STIMULATION OF 3′,5′-CYCLIC AMP AND TESTOSTERONE PRODUCTION IN RAT TESTIS *IN VITRO*

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1. Introduction

It has been shown that ICSH (LH) and HCG can stimulate testicular steroidogenesis both *in vitro* and *in vivo* and it has been suggested that 3′,5′-cyclic AMP is the intracellular mediator of this process [1]. However, the necessary experimental evidence for cAMP being the "second messenger" [2] in the testis has not been obtained. This is in contrast to other steroid producing tissues e.g. the ovary and adrenal gland where there is good evidence for cAMP being a mediator of trophic hormone action on steroidogenesis [3, 4]. The present communication describes experiments carried out to examine three criteria for the role of cAMP as second messenger in testosterone production in the testis. It has been found that:

1) The increase in cAMP levels precedes the increase in testosterone production in HCG stimulated tissue.
2) Dibutyryl-cAMP stimulates testosterone production.
3) Theophylline (with and without HCG) has a variable effect on testosterone production.

2. Materials and methods

HCG was obtained from N.V. Organon (Oss, The Netherlands) (3500 I.U./mg, rat seminal vesicle weight test) and N^6-2′-*O*-dibutyryl-cAMP from N.V. Boehringer, Mannheim. These compounds were dissolved in Krebs-Ringer-Bicarbonate buffer (KRB) immediately before use.

[1,2-^{3}H] Testosterone (45 Ci/mmole) was obtained from the Radiochemical Centre, Amersham and purified by paper chromatography (Bush A-2 system containing ligroin, methanol, water, 50:35:15, by vol and Bush B-1 system containing ligroin, benzene, methanol, water, 25:25:35:15, by vol). [^{3}H] cAMP (Adenosine-^{3}H(G) 3′,5′-cyclic phosphate, ammonium salt, 24 Ci/mmole) was obtained from New England Nuclear and checked for purity by paper chromatography (isopropanol, ammonium hydroxide, H_2O, 70:10:30 by vol); no impurities were found.

Wistar strain rats, 10 weeks old, weighing 200–250 g were killed by decapitation. The testes were removed, decapsulated, slightly teased and separately preincubated for 1 hr at 32° in 6 ml Krebs-Ringer-Bicarbonate (KRB) in open 50 ml beakers with shaking in an atmosphere of 95% O_2 and 5% CO_2. Each testis was then removed with forceps from the medium and teased into 12–20 pieces. One piece (approx. 100 mg wet weight) from both the left and the right testis from one rat was added to 0.5 ml KRB or KRB containing 1.5 mM dibutyryl-cAMP, 10 mM theophylline or 10 I.U. HCG per 0.5 ml as indicated. Incubations were carried out for 5–240 min at 32° in an atmosphere of 95% O_2 and 5% CO_2 and were stopped by cooling the vessels in ice immediately followed by addition of the internal standards [^{3}H]cAMP and [^{3}H]testosterone. The samples were sonicated (20 KHz, amplitude 5 μm) at 0° for 30 sec and then extracted with acetone (2 × 2 ml)

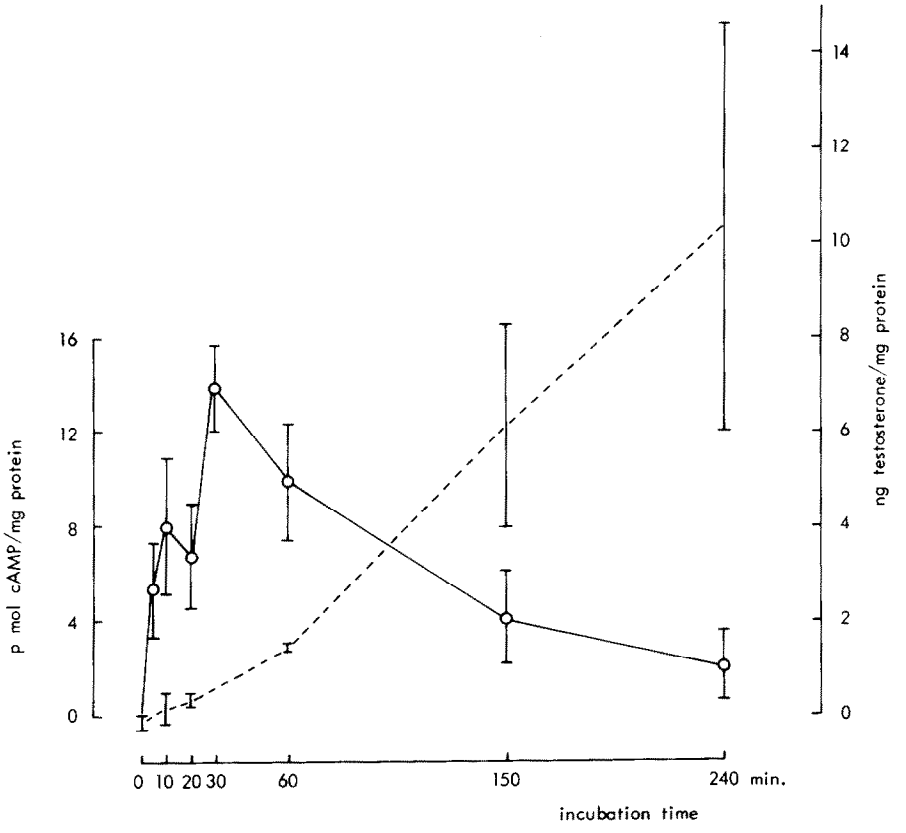

Fig. 1. Time course relationship of cAMP (——) and testosterone (- - - -) production in preincubated total rat testis tissue incubated with HCG (10 I.U.) *in vitro*. The values presented (means ± S.E.M., n = 3 to 6) are the difference between the levels of stimulated and unstimulated tissues at each time period. For incubation conditions see text.

