Regulation of the Synthesis of Lutropin-Induced Protein in Rat Testis Leydig Cells

By FELIX H. A. JANSZEN, BRIAN A. COOKE, MARIA J. A. VAN DRIEL
and HENK J. VAN DER MOLEN

*Department of Biochemistry (Division of Chemical Endocrinology), Medical Faculty,
Erasmus University Rotterdam, Rotterdam, The Netherlands*

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The mechanism of action of lutropin on the stimulation of the synthesis of a specific lutropin-induced protein in rat testis Leydig cells was investigated. Lutropin-induced protein has a mol.wt. of approx. 21 000 and is detected by labelling the Leydig-cell proteins with [^{35}S]methionine, followed by separation by polyacrylamide-gel electrophoresis and radioautography of the dried gel. The incorporation of ^{35}S into lutropin-induced protein was used as an estimate for the synthesis of the protein. Incubation of Leydig cells with dibutyryl cyclic AMP or cholera toxin also resulted in the stimulation of synthesis of the protein. Synthesis of lutropin-induced protein, when maximally stimulated with 100 ng of lutropin/ml, could not be stimulated further by addition of dibutyryl cyclic AMP. Addition of 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, further increased synthesis of the protein in the presence of a submaximal dose of lutropin (10 ng/ml) but not in the absence of lutropin or with maximal amounts of lutropin (100 and 1000 ng/ml). Actinomycin D prevented the effect of lutropin on the stimulation of lutropin-induced protein synthesis when added immediately or 1 h after the start of the incubation, but not when added after 5–6 h. This is interpreted as reflecting that, after induction of mRNA coding for lutropin-induced protein, lutropin had no influence on the synthesis of the protein in the presence of actinomycin D. Synthesis of the protein was also stimulated *in vivo* by injection of choriogonadotropin into rats 1 day after hypophysectomy, and the time course of this stimulation of lutropin-induced protein synthesis *in vivo* was similar to that obtained by incubating Leydig cells *in vitro* with lutropin. From these results it is concluded that stimulation of lutropin-induced protein synthesis by lutropin is most probably mediated by cyclic AMP and involves synthesis of mRNA.

It has been shown that protein synthesis is involved in stimulation by lutropin of testosterone production in rat testis Leydig cells (Hall & Eik-Nes, 1962; Cooke *et al.*, 1975b; Mendelson *et al.*, 1975). After maximal stimulation of testosterone synthesis in rat testis Leydig cells by lutropin, addition of cycloheximide decreases testosterone production to control values. This decrease follows first-order kinetics with a half-time of 13 min, indicating that continuous synthesis of a protein(s) with short half-life is necessary (Cooke *et al.*, 1975b). Inhibitors of RNA synthesis also cause at least a partial inhibition of lutropin-stimulated production of testosterone (Cooke *et al.*, 1975a; Mendelson *et al.*, 1975). These results may indicate that the synthesis of mRNA coding for this protein(s) may also be involved in lutropin regulation of testosterone production. From previous studies in our laboratory it is known that addition of lutropin to Leydig cells stimulates the synthesis of a specific protein (lutropin-induced protein) with a mol.wt. of approx. 21 000 (Janszen *et al.*, 1976b, 1977). Lutropin-induced protein synthesis

was not influenced by addition of testosterone or follitropin to the Leydig cells (Janszen *et al.*, 1976b). Addition of actinomycin D to Leydig cells prevented the stimulation of the synthesis of this protein (Janszen *et al.*, 1976b). The half-life of this protein, however, was found to be longer than 30 min (Janszen *et al.*, 1977), and increased synthesis of lutropin-induced protein could be observed only 2 h after addition of lutropin to Leydig cells, which is a long time after stimulation of testosterone production by lutropin can be detected (5–30 min; Cooke *et al.*, 1977).

