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Effect of Protein-Synthesis Inhibitors on Testosterone Production in Rat Testis Interstitial Tissue and Leydig-Cell Preparations

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Luteinizing-hormone-stimulated testosterone biosynthesis was inhibited by cycloheximide during incubation of rat testis interstitial tissue *in vitro* and also by puromycin and cycloheximide during incubation of Leydig-cell preparations, but not by chloramphenicol. These results suggest that a protein regulator(s) formed by cytoplasmic protein synthesis is involved in steroidogenesis in the rat testis. The specific effect of cycloheximide and puromycin on protein synthesis rather than on other non-specific processes is suggested by the inhibition of protein synthesis and steroidogenesis with different doses of the inhibitors and the lack of effect of cycloheximide on luteinizing-hormone-induced adenosine 3′:5′-cyclic monophosphate production. Stimulation of testosterone production by luteinizing hormone during superfusion of interstitial tissue was detectable within 10–20min and reached a maximum at 120min, and thereafter slowly decreased. Cycloheximide added at maximum steroid production caused a rapid decrease in testosterone synthesis which followed first-order kinetics (half-life 13min), thus indicating that the protein regulator(s) has a short half-life. No effect of cycloheximide, puromycin or chloramphenicol on testosterone production in the absence of added luteinizing hormone was found, suggesting that the basal production of testosterone is independent of protein synthesis.

Previous work has shown that luteinizing hormone specifically stimulates adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and testosterone production in rat testis interstitial tissue *in vitro* (Cooke *et al.*, 1972; Moyle & Ramachandran, 1973; Rommerts *et al.*, 1973; Cooke *et al.*, 1974*b*; Dorrington & Fritz, 1974), and cyclic AMP has been proposed as the intracellular mediator of luteinizing-hormone action in this tissue (see review by Rommerts *et al.*, 1974). However, the intracellular mechanism of stimulation of steroidogenesis in the testis is unknown. For the adrenal gland and ovary it has been shown that inhibitors of protein synthesis inhibit the stimulation of steroidogenesis by trophic hormones. This inhibition is very rapid and hence it has been suggested that a protein with a short half-life is required for the increase of steroidogenesis in the presence of trophic hormones (Garren *et al.*, 1971; Schulster *et al.*, 1970, 1974; Lowry & McMartin, 1974; Hermier *et al.*, 1971). The function of this protein or the mechanism by which its synthesis is controlled is unknown. In the present investigation the effect of cycloheximide on testosterone synthesis in testis interstitial tissue has been determined both in static and in continuous-flow incubation systems. In addition the effects of other protein-synthesis inhibitors, puromycin and chloramphenicol, as well as cycloheximide, on testosterone biosynthesis in testis Leydig-cell preparations have been investigated. A preliminary report of part of this work has been published (Cooke *et al.*, 1974*a*).

Materials and Methods

Materials

Ovine luteinizing hormone (NIH-LH-S18, 1.03 units/mg) was a gift from the Endocrinology Study Section, National Institute of Health, Bethesda, Md., U.S.A. L-[U-^{14}C]Leucine (348mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Static experiments in vitro

Interstitial tissue. In each experiment the interstitial tissue (approx. 300mg wet weight from two rats) obtained by wet dissection of total testis tissue from rats (Wistar strain), 3–4 months old, weighing 200–250g, was preincubated for 1h at 32°C in Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1964) (2ml), pH7.4, containing 0.2% glucose as described by Cooke *et al.* (1972). The tissue was then divided between eight incubation tubes containing (as indicated in the Figures) [^{14}C]leucine (1μCi), luteinizing hormone (100ng/ml) and cycloheximide,

