Effects of seminiferous tubule secreted factor(s) on Leydig cell cyclic AMP production in mature rat

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The effects of spent media from seminiferous tubules (STM) on Percoll-purified rat Leydig cells were investigated. Intracellular and extracellular cyclic AMP (cAMP) accumulation and testosterone production were measured. After a 5 h incubation period, STM reduces both the basal and LH-dependent cAMP levels (38 and 20%, respectively for intra- and extracellular cAMP) while, simultaneously, a stimulation of testosterone production is observed (47 to 50%, respectively in the absence or presence of LH). The reduction of cAMP levels observed after 5 h is likely to be due to the potentiating effect of the STM factor on the LH-dependent initial rise of the cAMP level which, in turn, induces a desensitization of the Leydig cell adenylate cyclase. This substance is a thermolabile protein ($M_r > 50000$) produced by the Sertoli cell, independent of FSH and testosterone controls, and different from the LHRH-like substance.

(Leydig cell, Sertoli cell) *Seminiferous tubule* *cyclic AMP* *Testosterone*

1. INTRODUCTION

There is now a considerable body of evidence to suggest that testicular factors from tubular origin regulate rat Leydig cell functions (reviews [1,2]). Seminiferous tubules from mature animals secrete factors which stimulate testosterone production [3–8] and aromatase activity [9] in the Leydig cell. An LHRH-like factor of Sertoli cell origin which modulates in vivo and in vitro Leydig cell steroidogenesis has been described by Sharpe [2]; however, it has been shown that its concentration is very low (1 pg/testis [10]). These findings prompted us to compare the effects of STM from mature rats on purified Leydig cell adenylate cyclase activity with those of an LHRH agonist.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Ovine luteinizing hormone (NIH-oLH 24) and ovine follicle-stimulating hormone (NIH-oFSH 15) were from NIADDK. The LHRH agonist (LHRHa: D-Ala^6,des-Gly^{10}–LHRH *N*-ethylamide), cholera toxin and 3-methyl-1-isobutylxanthine (MIX) were purchased from Sigma. The LHRH antagonist (LHRHanta: D-pGlu^1,D-Phe^2, D-Trp^{3,6}–LHRH) was obtained from Peninsula laboratories. Testosterone and estradiol antibodies were from Biomerieux; cyclic AMP kit used for radioimmunoassay (RIA) was from Amersham. All other reagents used for cell purification and culture were as described in [11].

2.2. *Leydig cell purification and incubation*

Leydig cells were purified from 70–80-day-old Sprague Dawley rat testes [11]. They were incubated for 5 h at 32°C under O_2/CO_2 (95:5, v/v) in 600 μl Ham F12/DME medium (1:1, v/v) supplemented with insulin (10 μg/ml), transferrin (5 μg/ml) to which were added the various spent media to be studied (400 μl) and the indicated hormones (LH, LHRHa, LHRHanta, cholera toxin).

2.3. *STM preparation*

Isolated segments (200 mm length) of

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seminiferous tubules were cultured for 20 h in 1 ml medium (Ham F12-DME plus insulin and transferrin) with or without FSH (5 μg/ml) and testosterone (200 ng/ml). The STM was collected after centrifugation (1000 × g, 5 min, 18°C) and the endogenous steroids removed by charcoal treatment (1 mg activated charcoal/mg protein, 12 h at 4°C). The purity of the seminiferous tubular preparations was examined as described by Parvinen et al. [4]. The STM steroid content (testosterone and estradiol) before and after charcoal treatment was determined by RIA and the amount of protein by the technique of Bradford [12]. Since there was no difference in Leydig cell function, irrespective of whether STM was treated with charcoal, routinely freshly prepared STM was used in each experiment.

2.4. *Sertoli cell medium (SCM)*

Sertoli cells were isolated from seminiferous tubules as in [13,14]. The cells were plated as small aggregates (5–15 cells) in the same medium as used for Leydig cells and cultured for 4 days to remove the germ cells; the medium was changed every 2 days. On day 4, a fresh medium supplemented or not with FSH (5 μg/ml) and testosterone (200 ng/ml) was added for a further 20 h incubation. Using morphological (light microscopy), biochemical (absence of specific hCG binding and testosterone synthesis) and histochemical (no 3β-hydroxysteroid dehydrogenase positive staining) parameters, it was concluded that the Sertoli cell preparation was free of Leydig cells on day 4. SCM was collected and concentrated 10-fold through an Amicon YM2 membrane; freshly prepared SCM was used in each experiment.

