ROLE OF CYCLIC AMP IN STEROIDOGENESIS IN LEYDIG CELLS: 'DISCREPANCIES' BETWEEN EFFECTS OF LUTEINIZING HORMONE AND CHOLERA TOXIN

B. A. COOKE, L. M. LINDH and F. H. A. JANSZEN

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

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1. Introduction

The mechanisms by which luteinizing hormone (LH) controls steroidogenesis in testis Leydig cells are not well defined. The available evidence suggests that in similarity with the adrenal gland, activation of adenylate cyclase [1–3], protein kinase [3,4] and protein synthesis [5–7] are involved. However, in contrast to the adrenal gland, steroidogenesis in trophic hormone (LH, hCG)-stimulated Leydig cells has been shown:

(1) To proceed more slowly (20–30 min is required before increases in steroidogenesis can be detected [2,8,9] (compared with 24 s – 3 min in the adrenal gland [10]).

(2) To be maximal without increases in cyclic AMP being detectable [1]. These data suggest that additional or different mechanisms not involving cyclic AMP may be operating in the Leydig cell.

In the present study the kinetics of cyclic AMP and testosterone production in LH-stimulated Leydig cells have been reinvestigated and compared with the effects of cholera toxin. The latter has previously been shown to mimic the action of hormones at their target cells through stimulation of cyclic AMP production [11–15] and thus is a useful tool in elucidating the role of this nucleotide in hormone action.

It has been found that by preincubating Leydig cells before addition of LH a rapid increase in testosterone can be obtained. After stimulation of testis Leydig cells with cholera toxin, in contrast to LH, cyclic AMP production was increased at all dose levels of the toxin which increased testosterone production. These results are discussed in relation to the mode of action of LH.

Address correspondence to: B. A. Cooke, Department of Biochemistry II, Medical Faculty, Erasmus University Rotterdam, P.O. Box 1738, Rotterdam, The Netherlands

2. Materials and methods

Cholera toxin was a gift from R. S. Northrup National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA and was prepared according to the procedure of Finkelstein and Lospalluto [16] under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, Texas, USA.

Ovine FSH (NIH-FSH-S9, 1 unit/mg) and ovine LH (NIH-LH-S18, 1 unit/mg) were gifts from the Endocrinology Study Section, National Institute of Health, Bethesda, Maryland, USA.

Leydig cell suspensions from rat testes were prepared, purified by centrifugation through Ficoll and Dextran solutions and incubated as previously described [8]. Cyclic AMP and testosterone were determined by saturation analysis [17,18] and protein kinase was assayed in Leydig cells incubated for 40 min at 32°C as previously described [3,4]. All incubations were carried out in the presence of 0.25 mM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity.

Table 1
Effect of preincubation on the time course of LH stimulation of testosterone production in Leydig cells

Time of incubation (min)	Testosterone production (ng/10^6/2 h)					
	Without preincubation			With preincubation (2 h)		
	Control	+ LH (100 ng/ml)	Effect of LH	Control	+ LH (100 ng/ml)	Effect of LH
5	0.06 ± 0.08	0.09 ± 0.14	N.S.	0.24 ± 0.36	1.10 ± 1.19	$P < 0.05$
10	0.09 ± 0.13	0.36 ± 0.53	N.S.	1.01 ± 0.84	3.47 ± 1.98	$P < 0.005$
20	0.25 ± 0.20	1.60 ± 1.94	N.S.	2.28 ± 0.92	9.27 ± 4.62	$P < 0.005$
30	0.77 ± 0.29	3.56 ± 2.45	$P < 0.05$	–	–	–
120	4.05 ± 1.19	83.60 ± 38.80	$P < 0.005$	6.36 ± 3.52	82.91 ± 38.83	$P < 0.005$

Results given are means ± S.D. (n = 6) obtained from 3 different experiments. In each experiment the Leydig cells were either incubated immediately (± LH) after preparation or preincubated for 2 h at 32°C and then further incubated (± LH) for 2 h without changing the incubation medium. N.S. = not significant (i.e. $P > 0.05$).

3. Results

3.1. *Time course of cyclic AMP and testosterone production*

It was found that by preincubating the Leydig cells for 2 h at 32°C before the addition of LH (100 ng/ml, a maximum stimulating dose [3]) increased production could be detected within 5 min (table 1) compared with 30 min for the non-preincubated cells. The production of testosterone during 2 h incubation was the same in both preincubated and non-preincubated cells. All further experiments carried out in this study were with cells preincubated for 2 h.