Acetone was evaporated under N_2 at 45° and the remaining water phase was extracted with ether (3 × 1 ml). Testosterone was assayed in the combined ether phases by gas–liquid chromatography as described by Brownie et al. [5]. For cAMP measurements 20 μl 50% (w/v) trichloroacetic acid was added to the water phase (made up to 1 ml) to precipitate residual protein.

cAMP was isolated by chromatography of the trichloroacetic acid/water mixture over Dowex (50W × 8, 200–400 mesh) ion exchange resin columns [6]. Eluted cAMP was assayed by saturation analysis [7]. Tissue samples were dissolved in M NaOH for estimating protein according to Lowry et al. [8].

3. Results and discussion

A reproducible stimulation of testosterone and cAMP production *in vitro* by HCG was found only when total testis tissue was preincubated for 1 hr at 32°. When the tissue was not preincubated, testosterone production was high and it was difficult to stimulate further production. It is possible therefore that inhibitors are removed from the tissue by the preincubation procedure.

In control incubations over a period of 4 hr cAMP levels decreased from 12 to 3 pmole/mg protein and testosterone levels increased from 2.4 to 4 ng/mg protein. Addition of HCG caused an increase in cAMP levels that preceded the increase in testosterone levels (fig. 1). A significant increase in

Table 1
Correlation between change in cAMP and testosterone levels in total rat testis tissue during stimulation with HCG *in vitro*.

Experiment number	cAMP (pmole/mg protein/20 min incubation)			Testosterone (ng/mg protein/240 min incubation)			X
	No additions (a)	HCG (10 I.U.) (b)	b−a (X)	No additions (c)	HCG (10 I.U.) (d)	d−c (Y)	Y
1	5.6	16.2	10.6	2.8	12.1	9.3	1.1
2	9.8	16.6	6.8	4.2	22.1	17.9	0.4
3	6.4	9.2	2.8	1.7	5.3	3.6	0.8
4	7.2	7.7	0.5	2.9	4.0	1.1	0.5
5	10.4	17.1	6.7	7.8	22.4	14.6	0.5

Table 2
Effect of dibutyryl-cAMP on testosterone levels in total rat testis during incubation *in vitro*.

Experiment number	Testosterone (ng/mg protein)					
	180 min incubation			240 min incubation		
	No additions (a)	Dibutyryl-cAMP (1.5 mM) (b)	b−a	No additions (c)	Dibutyryl-cAMP (1.5 mM) (d)	d−c
1	3.1	5.8	2.7	3.2	16.3	13.1
2	3.7	4.8	1.1	4.1	7.6	3.5
3	2.5	9.2	6.7	3.2	25.3	22.1
4	3.4	16.8	13.4	2.8	13.3	10.5
5	2.4	10.3	7.9	2.5	16.1	13.6

Table 3
Effect of theophylline on testosterone levels in total rat testis tissue during incubation *in vitro*.

Experiment	Testosterone (ng/mg protein/240 min incubation)					
	No additions (a)	Theophylline (10 mM) (b)	b−a	HCG (10 I.U.) (c)	HCG (10 I.U) + theophylline (10 mM) (d)	d−c
1	3.1	2.7	−0.4	4.8	4.0	−0.8
2	4.1	7.6	3.5	12.4	6.2	−6.2
3	2.9	2.6	−0.3	4.0	7.3	3.3
4	7.8	7.8	0	22.5	16.0	−6.5

cAMP levels ($P < 0.025$) was found during 10 min incubation whereas testosterone levels were not significantly increased until 60 min ($P < 0.001$).

Although a stimulation of cAMP and testosterone production was always observed, the degree of stimulation varied. A correlation was found, however,

between the change in cAMP levels during 20 min and the change in testosterone levels during 240 min (table 1). For example, when the cAMP increase during 20 min was small, there was also a small increase in the production of testosterone. In this respect the results of Dufau et al. [9] are of interest. They found that HCG stimulated testosterone production in decapsulated total testis *in vitro*, but if the testis was teased apart a much lower stimulation occurred. Additions of dibutyryl-cAMP (1.5 mM) resulted in a stimulation of testosterone production especially during incubation periods of more than 180 min (table 2). These results are in agreement with data published by other investigators [9–11]. In an attempt to increase testosterone production by inhibiting the breakdown of cAMP, theophylline (10 mM) was added to inhibit phosphodiesterase activity. However, a consistent effect of this compound on testosterone production when added alone or with HCG, could not be demonstrated. In some experiments an inhibition of testosterone production by theophylline was observed (table 3). Comparable inhibiting effects on corticosteroid production have also been reported by other workers for the adrenal gland [12, 13]. Therefore 10 mM theophylline is apparently unsuitable for testing the participation of cAMP in hormone action on steroid producing tissues.

It may be concluded from the present observations on the time course of cAMP and testosterone production during HCG stimulation and from the effect of dibutyryl-cAMP, that cAMP could be a mediator of trophic hormone action on the testis. However, because of the inhomogeneous nature of total testis tissue only tentative conclusions can be drawn and this work is therefore being extended to testis interstitial tissue and seminiferous tubules. Results already obtained show that HCG specifically stimulates cAMP production in interstitial tissue [6] and that testosterone production can also be stimulated in this tissue by HCG.

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