and incubated for 3h at 32°C in Krebs–Ringer bicarbonate–glucose buffer (0.5ml) in an atmosphere of O_2+CO_2 (95:5). The incubation tubes were then placed in ice and the volume was made up to 1ml with the Krebs–Ringer bicarbonate–glucose buffer, and the tissues were homogenized by sonication. One-half of the homogenate was removed for determination of testosterone by radioimmunoassay and in some experiments for cyclic AMP by competitive protein binding as described by Rommerts *et al.* (1973), and the remainder was used to determine the incorporation of the labelled leucine into protein. $HClO_4$ (0.5M, 0.5ml) was added and after standing for 10min at 0°C the samples were centrifuged, and the precipitated protein was washed four times with 0.2M-$HClO_4$. After the last wash the remaining $HClO_4$ was evaporated under N_2 and the precipitate was dissolved in NaOH (0.5ml, 1M), and the amount of ^{14}C was counted in a Nuclear–Chicago liquid-scintillation counter in a medium containing naphthalene (80g/litre), Permablend (Packard Instrument Co., La Grange, Ill., U.S.A.) (5g/litre) and toluene–methoxyethanol (10:9, v/v). Protein was determined by the method of Lowry *et al.* (1951). In control experiments in which tissue was not incubated, less than 1% of the added [^{14}C]leucine was recovered in the protein isolated.

Leydig-cell preparations. The method used to prepare the Leydig-cell preparations was essentially the same as that described by Moyle & Ramachandran (1973). Two decapsulated testes were incubated together in 7ml of Krebs–Ringer bicarbonate–glucose buffer containing collagenase [1mg of Sigma Chemical Co. (St. Louis, Mo., U.S.A.) type 1/ml], under an O_2+CO_2 (95:5) atmosphere for 18min at 37°C. Plastic incubator tubes (40ml) with tight-fitting caps were used and they were placed longitudinally in a water bath and shaken at 75 cycles/min. After incubation, 30ml of 0.9% NaCl at room temperature was added to each tube. The tubes were carefully inverted ten times and left for 10min at room temperature. The supernatant was siphoned off with the aid of Tygon tubing and filtered through nylon gauze (60μM). The filtrate was centrifuged at 100*g* for 10min at room temperature. The supernatant was discarded and the sediment suspended in 0.2ml of Krebs–Ringer bicarbonate–glucose buffer, pH7.4, containing 1% albumin (bovine, fraction V, Fluka A.G., Buchs SG, Switzerland) and lima-bean trypsin inhibitor (0.1mg/ml). The suspension was then adjusted to a cell density of 3×10^6–10×10^6 cells/ml and preincubated for 1h at 32°C under O_2+CO_2 (95:5). The suspensions were finally diluted to volumes of 0.25ml to give cell densities of about 3×10^6 cells/ml. Incubations were carried out in a water bath at 32°C shaking at 75 cycles/min in plastic tubes in a sloping position with an angle of about 60° under O_2+CO_2 (95:5). Luteinizing hormone, [^{14}C]leucine (0.5μCi) and other relevant compounds (as indicated in the Results section) were added in the Krebs–Ringer bicarbonate–glucose buffer containing 0.1% albumin. The extraction and determination of testosterone was as described by Rommerts *et al.* (1973). The protein pellet obtained after acetone extraction was washed four times with trichloroacetic acid (5%, w/v) and the incorporation of [^{14}C]leucine determined as described above for interstitial tissue.

Superfusion experiments with interstitial tissue

The apparatus used was essentially the same as that described by Lowry & McMartin (1974). Interstitial tissue (containing approx. 5mg of protein) was loosely packed between silicone-treated glass beads (0.5mm diam.) contained in a plastic syringe (total volume 0.5ml) and then superfused with oxygenated Krebs–Ringer bicarbonate–glucose buffer at 32°C containing γ-globulin (human immunoglobulin) (0.01%, w/v). The flow rate was 0.4ml/min. Polyethylene tubing was used to connect the perfusion pump to the superfusion syringe and the fraction collector. When [^3H]testosterone was perfused through the apparatus no adsorption occurred and this steroid was eluted from the apparatus within 1–2min.

Fractions (0.4ml) were collected from the superfused tissue and the testosterone content was determined by radioimmunoassay after extraction by hexane as described by Rommerts *et al.* (1973).

The concentrations of luteinizing hormone used in the experiments with interstitial tissue and Leydig-cell preparations were 100ng/ml and 1μg/ml respectively; both concentrations gave a maximum testosterone production with both preparations.