To obtain more information about the mechanism involved in the control of the synthesis of lutropin-induced protein, we have now studied the effects of dibutyryl cyclic AMP, 3-isobutyl-1-methylxanthine and actinomycin D on synthesis of the protein, because it is known that these compounds can influence testosterone production in Leydig cells (Catt *et al.*, 1972; Cooke *et al.*, 1975a). In addition, the production of lutropin-induced protein in Leydig cells after stimulation with choriogonadotropin *in*

vivo has been investigated. The results obtained are discussed in relation to the possible role of lutropin-induced protein in the regulation of steroidogenesis.

Materials and Methods

Cholera toxin was a gift from R. S. Northrup, National Institute of Allergy and Infectious Diseases, Bethesda, MD, U.S.A., and was prepared by the procedure of Finkelstein & Lospalluto (1970) under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by Dr. R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, TX, U.S.A. Sheep lutropin (NIH-LH-S18) was a gift from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A.

[³⁵S]Methionine (280 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and 3-isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Human choriogonadotropin was obtained from Organon, Oss, The Netherlands. Leydig-cell suspensions from adult rat testes were prepared and purified by centrifugation through Ficoll and dextran solutions as described previously (Janszen *et al.*, 1976a), except that testes were incubated in the collagenase solution (1 mg/ml) for 30 min instead of 18 min.

Leydig cells were incubated and proteins were labelled with [³⁵S]methionine, extracted, separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and detected by radioautography as described previously (Janszen *et al.*, 1976b), except that proteins were labelled with [³⁵S]methionine for 60 min instead of 30 min. Quantification of radioactivity in the lutropin-induced protein band was performed as described previously (Janszen *et al.*, 1977). The ratio of the density of the lutropin-induced protein band divided by the density of a protein band of mol.wt. approx. 23000 (which was shown to be unaffected by the different conditions used) was calculated. This ratio was used for a quantitative

evaluation of the lutropin-induced protein. For comparison between different experiments the amount of radioactivity in lutropin-induced protein, measured as described above, was expressed as a percentage of the amount of radioactivity in lutropin-induced protein when maximally stimulated with 100 ng of lutropin/ml.

To investigate if steroidogenesis was stimulated by the different reagents used, Leydig cells were incubated in parallel experiments for 2 h in the presence of these various reagents. After the incubation period testosterone was extracted and determined as described by Verjans *et al.* (1973).

For statistical analysis Student's *t* test for correlated data was used. Differences were considered significant when *P* < 0.025 (one-tailed).

Results

Role of cyclic AMP in the stimulation of the synthesis of lutropin-induced protein by lutropin

This was investigated by studying the effects of dibutyryl cyclic AMP, 3-isobutyl-1-methylxanthine and cholera toxin. The effect of dibutyryl cyclic AMP on the synthesis of lutropin-induced protein and testosterone was investigated in the presence and absence of maximum amounts of lutropin. The results in Table 1 show that the effect of 100 ng of lutropin/ml or of 1 mM-dibutyryl cyclic AMP on stimulation of testosterone production and lutropin-induced-protein synthesis are not significantly different from each other, and no further stimulation of synthesis of the protein was observed when these two substances were added together.

To estimate the effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine on lutropin-induced protein synthesis, the Leydig cells were first incubated for 3 h in the presence of different doses of lutropin with or without 3-isobutyl-1-methylxanthine, then [³⁵S]methionine was added and the incubation was continued for another hour. As shown in Table 2, lutropin-induced protein synthesis is significantly increased in the presence of 3-isobutyl-

Table 1. *Effect of lutropin (100 ng/ml) and dibutyryl cyclic AMP (1 mM) on synthesis of testosterone and lutropin-induced protein in testis Leydig cells*

Results are expressed as percentage of synthesis obtained in the presence of lutropin. The results for testosterone production are means $\pm$ S.E.M. for the numbers of experiments given in parentheses, and those for lutropin-induced protein synthesis are means $\pm$ S.D. n.d., Not determined. **P* < 0.005; †not significant (*P* > 0.025).