2.5. *Miscellaneous*

The testosterone levels were measured by RIA in the cell culture media as outlined in [11]. The inter- and intra-assay coefficients of variation were respectively 3 and 6% and the sensitivity 4 pg/tube. The intracellular and extracellular cAMP levels were determined as described by Schumacher et al. [15]. The inter- and intra-assay coefficients of variation were 6 and 8%, respectively, and the sensitivity 0.1 pmol/tube.

2.6. *Statistical analysis*

The data obtained were analyzed using the Student's t-test.

Table 1

Effects of STM on basal and LH-stimulated testosterone synthesis in purified rat Leydig cells

Treatment	Testosterone (ng/10^5 cells per 5 h)
Cells + control medium (CM)	1.68 ± 0.05
+ STM (40%)	2.47 ± 0.12*[a]
+ LH (25 ng/ml)	20.19 ± 0.48
+ LH + STM	30.19 ± 1.29*[b]

Results are means ± SE of 3 separate experiments performed in duplicate; * $p < 0.001$ vs respective controls ([a] CM, [b] LH); STM, seminiferous tubule medium

Table 2

Effects of STM, LHRHa and LHRHanta on basal, LH and cholera toxin stimulated adenylate cyclase activity, with or without MIX (0.5 mM) in purified Leydig cells

Treatment	Intracellular cAMP (pmol/10^5 cells per 5 h)	
	− MIX (4)	+ MIX (2)
Cells + CM	0.32 ± 0.03	0.35 ± 0.03
+ STM (40%)	0.20 ± 0.03*[a]	0.23 ± 0.02*[a]
+ LH (25 ng/ml)	2.44 ± 0.10	2.70 ± 0.04
+ LH + STM	1.52 ± 0.04*[b]	1.68 ± 0.11*[b]
+ LH + STM (+ FSH + T)	1.50 ± 0.06*[b]	–
+ LHRHa (10^7 M)	0.20 ± 0.01*[a]	0.32 ± 0.03[a] NS
+ LH + LHRHa	2.16 ± 0.05*[b]	2.66 ± 0.06[b] NS
+ LH + LHRHa + LHRHanta (10^6 M)	2.42 ± 0.10[b] NS	–
+ LH + STM + LHRHanta	1.86 ± 0.03*[b]	–
+ Cholera toxin (10 μg/ml)	2.35 ± 0.06	6.17 ± 0.34
+ Cholera toxin + STM	1.11 ± 0.03*[c]	1.62 ± 0.09*[c]

Results are means ± SE (number of experiments in parentheses). NS, not significant; * $p < 0.001$ vs respective controls ([a] CM, [b] LH, [c] cholera toxin). T, testosterone; STM, seminiferous tubule medium; CM, control medium

3. RESULTS

In purified rat Leydig cells, basal (1.68 ng/10^5 cells) and LH-stimulated (20.19 ng) testosterone productions are significantly increased (1.5-fold; $p < 0.001$) by STM (table 1) after a 5 h incubation period. By contrast, the intracellular cAMP production is diminished (table 2): the addition of STM, with or without MIX, leads to a significant diminution of the basal intracellular cAMP levels (0.2 and 0.3 pmol, respectively in the presence and absence of STM). Under LH stimulation (25 ng/ml), the cAMP accumulation is increased 7.6-fold (2.44 pmoles) when compared to control medium; this effect is reduced 38% by STM and MIX is ineffective. The STM effect on cAMP production is dose-dependent (table 3) and the maximal decrease of cAMP levels is obtained using 40% (v/v) STM. It is noteworthy that the addition of FSH + testosterone does not increase the production of the STM inhibitory factor. When the Leydig cells are incubated with LHRHa (10^{-7} M), the basal cAMP level is decreased to a similar extent to that in the presence of STM (0.2 pmol); however, when LH is added, the adenylate cyclase activity is weakly inhibited by LHRHa (11%) when compared to the STM effect. Under these conditions, the presence of MIX completely blocks the LHRHa inhibitory effect; similarly, the addition of LHRHanta (10^{-6} M) abolished specifically the LHRHa action while the STM inhibitory effect is slightly diminished (2.24 and 1.86 pmol, respectively). In the presence of cholera toxin (10 μg/ml), the cAMP levels are increased (2.35 pmol) and MIX further enhances (3-fold) the cAMP accumulation. The addition of STM (40%) induces a significant ($p < 0.001$) decrease in cAMP production which is again not influenced by MIX. In fig.1, we report the kinetics of the cAMP responses