Results

Incubations of interstitial tissue were carried out with different amounts of cycloheximide (0–89nmol/ml) and a constant amount of luteinizing hormone (100ng/ml). It can be seen from Table 1 that in the absence of cycloheximide luteinizing hormone had no detectable effect on the total amount of [^{14}C]leucine incorporated into protein when compared with the control, although it produced a 2.5-fold increase in testosterone biosynthesis. Addition of 89nmol of cycloheximide/ml caused an 88% inhibition of protein synthesis and a 97% inhibition of luteinizing-hormone-stimulated testosterone biosynthesis. It did not, however, inhibit the amounts of testosterone formed to below basal production found in the absence of added luteinizing hormone. The degree of inhibition of both protein synthesis and luteinizing-hormone-stimulated testosterone synthesis depended on the amount of cycloheximide added, e.g. 0.89nmol

Table 1. *Effect of cycloheximide on testosterone, cyclic AMP and protein synthesis in rat testis interstitial tissue*

The means ± S.E.M. of four to ten experiments with tissue from different rats are given. The zero incubation time values for testosterone and cyclic AMP in the tissue have been subtracted (4.9ng/mg of protein and 8.0pmol/mg of protein respectively). The mean percentage inhibition of luteinizing-hormone-stimulated testosterone and cyclic AMP production (basal production was first subtracted) and the inhibition of [^{14}C]leucine into protein are given in parentheses.

Luteinizing hormone (100ng/ml)	0	+	+	+	+	+	+
Cycloheximide (nmol/ml)	0	0	89	8.9	0.89	0.089	0.0089
Testosterone (ng/4h per mg of protein)	9.6±1.3	26.8±3.1	9.7±1.3 (97%)	14.0±1.6 (73%)	15.3±3.2 (65%)	23.8±7.0 (17%)	21.1±4.0 (33%)
10^{-3} × [^{14}C]Leucine incorporation (d.p.m./mg of protein)	26.1±2.0	25.2±1.6	3.0±0.5 (88%)	4.1±1.0 (87%)	10.0±1.5 (60%)	20.9±3.4 (17%)	25.9±6.0 (0%)
Cyclic AMP (pmol/4h per mg of protein)	4.3±1.9	39.0±4.6	37.3±6.8 (4.9%)	38.3±10.3 (2.0%)	42.0±9.5 (−9.0%)	33.5±13.0 (15.6%)	— (—)

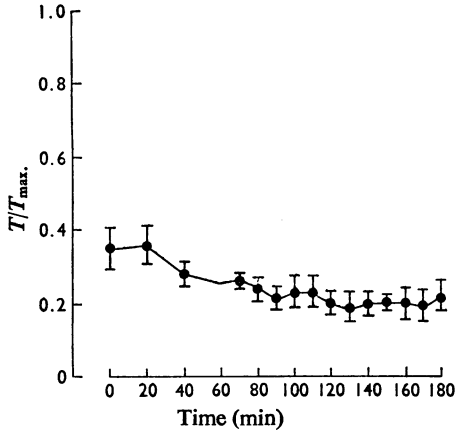

Fig. 1. *Basal production of testosterone from superfused rat testis interstitial tissue*

To compare values obtained with tissue from different rats the results are expressed as the testosterone formed (in pg/min per mg of protein, T) divided by the maximum luteinizing-hormone-stimulated testosterone formed (in pg/min per mg of protein, $T_{max.}$). The latter was measured in another series of experiments (Fig. 3). The mean testosterone production after 60min superfusion of the tissue was 58±6S.E.M. pg/min per mg of protein ($n = 8$). Other details are given in the Materials and Methods section.

