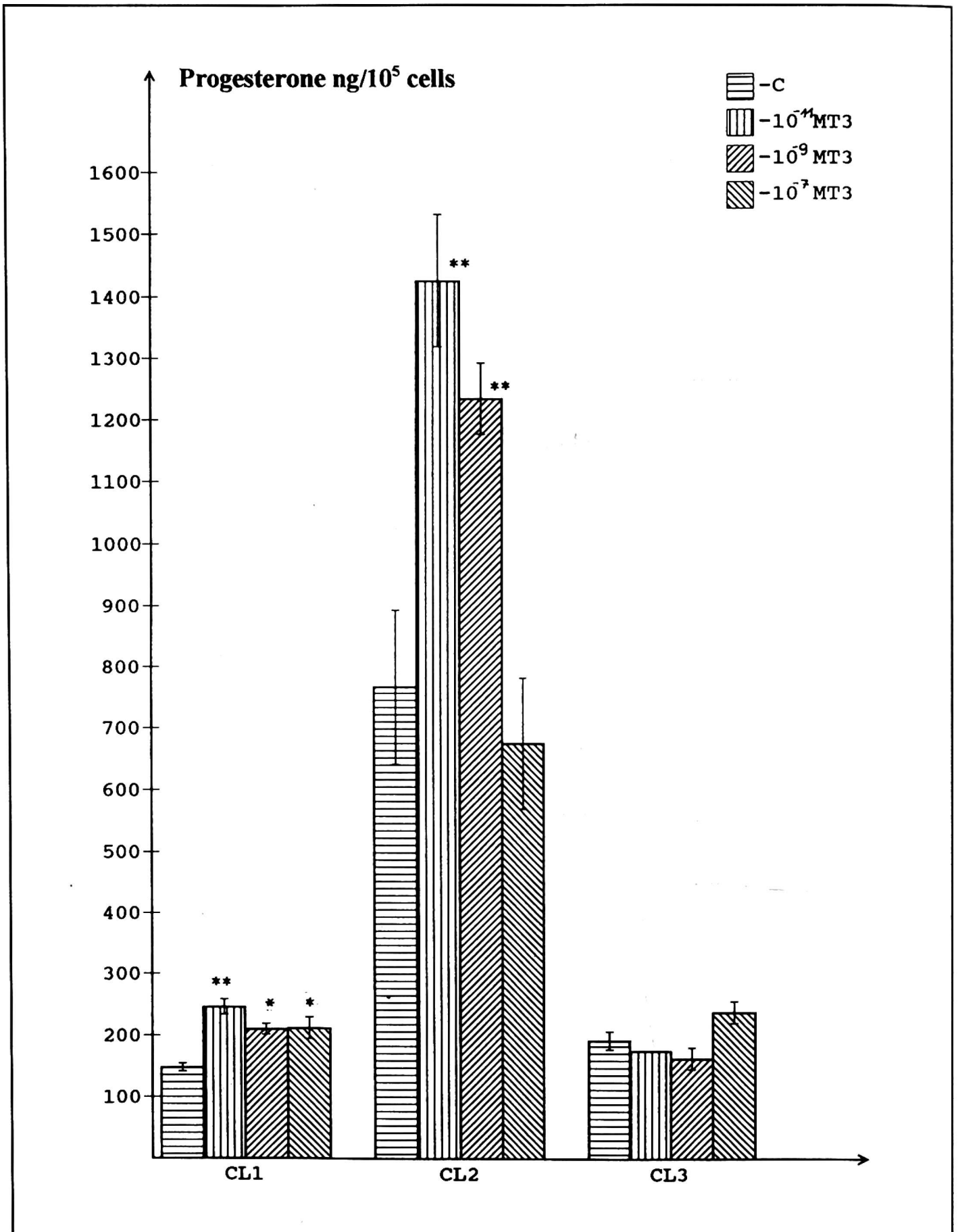
Fig. 1. Effect of triiodothyronine $(10^{-7}$—10^{-9} — and 10^{-11} MT) on progesterone secretion measured following culture for 24 h of porcine luteal cells collected from: newly developed corpora lutea (CL1; 1—3 days after ovulation), mature corpora lutea (CL3; 7—10 days after ovulation) and regressing corpora lutea (CL4; 12—14 days after ovulation). Each bar represents the mean $\pm$ SE of 3 replicate cultures each in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001.
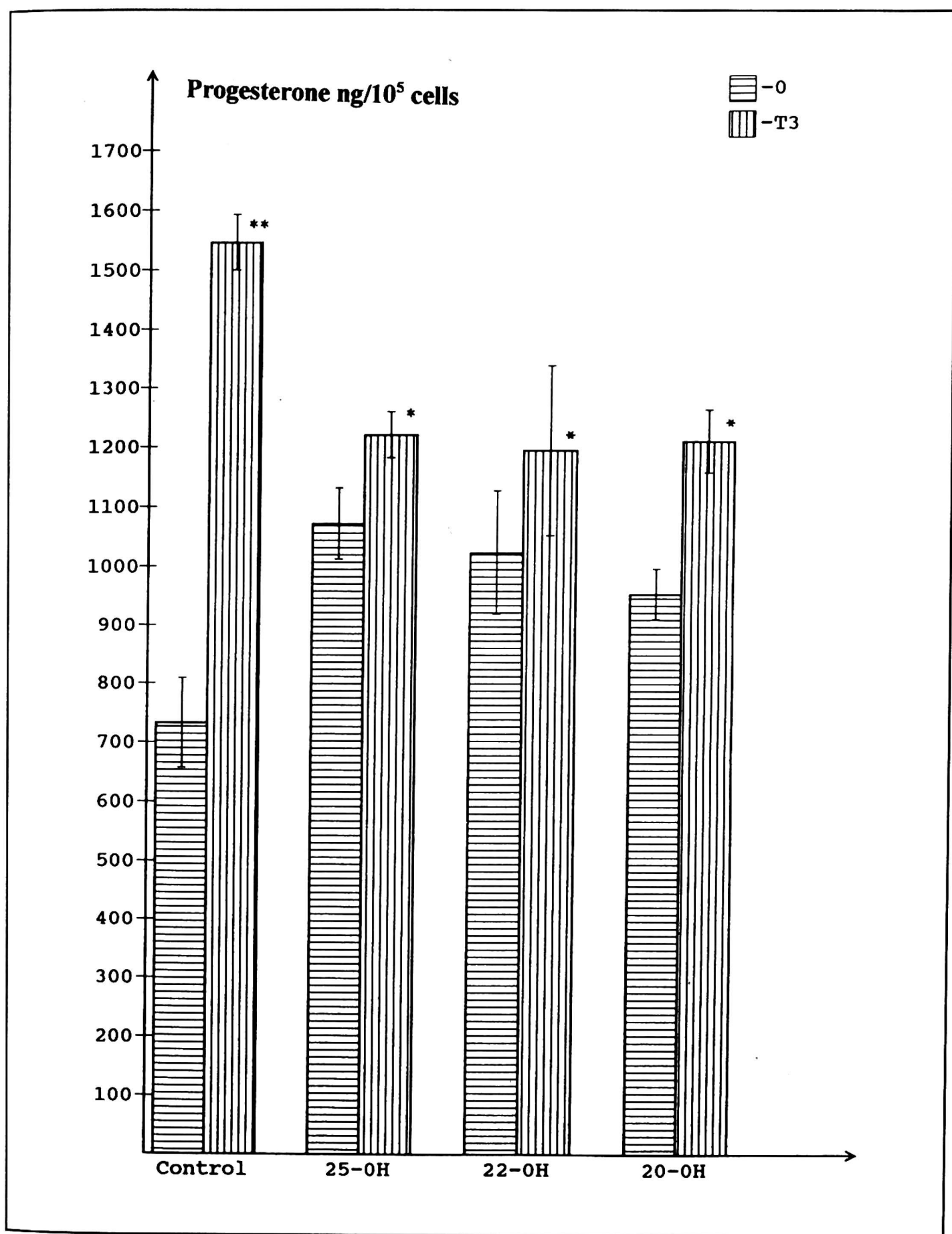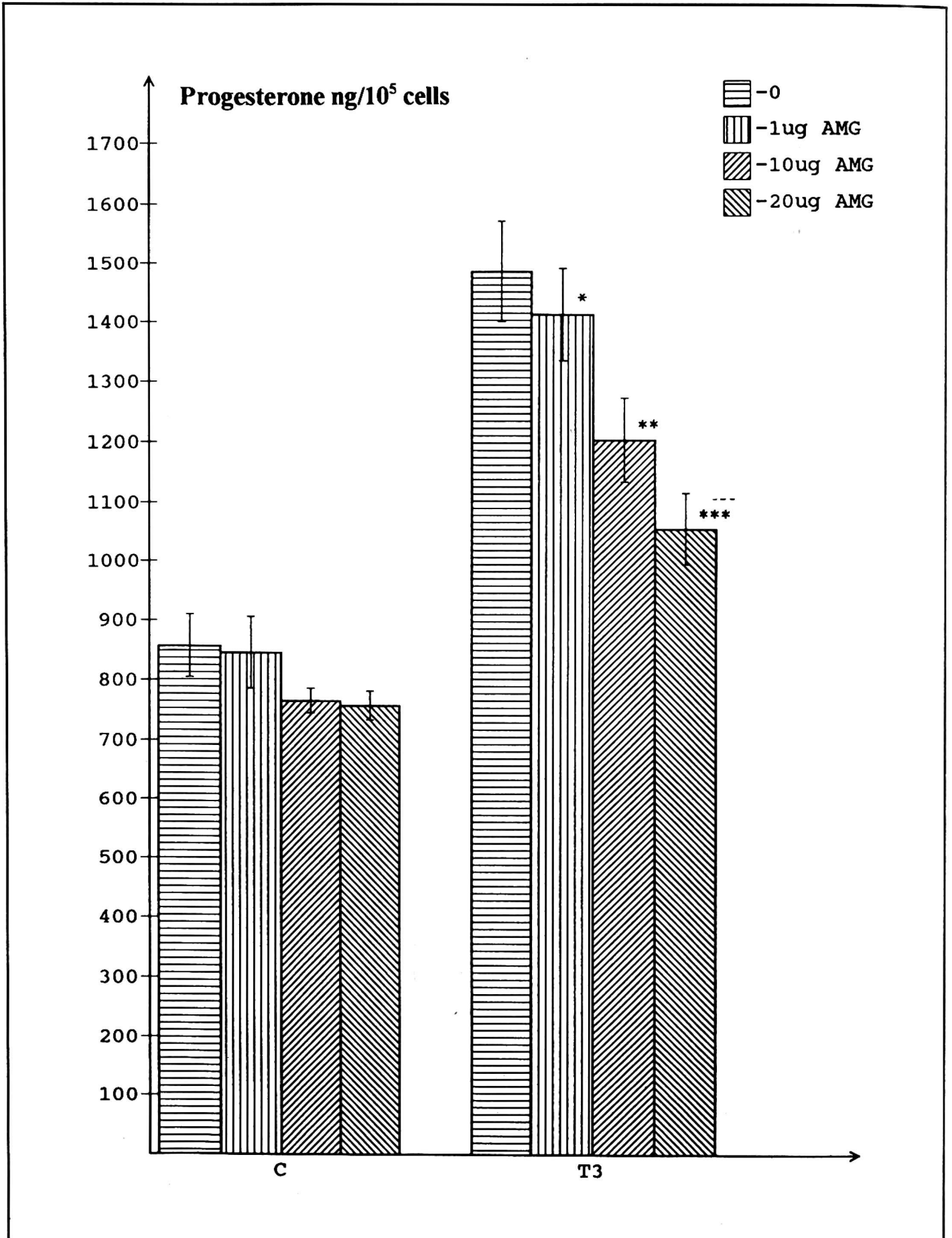
Fig. 2. Progesterone production by luteal cells isolated from mature (CL3) corpora lutea. Cells were plated into 24-well plates for 18 h incubation with M199 supplemented with 5% of calf serum. The plates were then washed with fresh M199 and 25-hydroxylated cholesterol (25-OH); 20-hydroxylated cholesterol (20-OH), and 22-hydroxylated cholesterol (22-OH) was added to the control (not-T3-treated) and T3-treated cells. Two hours later, all cultures were terminated and the media were frozen until further progesterone analysis. Each bar represents the mean ± SE of replicate cultures each in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 3. Progesterone production by luteal cells mature (CL2) corpora lutea. Cells were cultured for 18 h with M199 supplemented with 5% of calf serum. The plates were then washed with fresh medium and aminoglutethimide, the inhibitor $P450_{scc}$ was added to the control (not-T3-treated cells). Two hours later, all cultures were terminated and the media were frozen for further progesterone analysis. Each bar represents the mean $\pm$ SE of 3 replicate cultures each in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001.

