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LUTROPIN STIMULATES *DE NOVO* SYNTHESIS OF SHORT-LIVED PROTEINS REQUIRED FOR LUTROPIN-DEPENDENT STEROID PRODUCTION IN TUMOUR LEYDIG CELLS

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Summary—Continuous protein synthesis is required for the hormonal regulation of cholesterol side chain cleavage activity. A protein with a short half-life ($t_{1/2} = 2$–13 min) is believed to play an important role, but the regulation of the synthesis of this putative rapidly-turning-over protein is largely unknown. The steroid production rate in tumour Leydig cells can be increased more than 4-fold after addition of lutropin. However, steroid production by cells preincubated for 60 min with medium containing cycloheximide (89 μM) could not be stimulated when lutropin was added to the medium. Thus, the putative protein with the short half-life is apparently not derived from a stable precursor protein. Moreover, in tumour Leydig cells incubated with low concentrations of cycloheximide (0.2–0.8 μM), inhibition of steroid production was significantly greater in lutropin-stimulated cells than in control cells. These results support the hypothesis that lutropin regulates the *de novo* synthesis of rapidly-turning-over proteins by increasing the rate of initiation of the translation step of protein synthesis.

INTRODUCTION

Short-term hormonal stimulation of steroid production in steroidogenic cells requires continuous protein synthesis. The stimulatory effects of tropic hormones on such (testicular, ovarian, adrenal) cells decay within a few minutes after administration of cycloheximide [1–6]. In this regard several investigators postulated the hormone-dependent synthesis of rapidly-turning-over proteins which are directly or indirectly involved in the mitochondrial cholesterol side-chain cleavage enzyme activity. The estimated half-lifes for such proteins in testis Leydig cells [3] and tumour Leydig cells [5] were 13 and 6 min respectively.

Studies with rat Leydig cells by Janszen *et al.*[7] and with rabbit ovarian cells by Losier and YoungLai[8] and YoungLai and Osoko[9] failed to demonstrate the synthesis of a rapidly-turning-over protein. In Leydig cells Janszen *et al.*[7] demonstrated the presence of a cycloheximide-sensitive protein of 33,000 Da with a half-life of approx. 11 min, but its synthesis was not controlled by lutropin, although a lutropin-induced protein could be demonstrated 2 h after the addition of lutropin.

Experiments with actinomycin D [1, 8, 10, 11] showed that acute effects of corticotropin or lutropin on mRNA synthesis are not required for increased steroid production. Therefore, the observation of rapidly-turning-over (or labile) proteins might reflect either regulation of *de novo* protein synthesis, or metabolism of stable precursor proteins. Kinetic experiments with Leydig cells preincubated for various time periods with cycloheximide suggested that lutropin stimulated the production of a labile protein by conversion of a stable (inactive) protein into an unstable (active) protein [12]. Recently, it was shown that hormones or cyclic AMP can also stimulate the production of specific proteins via an effect on *de novo* protein synthesis (rat hepatoma cells, 13; rat adrenal cells, 14).

In the present study we have investigated whether labile proteins produced in tumour Leydig cells are derived from either conversion of a stable regulator protein, or *de novo* protein synthesis.

EXPERIMENTAL

Materials and methods

Chemicals used, procedures for isolation of tumour Leydig cells, incubation conditions for incorporation of ^{3}H and ^{14}C-labelled amino acids into proteins, and the procedure for estimation of pregnenolone production have been described previously [5, 15].

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Trivial names: Lutropin, LH, luteinizing hormone; adrenocorticotropin, ACTH, adrenocorticotropic hormone; dibutyryl cyclic AMP, (N^6-2′-*O*-dibutyryl) adenosine cyclic-3′,5′-monophosphate.

Lutropin (LH-NIH-S20, 1.9 I.U. NIH-S1/mg) was a gift from the National Institute of Health, Endocrinology Study Section, Bethesda, U.S.A.

Leydig cells were preincubated at 32°C for 1 h and during this period Leydig cells attached to the plastic dish. In all experiments $1–2 \times 10^6$ attached tumour Leydig cells were used. Stimulation of tumour Leydig cells was performed with a solution containing lutropin (1000 ng/ml) and 3-isobutyl-1-methylxanthine (0.25 mM).