	Additions			
	None	Lutropin	Dibutyryl cyclic AMP	Lutropin + dibutyryl cyclic AMP
Testosterone production (ng/2h per 10 ⁶ cells)	5 $\pm$ 1 (12)	114 $\pm$ 16 (11)	126 $\pm$ 28 (7)	n.d.
Lutropin-induced protein synthesis	22 $\pm$ 24 (4)*	100 (4)*	100 $\pm$ 23 (4)†	100 $\pm$ 23 (4)†

1-methylxanthine with a submaximal dose of lutropin, but not in the absence of lutropin or with a maximal dose (100 and 1000ng/ml). In these experiments testosterone production in the presence of 1 ng of lutropin/ml increased from 6 ± 2 ng/2h per 10^6 cells in the absence of 3-isobutyl-1-methylxanthine to 26 ± 16 ng/2h per 10^6 cells in its presence (means $\pm$ s.d., $n = 4$, $P < 0.025$).

The Leydig cells were incubated in the presence of cholera toxin, which has previously been shown to stimulate production of both cyclic AMP and testosterone in Leydig cells in a parallel fashion (Cooke *et al.*, 1977). Leydig cells were incubated for 3h with or without lutropin or with 40 μ g of cholera toxin/ml, followed by incubation with [³⁵S]methionine for 1h. For measurement of testosterone production, Leydig cells were incubated for 2h. Table 3 shows that in the presence of cholera toxin both testosterone production and lutropin-induced protein synthesis were increased, although a lower stimulation was found compared with the effect of lutropin. It has been previously demonstrated that stimulation of production of cyclic AMP and testosterone is delayed in the presence of cholera toxin compared with the stimulation obtained in the presence of lutropin (Cooke *et al.*, 1977). To investi-

gate if there was also a delay in the stimulation of lutropin-induced protein synthesis by cholera toxin, the time course of this stimulation was investigated. With cholera toxin as well as with lutropin, the first stimulation of synthesis of the protein was observed 2h after the start of the incubation, and synthesis was maximal after about 4h (Fig. 1).

Role of mRNA synthesis in the stimulation of the synthesis of lutropin-induced protein by lutropin

The effect of actinomycin D on the stimulation by lutropin of lutropin-induced protein synthesis was

Table 2. Effect of 0.25 mM-3-isobutyl-1-methylxanthine on lutropin-induced protein synthesis in Leydig cells in the presence of different doses of lutropin

Results are expressed as percentage of lutropin-induced-protein synthesis, in the presence of 100ng of lutropin, and are means $\pm$ s.d. for the numbers of experiments in parentheses. N.S., Not significant ($P > 0.025$).

Lutropin (ng/ml)	Lutropin-induced protein synthesis		Effect of inhibitor
	-Inhibitor	+Inhibitor	
0	14 $\pm$ 22	15 $\pm$ 10 (4)	N.S.
1	14 $\pm$ 22	34 $\pm$ 26 (4)	N.S.
10	35 $\pm$ 23	80 $\pm$ 16 (3)	$P < 0.005$
100	100	89 $\pm$ 12 (3)	N.S.
1000	95 $\pm$ 9	89 $\pm$ 23 (3)	N.S.

Fig. 1. Time course of lutropin-induced protein synthesis in the presence of lutropin or cholera toxin

Leydig cells were incubated for different time periods in the absence ($\blacktriangle$) or presence of lutropin ($\blacksquare$) or cholera toxin ($\bullet$). [³⁵S]Methionine was then added and the incubation was continued for 1h. Total cell protein was extracted and separated by electrophoresis and the amount of radioactivity in the lutropin-induced protein band was measured as described in the Materials and Methods section. Lutropin-induced protein synthesis is expressed as a percentage of that 5h after addition of lutropin.

Table 3. Effect of cholera toxin on testosterone production and lutropin-induced protein synthesis in Leydig cells
Lutropin-induced protein synthesis is expressed as a percentage of that in the presence of lutropin (100ng/ml).