Table 3

Effect of different concentrations of STM on LH-stimulated cAMP accumulation in purified rat Leydig cells

Treatment	cAMP
LH (25 ng/ml)	100.0 ± 4.5
+ STM (5%)	93.0 ± 2.6 NS
+ STM (10%)	86.2 ± 3.8[a]
+ STM (20%)	73.0 ± 3.0[b]
+ STM (40%)	69.0 ± 3.4[b]
+ STM (60%)	66.0 ± 2.5[b]
+ STM (80%)	65.2 ± 3.5[b]

The results (mean of two separate experiments) are expressed as percentage of control (LH alone). NS, not significant; [a] $p < 0.01$; [b] $p < 0.001$ vs control (LH alone)

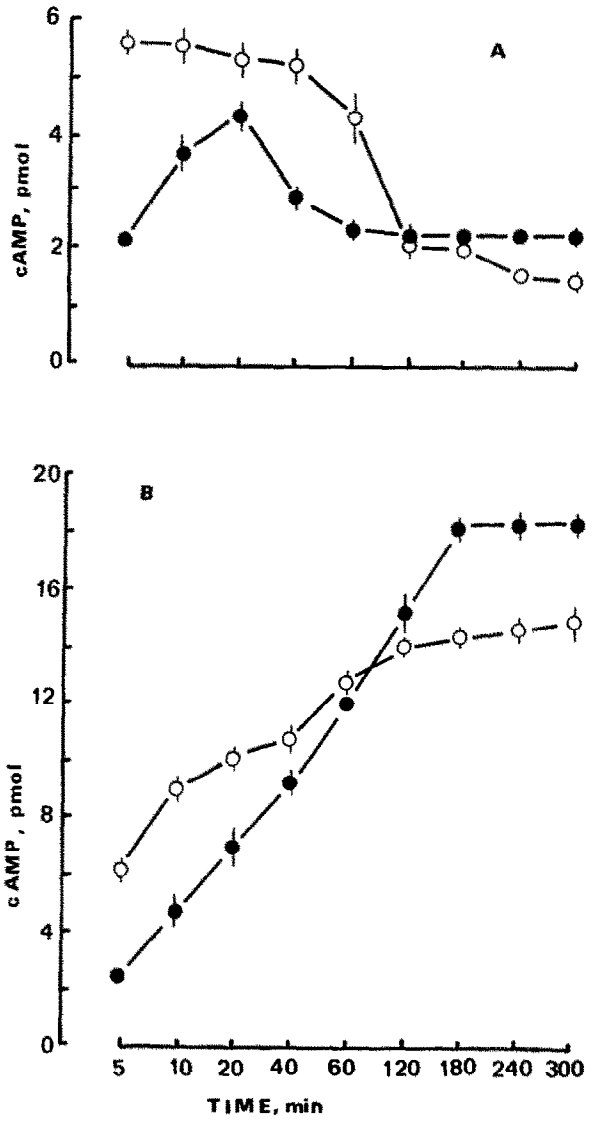

Fig.1. Leydig cell intracellular (A) and extracellular (B) cAMP accumulation in the presence of LH (25 ng/ml) (●—●) or LH + STM (40%) (○—○). The results (pmol/10^5 Leydig cells) are means ± SE of 2 separate experiments performed in duplicate.