RESULTS AND DISCUSSION

Hormonal stimulation of steroid production in Leydig cells may involve transformation of an inactive, stable precursor protein into an active protein with a short half-life required for cholesterol side-chain cleavage. Owing to the proposed stability of this regulator protein, preincubation of cells with cycloheximide should not significantly reduce the initial effect of lutropin on steroidogenesis [12]. To test this hypothesis, pregnenolone production was measured during consecutive 10 min periods in cells incubated each time with fresh medium. With this approach the determination of lutropin-induced changes in steroid production is more accurate than in static incubations. The results in Fig. 1A show that the rate of pregnenolone production by cells preincubated for 60 min with cycloheximide was not increased by lutropin/3-isobutyl-1-methylxanthine in the continued presence of cycloheximide. No transient steroid response could be observed. The marginal increase in steroid production which was observed occasionally during incubation with lutropin/3-isobutyl-1-methylxanthine and cycloheximide was not caused by lutropin or 3-isobutyl-1-methylxanthine, since the same small increase was observed in the presence of cycloheximide alone (data not shown). These results indicate no evidence for the presence of a stable precursor protein which is required for lutropin-stimulation of steroidogenesis. In addition, the absence of lutropin-stimulation of steroid production in the presence of cycloheximide cannot be explained by aspecific toxic effects of cycloheximide, because after removal of cycloheximide and incubation of cells with lutropin/3-isobutyl-1-methylxanthine, steroid production could be stimulated more than 20-fold indicating that the cells were still highly active (Fig. 1B). This is in support of results by Mason and Robidoux[16] in showing no adverse effects on steroid production due to previous incubation with cycloheximide. Moreover, non-specific effects of cycloheximide apart from inhibition of protein synthesis appear unlikely in view of the concentrations of cycloheximide used [see: 16, 17].

Cooke *et al.*[12] have reported that lutropin-dependent steroid production in rat Leydig cells was not affected by previous incubation with cycloheximide, intended to remove putative rapidly-turning-over proteins. However, in their experiments Cooke *et al.*[12] employed a washing procedure (at 4°C) to remove cycloheximide prior to incubation of cells with lutropin, which did not eliminate possible resynthesis of rapidly-turning-over proteins during the warming-up period and during incubation with lutropin. In the present experiments cylcoheximide was present continuously, thus continuously abolishing protein synthesis [cf. Bakker *et al.*, 5]. The absence of an effect of lutropin on steroid production under these conditions strongly suggests that stable

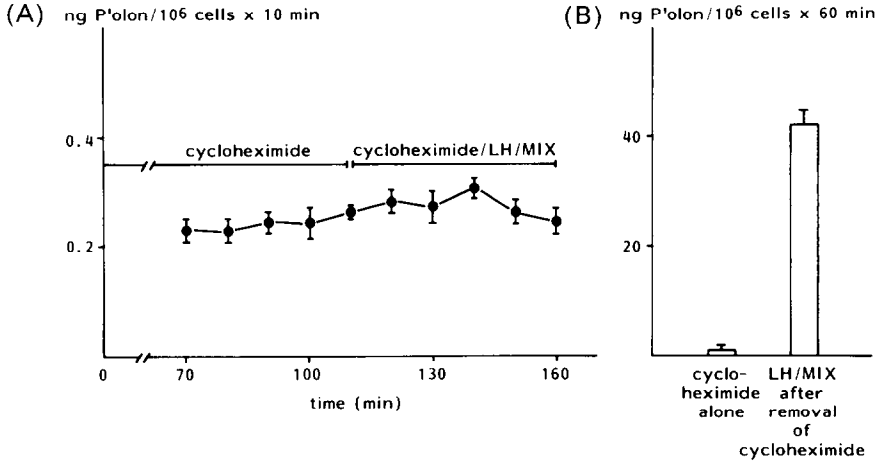

Fig. 1. Effects of lutropin/3-isobutyl-1-methylxanthine and cycloheximide (89 μM) on pregnenolone production of tumour Leydig cells. *A* Cells were incubated in Petri dishes in the presence of cycloheximide and lutropin/3-isobutyl-1-methylxanthine (LH/MIX), as indicated. Pregnenolone production was determined in media collected in 10 min periods with fresh medium. The effects of addition of lutropin/3-isobutyl-1-methylxanthine was not significant. *B* Pregnenolone production in cells after washing (three times) with fresh medium to remove cycloheximide and addition of lutropin/3-isobutyl-1-methylxanthine, or in cells in the continued presence of cycloheximide. Results shown are means ± SD ($n = 3$).