Cholera toxin (2 μg/ml) was found to stimulate both cyclic AMP and testosterone production in the Leydig cells (figs.1 and 2). There was a delayed response to the cholera toxin; it was 20 min before increased cyclic AMP could be detected, which is in contrast to the rapid increase caused by LH. In three experiments carried out it was found that similar amounts of cyclic AMP were formed during 3 h incubation with maximum amounts of LH and cholera toxin. There was a time delay of approximately 40 min before a stimulation of testosterone production could be detected in the presence of cholera toxin (fig.2). After 3 h similar amounts of testosterone were formed during incubations with cholera toxin and LH.

3.2. *Effect of varying the amounts of cholera toxin*

The effects of different amounts of cholera toxin were also investigated and the results of a representative experiment are given in fig.3. With 0.002 μg/ml

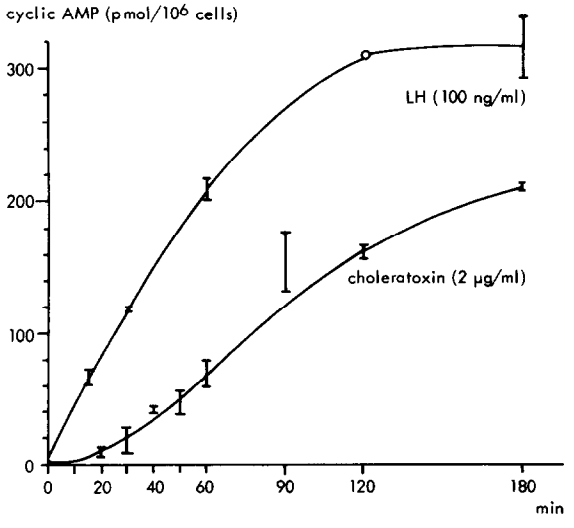

Fig.1. Time study of effect of cholera toxin and LH on cyclic AMP production in Leydig cells. Suspensions of Leydig cells (3 × 10^6/ml) obtained from 8 rat testes were incubated for the times indicated at 32°C in a Krebs Ringer bicarbonate buffer, pH 7.4, containing 0.2% glucose and 0.25 mM 3-isobutyl-1-methylxanthine with LH (100 ng/ml) or cholera toxin (2 μg/ml). Results given are means and ranges from duplicate incubations.

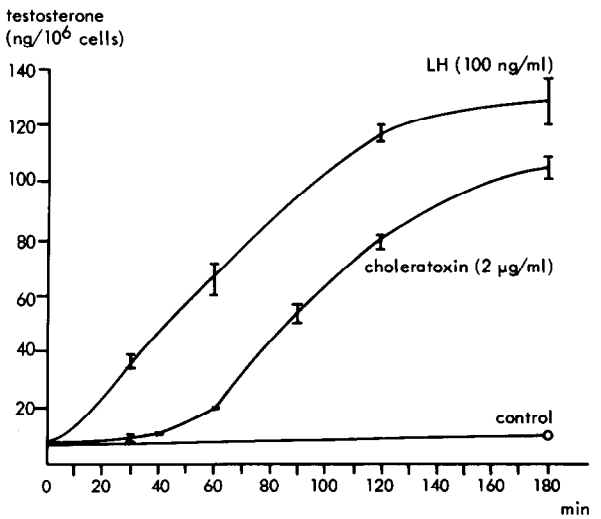

Fig.2. Time study of effects of choleratoxin and LH on testosterone production in Leydig cells. See legend to fig.1 for experimental details.

no detectable changes were found in any of the parameters measured. With 0.02 μg/ml protein kinase activation and cyclic AMP (but not testosterone) production were stimulated. Protein kinase activation reached a maximum with 2.0 μg/ml. With 0.2 μg/ml and larger amounts of cholera toxin, testosterone and cyclic AMP production increased in parallel. Maximum productions during 2 h incubation were not reached in the experiment shown in fig.3 with the amounts of cholera toxin used, but in other incubations carried out for 3 h maximum productions were obtained with 2.0 μg/ml.

3.3. *Specificity of the effects of cholera toxin*

The only other cell known to produce cyclic AMP in the testis is the Sertoli cell, which responds to FSH [19–21]. However, FSH at doses up to 1000 ng/ml was found to have no effect on cyclic AMP production. It is concluded that no Sertoli cells were present in the Leydig cell preparation. Erythrocytes at the same concentration present in the Leydig cell preparation were found not to form cyclic AMP during 3 h incubation with cholera toxin (2 μg/ml). Thus it is probable that cholera toxin specifically increases cyclic AMP in Leydig cells.