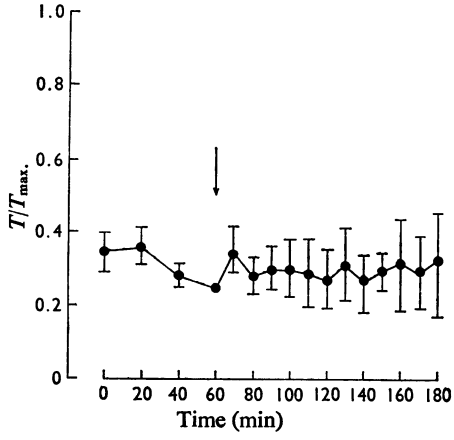

Fig. 2. *Effect of cycloheximide on basal testosterone production in superfused rat testis interstitial tissue*

Results ($n = 8$, ±S.E.M.) were calculated as described in the legend for Fig. 1. Cycloheximide (89nmol/ml) was added to the superfusion medium after 60min (at the arrow) and thereafter remained in the medium until the end of the experiment.

of cycloheximide/ml gave 60 and 65% inhibition of protein synthesis and testosterone synthesis respectively. The eightfold stimulation of cyclic AMP production by luteinizing hormone was not significantly inhibited by the different amounts of cycloheximide added (Table 1).

To determine the kinetics of testosterone production and inhibition by cyloheximide, the interstitial tissue was placed in a continuous-flow (superfusion) apparatus and superfused with medium containing Krebs–Ringer bicarbonate–glucose buffer. Fig. 1 shows that testosterone production decreased during the first 60min and thereafter remained fairly constant. This initial decrease in testosterone production during the first 60min can also be seen in Fig. 3 (results from a different series of experiments). When cycloheximide was added to the superfusion medium in the absence of added luteinizing hormone no decrease in the testosterone production was observed (Fig. 2).

Addition of luteinizing hormone to the medium after 60min superfusion caused a rapid increase in testosterone production (testosterone concentrations were significantly increased above the control between 10 and 20min after addition of luteinizing hormone, $P < 0.025$), which reached a maximum 120

min after the addition of luteinizing hormone and thereafter slowly decreased (Fig. 3); the decrease followed first-order kinetics with a half-life of 47 min. When cycloheximide was added at maximum testosterone production a rapid decline in steroid synthesis occurred, which followed first-order kinetics with a half-life of 13 min (this was calculated after correction for the decline of testosterone synthesis obtained in the absence of cycloheximide). The steroid synthesis in the presence of cycloheximide did not decrease below the basal production obtained after 60 min superfusion.

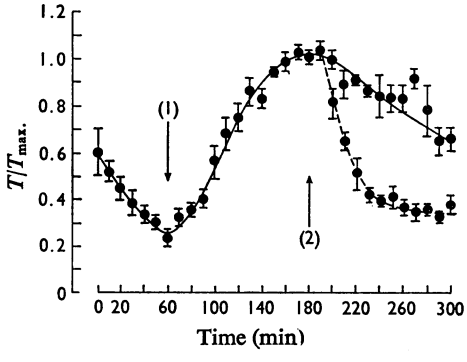

Fig. 3. *Effect of luteinizing hormone and cycloheximide on testosterone production from superfused rat testis interstitial tissue*

After 60 min superfusion with medium only, luteinizing hormone (100 ng/ml) was added to the medium (at arrow 1) and continuously superfused until the end of the experiment. The results ($n = 12$, ±S.E.M.) (——) are expressed as the testosterone formed (in pg/min per mg of tissue protein, T) divided by the maximum luteinizing-hormone-stimulated testosterone production (in pg/min per mg of tissue, $T_{max.}$). The maximum production at 180 min was 191 ± 17 pg/min per mg of protein. ----, Another series of experiments in which cycloheximide (89 nmol/ml) was added (at arrow 2) after 180 min as indicated in addition to the luteinizing hormone ($n = 4$, ±S.E.M.).

The effects of cycloheximide on testosterone biosynthesis were also investigated by using a Leydig-cell preparation. In addition the effects of two other protein-synthesis inhibitors, puromycin and chloramphenicol, were determined. The results from two experiments given in Table 2 are calculated as percentage inhibition of testosterone production and incorporation of [^{14}C]leucine into protein with and without added luteinizing hormone (1 μg/ml). The testosterone production in these experiments was (in ng/3 h per mg of protein, means of duplicate incubations): basal production 9.3, 11.5; with luteinizing hormone 25.0, 43.8. The zero-time incubation values (which have been subtracted) were 10.4 and 11.8 respectively. The mean incorporation of [^{14}C]-leucine into protein in the two experiments with and without added luteinizing hormone was 45×10^4 and 87.5×10^4 d.p.m./3 h per mg of protein.