The effect of T3 on 25-, 20-, and 22-hydroxylated cholesterol-stimulated progesterone secretion by cells isolated mature corpora lutea.

Triiodothyronine added to the culture medium of cells isolated from mature corpora lutea increased both basal (1546.6 ± 46.1 ng vs 733.3 ± 76.3 ng/10^5 cells in control culture), $p < 0.01$, and hydroxylated cholesterol derivative-stimulated cells (1220.6 ± 39.0 ng vs 1071.6 ± 59.2 ng/10^5 cells in 25-OH-stimulated cells; 1195 ± 14.2 ng vs 1022.6 ± 10.3 ng/10^5 cells in 22-OH-stimulated cells and 1210 ± 53.7 ng vs 953.3 ng/10^5 cells in 20-OH-stimulated cells), $p < 0.05$. (*Fig. 2*).

The effect of aminoglutethimide on basal and T-3 stimulated progesterone secretion

Aminoglutethimide added to the control cultures in all the doses used, had no effect on progesterone secretion (845.5 ± 60.3 ng; 764.9 ± 20.0 ng and 756.3 ± 23.1 ng/10^5 cells as the effect of 1, 10, and 20 µg AG respectively vs 857.0 ± 52.0 ng/10^5 cells in the control culture) (*Fig. 3*).

Aminoglutethimide added to the T-3 treated cells in a dose dependent manner reduced T3-stimulated progesterone production (1412.5 ± 36.1 ng, 1203.0 ± 10.0 ng and 1054.0 ± 17.2 ng/10^5 cells as the effect of 1, 10, and 20 µg AG respectively as 11485.3 ± 34.1 ng/10^3 cells in the T3-stimulated cells). (*Fig. 3*).

DISCUSSION

The effects of thyroid hormones on mitochondria and energy metabolism have been the subject of intensive research and are well documented (3, 8, 17—19). Demonacos *et al.*, (20) proposed that thyroid hormone effects on mitochondria gene(s) are direct, concomitant with the effects on nuclear genes and involving similar molecular mechanisms to those mediating steroid--thyroid hormone actions on nuclear gene transcription. In luteal cells the activity of P450$_{scc}$ has long been proposed as the rate-limiting step in the production of progesterone. It has been found that one catochrome P450 enzyme, localized on the inner mitochondrial membrane, is responsible for both the 20- and 22-hydroxylation of cholesterol and for the final oxidation step which causes cleavage of the side chain (21, 22). In the present study hydroxylated cholesterol derivatives were used to monitor P450$_{scc}$ activity as an effect of triiodothyronine. Triiodothyronine added to the culture medium increased both basal and hydroxylated cholesterol derivative-stimulated cells.

This study suggested that all the 20 and 22 hydroxylation steps and the side chain cleavage step of the 450_{scc} are acutely regulated by triiodothyronine. The cholesterol content, phospholipid composition, and degree of fatty acid saturation all change in the mitochondrial inner membrane with the thyroid state (23). The possibility that T3 acted as an inhibitor of $P450_{scc}$ was examined by using aminoglutethimide, the main inhibitor of P450 cytochrome (11). Aminoglutethimide, added to the culture medium in all doses used, had no effect on basal progesterone secretion while added to the T3-treated cells reduced progesterone production. In conclusion, this study suggested that cytochrome $P450_{scc}$ — catalysing side-chain cleavage of cholesterol may be a key target of T3 action on the mitochondria in luteal cells. Additional studies of the mitochondrial receptors will help clarify the role of thyroid hormones in the regulation of mitochondrial activity in the luteal cells.

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