4. Discussion

The data presented in this communication demonstrate that detectable increases in testosterone after LH stimulation occur within 5 min if the cells are preincubated for 2 h before addition of LH. The

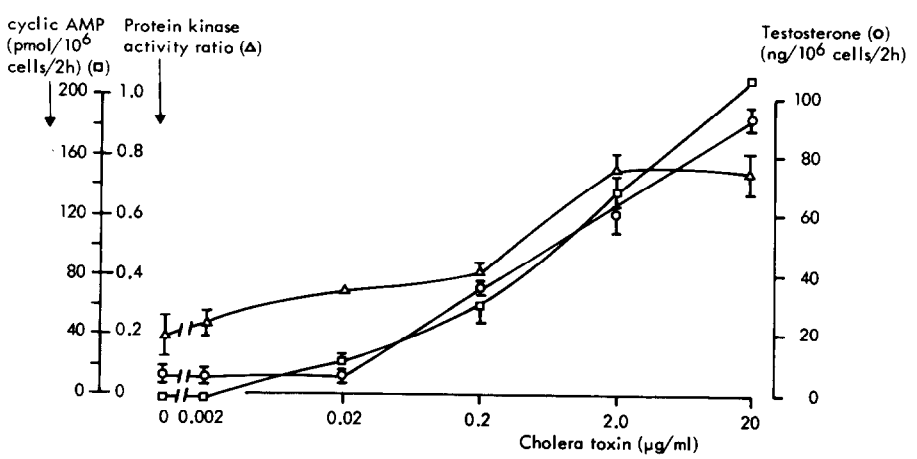

Fig.3. Dose–response curves for effects of cholera toxin on protein kinase activation, cyclic AMP and testosterone production in Leydig cells. Suspensions of Leydig cells (3×10^6/ml) obtained from 8 rat testes were incubated at 32°C in a Krebs Ringer bicarbonate buffer, pH 7.4, containing 0.2% glucose and 0.25 mM 3-isobutyl-1-methylxanthine with different amounts of cholera toxin for 40 min for protein kinase assay (Δ) and 2 h for cyclic AMP (□) and testosterone assay (○). Results given are means and ranges from duplicate incubations. The degree of protein kinase activation is expressed as the protein kinase activity ratio (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP).

reasons for this are not known but it may be that time is required for the synthesis of factor(s) lost during preparation of the tissue and cells.

The effect of cholera toxin on Leydig cells with regard to the time course of production of cyclic AMP and testosterone and the superimposable and parallel dose response curves for cyclic AMP and testosterone are very similar to the data reported for the effect of cholera toxin on the adrenal cell [12,13]. However, the difference found was that the total amount of cyclic AMP formed after stimulation of the Leydig cells with cholera toxin were comparable with that formed by LH whereas in the adrenal cell the maximum production of cyclic AMP after cholera toxin stimulation was only 10% or less that stimulated by ACTH.

The results obtained in the present study disagree with those previously reported by Sato et al. [15] using testis slices. These authors found that approximately 3 times more cyclic AMP was formed after cholera toxin than with trophic hormone (hCG) and also that testosterone production could be stimulated with low amounts of cholera toxin without being able to detect any changes in cyclic AMP production. These results may well be due to the presence of other testis cell types and the low percentage of Leydig cells present in testis slices.

The data obtained with cholera toxin and LH might reflect that cyclic AMP is involved in LH action but that at low levels of LH there is another factor involved in stimulating steroidogenesis in addition to cyclic AMP. However, there are several factors which argue against this. With cholera toxin there was a delay of approximately 20 min between the detection of increases in cyclic AMP production and subsequent testosterone production; this is inconsistent with the much shorter times required for cyclic AMP and testosterone production after LH stimulation (less than 5 min).

This raises the question of the availability of the cyclic AMP produced after stimulation with cholera toxin to the steroidogenic system, i.e., is the cyclic AMP formed in a compartment of the cell which is not immediately accessible and if so how much of the cyclic AMP formed eventually does reach the steroidogenic system? The amounts measured may therefore be far in excess of those actually involved in stimulating steroidogenesis.

Indirect evidence indicates that the apparent lack of cyclic AMP production with low amounts of LH may well be due to the inability of the methods available to detect small changes in cyclic AMP production. For example, theophylline (an inhibitor of cyclic AMP metabolism) potentiates the stimulating effect of hCG at levels of the hormone where changes in cyclic AMP are undetectable [1]. In addition cyclic AMP dependent protein kinase activation is detectable with all amounts of LH which stimulate testosterone synthesis in Leydig cells [3]. Further preliminary studies (unpublished) indicate that phosphorylation of endogenous protein substrates in the Leydig cell after LH stimulation also closely correlate with increases in testosterone synthesis.

To conclude, with respect to the time course of cyclic AMP and steroid production after stimulation with trophic hormone and cholera toxin, the Leydig cell has now been shown to be similar to the adrenal cell. The apparent discrepancy between the effects of cholera toxin and LH are most probably a result of the lack of sensitivity of the methods for detecting cyclic AMP after stimulation with low amounts of LH, and compartmentalization of the cyclic AMP produced by cholera toxin. These data are therefore not inconsistent with an obligatory role for cyclic AMP in LH action on steroidogenesis in Leydig cells.

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