	Lutropin-induced protein synthesis		
	Control	+Lutropin	+Cholera toxin
Testosterone production (ng/2h per 10^6 cells)*	4 $\pm$ 1	141 $\pm$ 35	45 $\pm$ 14
Lutropin-induced protein synthesis†	4 $\pm$ 5	100	80 $\pm$ 18‡

* Means $\pm$ s.d. for four different experiments.
† Means $\pm$ s.d. for six different observations in four different experiments.
‡ $P < 0.005$ compared with control.

Fig. 2. Effect of actinomycin D on lutropin-induced protein synthesis

Actinomycin D was added at different times after the start of incubation in the presence of lutropin. Leydig cells were incubated with lutropin (100 ng/ml) for different time periods, then actinomycin D (46 μ M) was added and the incubations were continued. The total incubation time was 8 h. [35 S]Methionine was then added and the incubation was continued for another hour. Lutropin induced-protein synthesis is expressed as a percentage of that in Leydig cells incubated for 9 h with lutropin and without actinomycin D.

investigated at different times after the start of the incubation. Stimulation of the synthesis of this protein was prevented completely when actinomycin D was added immediately or 1 h after the start of the incubation. Inhibition became progressively less when actinomycin D was added at later times until no inhibition was detectable when actinomycin D was added 4–5 h after the start of the incubation with lutropin (Fig. 2). The stability of mRNA was investigated in Leydig cells that were incubated with dibutyryl cyclic AMP for 3 h to stimulate lutropin-induced protein synthesis. The synthesis of lutropin-induced protein in the control cells was $5 \pm 6\%$ of that in the dibutyryl cyclic AMP-stimulated Leydig cells ($=100\%$) (mean $\pm$ s.d. for five experiments). The dibutyryl cyclic AMP was then removed from the cells by washing with the incubation buffer followed by centrifugation (twice) and the incubation was then continued for 3 h in the presence of actinomycin D with or without lutropin. After this period [35 S]-methionine was added and the incubation was continued for another hour. In this case the production of lutropin-induced protein in the Leydig cells that were incubated during the last 4 h with or without lutropin was $106 \pm 38\%$ and $91 \pm 25\%$ respectively of the synthesis of lutropin-induced protein in Leydig cells only incubated for 4 h with

dibutyryl cyclic AMP (mean $\pm$ s.d. for five experiments). In separate experiments it was demonstrated that with this washing procedure testosterone production in Leydig cells preincubated for 3 h in the presence of dibutyryl cyclic AMP fell to basal values after removal of dibutyryl cyclic AMP (7 ± 6 and 6 ± 3 ng of testosterone/2 h per 10^6 cells respectively for washed cells and control cells, mean $\pm$ s.d., $n = 3$).

Lutropin-induced protein synthesis in Leydig cells stimulated *in vivo* with choriogonadotropin

To investigate if lutropin-induced protein synthesis could be stimulated also *in vivo*, choriogonadotropin (100 i.u.*) was injected into the tail vein of rats 1 day after hypophysectomy. From previous work it was known that this amount of choriogonadotropin would stimulate testosterone secretion *in vivo* (de Jong *et al.*, 1973). Control rats were injected with 0.9% NaCl 3 h before decapitation. After decapitation Leydig cells were prepared and incubated *in vitro* for 3 h with or without lutropin in the presence or absence of actinomycin D. [35 S]Methionine was then added and the incubation was continued for another 1 h. Testosterone production in the presence or absence of lutropin was measured in parallel incubations (Table 4). In control rats lutropin-induced protein synthesis increased in the presence of lutropin, and this increase was prevented by addition of actinomycin D to the cells (Table 5 and Fig. 3). From Leydig cells of choriogonadotropin-injected rats a prominent 35 S-labelled protein with the same molecular weight as lutropin-induced protein was isolated (Fig. 3). Incorporation of 35 S into this

* One i.u. is defined as the activity in a bioassay of 0.001279 mg of the Second International Standard (1963) (Biological Substances International Standard and Reference Preparations, World Health Organization, Geneva, 1975).