With cycloheximide and puromycin similar results were obtained; the degree of inhibition of luteinizing-hormone-stimulated testosterone biosynthesis and protein synthesis was similar with the different amounts of inhibitors used. In confirmation of the superfusion results it was also shown that no inhibition occurred of the basal steroid production with either inhibitor in the absence of added luteinizing hormone. Chloramphenicol, an inhibitor of mitochondrial protein biosynthesis, had little or no effect on steroidogenesis with or without added luteinizing hormone with the different amounts used.

Discussion

The results of the present investigation show that cycloheximide inhibits luteinizing-hormone-stimulated testosterone biosynthesis in rat testis interstitial tissue. This was confirmed by using Leydig-cell

Table 2. *Effect of protein synthesis inhibitors on testosterone and protein synthesis in Leydig-cell preparations*

The percentage inhibition of the basal production of testosterone and protein synthesis in the absence of added luteinizing hormone (column 1) and the percentage of inhibition of luteinizing-hormone-stimulated testosterone and protein synthesis (columns 2, 3 and 4) are given (means ± S.E.M. from duplicate incubations in two experiments).

Luteinizing hormone (1 μg/ml)	0	+	+	+
Cycloheximide (nmol/ml)	89	89	0.89	0.0089
% inhibition of testosterone production	−2.9 ± 23.0	89.1 ± 2.0	47.0 ± 5.2	3.2 ± 11.6
% inhibition of [^{14}C]leucine incorporation into protein	94.1 ± 0.3	94.2 ± 0.2	70.1 ± 0.5	1.6 ± 1.4
Luteinizing hormone (1 μg/ml)	0	+	+	+
Puromycin (nmol/ml)	120	120	12	2
% inhibition of testosterone production	15.1 ± 7.5	93.5 ± 1.6	62.5 ± 4.4	10.3 ± 13.0
% inhibition of [^{14}C]leucine incorporation into protein	94.9 ± 0.3	95.2 ± 0.3	56.0 ± 3.3	25.1 ± 2.0
Luteinizing hormone (1 μg/ml)	0	+	+	+
Chloramphenicol (nmol/ml)	310	310	62	15
% inhibition of testosterone production	−13.9 ± 10.6	6.3 ± 4.3	5.8 ± 10.0	11.0 ± 21.2
% inhibition of [^{14}C]leucine incorporation into protein	5.4 ± 2.7	10.9 ± 2.2	4.7 ± 4.0	0.9 ± 0.8

suspensions. In addition it was found that puromycin, another protein-synthesis inhibitor, but with a different site of action from cycloheximide (Beard *et al.*, 1969; Williamson & Schweet, 1965), also inhibits steroidogenesis in Leydig-cell preparations. In contrast chloramphenicol, a mitochondrial protein-synthesis inhibitor, had little or no effect. This evidence clearly indicates that a protein regulator (or regulators) formed by cytoplasmic protein synthesis is involved in the luteinizing-hormone stimulation of testosterone production in the testis. Further evidence that the effects of the inhibitors observed were not due to non-specific effects came from the parallel inhibitions of protein synthesis and luteinizing-hormone-stimulated testosterone biosynthesis with different amounts of inhibitors added. It was also shown that cycloheximide did not decrease the luteinizing-hormone stimulation of cyclic AMP production in the interstitial tissue; this suggests that cycloheximide does not affect the interaction of luteinizing hormone with the cell membrane receptor. Marsh *et al.* (1966) have also shown that puromycin in the presence of luteinizing hormone does not inhibit the accumulation of cyclic AMP in corpus luteum slices.