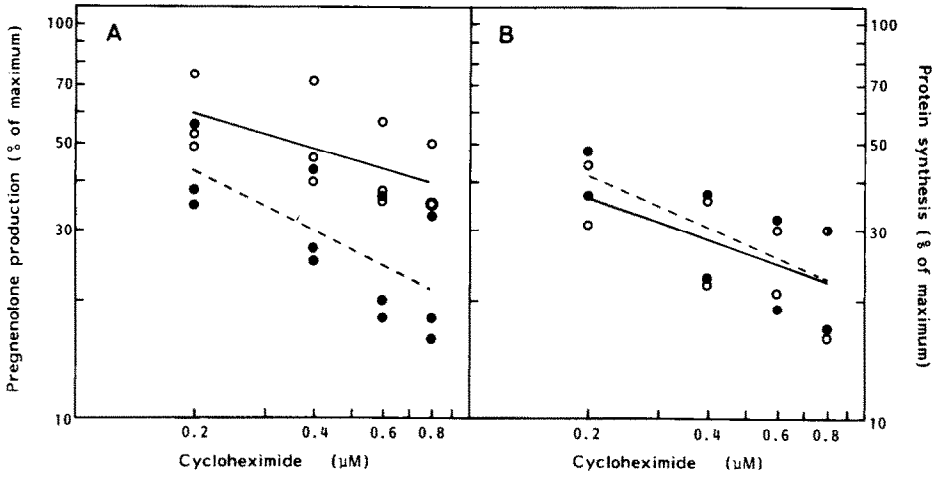

Fig. 2. Effects of low concentrations of cycloheximide on pregnenolone production (A) and incorporation of ^{14}C-labelled amino acids into proteins (B) in tumour Leydig cells. Tumour Leydig cells were preincubated for 1 h and incubated with various concentrations of cycloheximide with (dashed lines, ●) or without (solid lines, ○) lutropin/3-isobutyl-1-methylxanthine. Incubations were carried out: *A* for 60 min for estimation of pregnenolone production; *B* for 30 min with ^{14}C-labelled amino acids. Results are expressed as the percentage of the activity in the absence of cycloheximide. Mean values of duplicate incubations from three (A) or two (B) different cell preparations are shown. Inhibition of lutropin-dependent pregnenolone production was significantly higher than inhibition of control pregnenolone production ($P < 0.005$). Lines drawn in panel A do not run in parallel ($P < 0.01$). One hundred percent values: *A* control, 53.1 ± 4.9 (6) ng h^{-1}.mg $protein^{-1}$; stimulated, 453.3 ± 58.1 (6) ng h^{-1}.mg $protein^{-1}$. *B* control and stimulated (cf. ref. 5), 250.9 ± 27.4 (4) dpm. 30 min^{-1}.mg $protein^{-1}$ in Expt 1; 776.0 ± 65.3 (4) dpm. 30 min^{-1}.mg $protein^{-1}$ in Expt 2. Results are means ± SD (*n*).

precursor proteins are of minor importance for lutropin-dependent steroidogenesis (Fig. 1A).

Recently, Snoek *et al.*[13] showed that dibutyryl cyclic AMP can increase the rate of initiation of translation of the mRNA coding for tyrosine aminotransferase in rat hepatoma cells, without stimulation of general protein synthesis. This specific effect of dibutyryl cyclic AMP on the rate of initiation could also be inferred from investigations on the effects of low concentrations of cycloheximide on synthesis of tyrosine aminotransferase. Cycloheximide inhibits the elongation step of translation [18]. Inhibition of protein synthesis in the presence of cycloheximide occurs when the rate of elongation becomes smaller than the rate of initiation, and an apparently greater inhibition of protein synthesis will be observed when at the same low concentration of cycloheximide the rate of initiation of translation is increased, e.g. by hormonal stimulation. Snoek *et al.*[13] showed that relative to control synthesis, the inhibition of dibutyryl cyclic AMP-stimulated synthesis of tyrosine aminotransferase was higher with low concentrations of cycloheximide (0.06–3 μM).

Lutropin-stimulated steroid production can be envisaged as the consequence of direct of indirect stimulation of the cholesterol side-chain cleavage enzyme activity by the putative rapidly-turning-over protein. The degree of stimulation of steroid production may reflect therefore specifically the steady-state concentration of the rapidly-turning-over protein in the cell. In view of this, the effects of low concentrations of cycloheximide on steroid production and general protein synthesis were investigated. Incubation of tumour Leydig cells with low concentrations of cycloheximide resulted in a significantly higher inhibition of lutropin-stimulated pregnenolone production when compared to pregnenolone production by cells without lutropin/3-isobutyl-1-methylxanthine (Fig. 2A). However, there was no difference in inhibition of general protein synthesis in control and stimulated cells (Fig. 2B). In analogy with the results described by Snoek *et al.*[13], the significantly higher inhibition of lutropin/3-isobutyl-1-methylxanthine-dependent pregnenolone production by cycloheximide may indicate, although indirectly, increased initiation of translation of a specific mRNA coding for a rapidly-turning-over protein [see also: 19]. The increase in the extent of inhibition of lutropin-dependent pregnenolone production with increasing concentrations of cycloheximide is significantly higher than the increase in the extent of inhibition of unstimulated pregenolone production. It has been shown, that the relative effect of cycloheximide on translation-controlled synthesis of a single, specific protein under control and stimulated conditions is the same [13; see also: 20, 21]. Hence, the aberrant effects of cycloheximide on pregnenolone production may indicate that lutropin influences the *de novo* synthesis of more than one specific protein required for steroid production. Support for this hypothesis can be derived from experiments with adrenal cells [14]. In these experiments [14] it was shown that adrenocorticotropin could rapidly stimulate the synthesis

of one protein, and inhibit the synthesis of another protein which appeared not to be a precursor for the former protein.

Additional experiments, such as isolation of the protein(s) from stimulatory cytosol fractions [cf. 22, 23, 24] are required for further elucidation of the mechanisms of the hormonal control of cholesterol side-chain cleavage activity in Leydig cells.

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