Table 4. Effect of choriogonadotropin on testosterone production in Leydig cells from 1-day-hypophysectomized rats

	Testosterone production (ng/2 h per 10^6 cells)	
	–Lutropin	+Lutropin
Control rats	6 ± 3	263 ± 65
Choriogonadotropin-treated rats	57 ± 17	53 ± 7

Fig. 3. Effect of injection of human choriogonadotropin on lutropin-induced protein synthesis by Leydig cells in vitro. Rats, 1 day after hypophysectomy, were injected with 100i.u. of human choriogonadotropin (b) or with 0.9% NaCl (control, a) 3 h before decapitation. Leydig cells were prepared and incubated for 3 h in the absence or presence of lutropin (100ng/ml) and in the absence or presence of actinomycin D. Then [³⁵S]methionine was added and the incubation was continued for 1 h. After extraction total cell protein was separated on a 10–15% discontinuous sodium dodecyl sulphate/polyacrylamide gel. Only the radioautogram of the 15% part of the gel is shown. Standards used for the calculation of molecular weights were: phosphorylase a (mol.wt. 93 000); catalase (mol.wt. 60 000); ovalbumin (mol.wt. 43 000); chymotrypsinogen (mol.wt. 25 700); cytochrome c (mol.wt. 11 700).

Table 5. Effect of choriogonadotropin on lutropin-induced protein synthesis in Leydig cells from 1-day-hypophysectomized rats. Rats were injected 3 h before decapitation with 0.9% NaCl or with 100i.u. of choriogonadotropin. Synthesis of lutropin-induced protein is expressed as percentage of synthesis in cells incubated *in vitro* with lutropin (100ng/ml), without actinomycin D. Results are given as means $\pm$ s.d. for three experiments.

	Synthesis of lutropin-induced protein			
	–Lutropin		+Lutropin	
	Actinomycin D		Actinomycin D	
	–	+	–	+
Control rats	14 $\pm$ 12	7 $\pm$ 7	100	10 $\pm$ 12
Choriogonadotropin-treated rats	91 $\pm$ 23	98 $\pm$ 34	100	106 $\pm$ 6

protein was not affected by either lutropin or actinomycin D added during incubation of the cells (Table 5).

In other experiments, rats that had been hypophysectomized for 1 day were injected with 100i.u. of choriogonadotropin at various times before decapitation. Leydig cells were prepared and then incubated *in vitro* as described above. In the cells isolated 3 h after injection of choriogonadotropin, again maximal incorporation of ³⁵S was obtained in a protein band of mol.wt. 21 000. This was observed in incubations with and without added lutropin and was unaffected by the presence of actinomycin D. When rats were injected with choriogonadotropin 1 and 2 h before

decapitation, addition of actinomycin D to the isolated cells resulted in a decrease in ³⁵S incorporation into this protein band (Table 6).

Discussion

In the present study it has been demonstrated that dibutyryl cyclic AMP, cholera toxin and 3-isobutyl-1-methylxanthine can stimulate the synthesis of lutropin-induced protein in testis Leydig cells. These results may support a possible role of cyclic AMP in the stimulation of synthesis of this protein, because it has been demonstrated that cyclic AMP production in Leydig cells is stimulated by lutropin (see reviews by

Table 6. *Lutropin-induced protein synthesis in isolated Leydig cells from rats injected with human choriogonadotropin at different times before decapitation*

Synthesis of lutropin-induced protein in the presence of lutropin was taken as 100% and the synthesis of lutropin-induced protein in the presence of actinomycin D was expressed as a percentage of the synthesis in the presence of lutropin. Results are means $\pm$ range for two determinations.