From the superfusion experiments it was calculated that the half-life of the proposed protein regulator is 13min. The actual value may be much less than this because this time also includes the time taken for steroidogenesis to decrease. Similar results have been obtained for the adrenal gland. Garren *et al.* (1971) observed a half-life of 7–10min for the decay of adrenocorticotrophic hormone-stimulated adrenal steroid production after administration of cycloheximide to hypophysectomized rats *in vivo*. Schulster *et al.* (1970) reported a value of 45–49min for superfused bisected adrenal glands *in vitro*, and for adrenal-cell suspensions a half-life of 2–4min was found (Schulster *et al.*, 1974). The lower values obtained with the cell suspensions were attributed to the quicker penetration of inhibitors into, and of products out of, the adrenal cells. The synthesis of progesterone in the corpus luteum is also inhibited by cycloheximide; the half-life of the proposed protein regulator in this tissue was calculated to be 8–10min (Hermier *et al.*, 1971).

Previous work on the involvement of protein synthesis in steroidogenesis in the testis by Hall & Eik-Nes (1962) showed that both puromycin and chloramphenicol (the amounts used were in the same range as that used for the present experiments) inhibited the incorporation of [1-^{14}C]acetate into testosterone in slices of rabbit testis. The effect of chloramphenicol is at variance with the results of the present study. This may represent a species difference or reflect that these authors were studying steroidogenesis *de novo* with radioactive precursors. Chloramphenicol via inhibition of mitochondrial protein synthesis may inhibit the conversion of [^{14}C]acetate into cholesterol; this may not be detected when endogenous production of testosterone is measured if there are sufficient quantities of steroid precursors, e.g. cholesterol, available.

Moyle *et al.* (1971) have also shown that cycloheximide (1070nmol/ml) inhibited luteinizing-hormone-stimulated steroidogenesis in a Leydig-cell tumour. The cyclic AMP-induced steroid synthesis *de novo* in a clonal epithelial culture line derived from a mouse testicular interstitial cell tumour was found by Shin & Sato (1971) to be inhibited by cycloheximide, puromycin and also actinomycin D. In the present experiments no effect of cycloheximide was found on the basal production of testosterone in the absence of added luteinizing hormone. In this respect the testis appears to be different from the adrenal gland and ovarian follicle; Schulster *et al.* (1970) found that cycloheximide decreased basal production of corticosterone from quartered rat adrenal glands, and Tsafriti *et al.* (1973) showed that protein and RNA synthesis inhibitors decreased the basal production of progesterone from rat follicles. It has been suggested that one of the roles of the protein regulator(s) is to transport substrates used for steroid synthesis to the mitochondria (Garren *et al.*, 1971). It is possible that in the testis Leydig cells there is enough of the rate-limiting substrate (possibly cholesterol; van der Molen *et al.*, 1972) within the mitochondria for this basal testosterone biosynthesis.

van der Vusse *et al.* (1974) have shown that mitochondrial preparations from rat testis interstitial tissue produce large amounts of pregnenolone and that this production can be further increased if the animals are given luteinizing hormone *in vivo* before isolation of the mitochondria. As in the present experiments it has also been shown that the luteinizing hormone-stimulated testosterone synthesis *in vivo* but not the basal synthesis was inhibited by cycloheximide injected *in vivo* (van der Vusse *et al.*, 1975).

It was found that the luteinizing hormone had no detectable effect on total protein synthesis in the interstitial tissue and Leydig-cell preparations *in vitro*. This suggests therefore that luteinizing hormone may be stimulating the synthesis of small amounts of a specific protein(s) without making a quantitatively important overall contribution to protein synthesis during incubation of this tissue *in vitro*. Whether luteinizing hormone actually stimulates the complete synthesis of this protein regulator(s) or merely activates an existing precursor as has been suggested for the steroidogenic action of adrenocorticotrophic hormone (Schulster *et al.*, 1974; Lowry & McMartin, 1974) has yet to be determined. Irby & Hall (1971) found that little or no increase in protein synthesis occurred in rat testis Leydig-cell preparations isolated from animals treated with luteinizing hormone *in*

vivo. They did, however, find that 5 days after hypophysectomy luteinizing hormone increased protein synthesis in these cells 5h after injection of the hormone, indicating that luteinizing hormone does have a long-term effect on protein synthesis. These results suggest therefore that luteinizing hormone has at least two effects on protein synthesis in the Leydig cell; a short one on the synthesis of rapidly turning over protein(s) involved in steroidogenesis and a longer effect on general protein synthesis.

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