Substance injected	Time before decapitation (h)	Synthesis of lutropin-induced protein
0.9% NaCl	3	11 $\pm$ 3
Choriogonadotropin	1	26 $\pm$ 10
	2	58 $\pm$ 2
	3	91 $\pm$ 19

Rommerts *et al.*, 1974; Catt & Dufau, 1976; Marsh, 1976) and by cholera toxin (Cooke *et al.*, 1977). Phosphodiesterase inhibitors, such as 3-isobutyl-1-methylxanthine or theophylline, have been shown to potentiate lutropin action most probably by inhibition of the degradation of cyclic AMP by phosphodiesterase (Catt *et al.*, 1974). Previously it has been shown that addition of actinomycin D to Leydig cells at the start of the incubation prevents stimulation of lutropin-induced protein synthesis by lutropin (Janzsen *et al.*, 1976b). From the lack of effect of actinomycin D on the lutropin-stimulated synthesis of lutropin-induced protein, when added to the Leydig cells 5 h after the start of the incubation (Fig. 2), it may be concluded that actinomycin D does not interfere with the synthesis of this protein as such. Hence, the inhibitory effect of actinomycin D on lutropin stimulation of the synthesis of this protein, when added earlier, may be explained by prevention of the accumulation of mRNA coding for lutropin-induced protein. This accumulation of mRNA may result from either stabilization of existing mRNA in the presence of lutropin or increased synthesis of mRNA. The present results demonstrate that after stimulation with dibutyl cyclic AMP the synthesis of lutropin-induced protein remains elevated for several hours after removal of the dibutyl cyclic AMP. This could indicate that under these conditions no marked degradation of mRNA occurs. However, these experiments were performed in the presence of actinomycin D in order to prevent new mRNA synthesis and secondary effects of this inhibitor on the synthesis of lutropin-induced protein cannot be excluded. Therefore more research is needed to determine the stability of the mRNA in the presence and in the absence of the hormone.

When hypophysectomized rats were injected with choriogonadotropin 3 h before decapitation no

subsequent inhibition of lutropin-induced protein synthesis occurred in the presence of actinomycin D. This indicates that in this situation synthesis of this protein was already maximally stimulated before addition of the actinomycin D. Testosterone production by Leydig cells from choriogonadotropin-treated rats could not be stimulated further by addition of lutropin to the isolated cells *in vitro*. However, stimulation of Leydig cells *in vitro* with dibutyl cyclic AMP and subsequent removal of the dibutyl cyclic AMP resulted in a low basal testosterone production. These results may be explained by assuming that choriogonadotropin, injected *in vivo*, remains bound to its receptor after isolation of the Leydig cells. Survival of the lutropin-receptor complex after washing Leydig cells by centrifugation has been described by Moyle & Ramachandran (1973). Hsueh *et al.* (1976) demonstrated occupancy of receptors by endogenous lutropin after preparation of Leydig cells. To study the time course of induction of lutropin-induced protein synthesis *in vivo*, actinomycin D was added to the Leydig cells from animals stimulated *in vivo* to prevent further stimulation of lutropin-induced protein. Under these conditions only a small stimulation of lutropin-induced protein synthesis was found in Leydig cells from rats injected with choriogonadotropin 1 h before decapitation (the total time of stimulation was 2.5 h; 1 h *in vivo* and 1.5 h during cell preparation). Maximum stimulation of lutropin-induced protein synthesis was observed when the Leydig cells were exposed to choriogonadotropin for 4.5 h. It may be concluded therefore that the time course of stimulation of lutropin-induced protein synthesis *in vivo* and *in vitro* are very similar.

To summarize, the present evidence indicates that the synthesis of both lutropin-induced protein and testosterone is regulated by similar mechanisms involving cyclic AMP and mRNA under the control of lutropin. However, it is still difficult to conclude that lutropin-induced protein is obligatorily involved in lutropin stimulation of testosterone biosynthesis, because a long time (2 h) is needed after lutropin addition before increased synthesis of the protein is detectable compared with 5–30 min for stimulation of testosterone production. Thus maximum testosterone synthesis can occur in the presence of low lutropin-induced protein synthesis. Therefore, if lutropin-induced protein is involved in steroid production, it may not be the only lutropin-induced factor; theoretically the protein could even be an inhibitor of steroidogenesis. It is also possible that stimulation of lutropin-induced protein synthesis is unrelated to steroidogenesis and that it is only one of the first trophic effects of lutropin on Leydig cells irrespective of steroid production. These questions cannot be answered at the moment and must await further studies with purified lutropin-induced protein.

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