to LH (25 ng/ml). The intracellular cAMP level increases very rapidly (20 min), then declines to the same levels as those obtained after a 5 min incubation period (fig.1A) and remains unchanged afterwards. Within 5 min, it is stimulated 2.6-fold by the addition of STM (5.6 vs 2.1 pmol/10^5 Leydig cells; fig.1A); no changes are observed until 1 h, then a sharp decrease occurs and the cAMP levels become lower than those obtained in the presence of LH alone. The extracellular cAMP productions are also enhanced within the first incubation hour when STM is added together with LH (fig.1B). After 2 h, the pattern observed is similar to that of fig.1A and the levels are diminished when compared to LH alone. It is of note that the basal cAMP levels are similarly affected by STM (not shown). Heating at 100°C and trypsin treatment completely destroy the STM factor (table 4), while freezing (−20 or −70°C) is ineffective (not shown); furthermore, rat serum is without effect on the adenylate cyclase activity. After filtration through an Amicon XM 50 membrane (M_r cut-off 50000), the STM-inhibiting activity is lost. Using a 10-fold concentrated SCM, the Leydig cell cAMP levels are diminished to the same extent as with STM; the addition of FSH plus testosterone to the Sertoli cell culture medium does not further enhance the SCM inhibitory activity.

Table 4

Main characteristics of the STM inhibitory factor (3 different experiments)

Treatment	cAMP (% control)
Cells + LH + CM	100
+ LH + STM	62 ± 0.4[a]
+ LH + STM (heated at 100°C, 30 min)	103 ± 1
+ LH + STM (treated with trypsin: 250 μg/300 μg protein)	98 ± 2.2
+ LH + STM (filtered through Amicon XM 50)	100 ± 0.6
+ LH + rat serum	93 ± 3
+ LH + SCM (10-fold concentrated)	65 ± 1.2[a]
+ LH + SCM (+ FSH + testosterone)	68 ± 5[a]

[a] $p < 0.001$ vs LH. All other comparisons are not statistically different; CM, control medium; STM, seminiferous tubule medium; SCM, Sertoli cell medium

4. DISCUSSION

These findings demonstrate that several secretory products from mature rat seminiferous tubules regulate Leydig cell functions: (i) a factor which affects the adenylate cyclase activity and (ii) another which acts on testosterone synthesis. Several authors [3–8] have demonstrated that rat interstitial fluid, seminiferous tubule and immature Sertoli cell culture media enhance Leydig cell testosterone production; the proteic factor involved ($M_r > 10000$) is different from the LHRH-like substance [16] and absent in rat serum. Besides this STM-stimulating factor, we also report the presence of an STM factor which depresses the Leydig cell adenylate cyclase activity after a 5 h incubation period. Our studies being performed in a medium containing 2 mM Ca^{2+}, the LHRHa inhibitory effects observed on the cyclase are in agreement with previous data [17]; however, the action of LHRHa is clearly different from that of the STM factor. The potent phosphodiesterase inhibitor, MIX, which blocks the LHRH effect as well as the LHRH antagonist are ineffective on the STM-induced reduction of cAMP levels. These results demonstrate that the STM factor which regulates the Leydig cell adenylate cyclase activity is not the LHRH like substance and, moreover, that its mechanism of action is different.

Cholera toxin is known to increase intracellular cAMP accumulation by causing ADP-ribosylation of the regulatory guanine nucleotide-binding protein [18]. In our experiments, the stimulatory effect of cholera toxin on Leydig cell adenylate cyclase activity is inhibited by STM; this result suggests that the STM factor acts either on the regulatory protein or on the catalytic subunit of the adenylate cyclase. It has been shown that an increase of cAMP induced by different compounds (dbcAMP, hCG, cholera toxin) leads to a desensitization of the Leydig cell adenylate cyclase; this effect depends on the initial rise of cAMP [15]. As we have shown that STM contained factor(s) which potentiate(s) the effect of LH on initial cAMP production, the following desensitization can explain the decrease in cAMP observed after a 5 h incubation period.

Using 10-fold concentrated SCM instead of STM, a similar decrease in cAMP accumulation is observed which demonstrates the Sertoli cell origin

of this factor, of which the secretion is independent of FSH and testosterone controls. This compound is of proteic nature, with an M_r higher than 50000 while the STM-stimulating factor of Leydig cell testosterone synthesis has an $M_r < 50000$ (unpublished). The differential effects of STM on cAMP and testosterone productions in rat Leydig cells raise the question concerning the role of cAMP as the only second messenger of LH action [11–19].

In conclusion, rat Leydig cell adenylate cyclase activity and testosterone synthesis are regulated by several Sertoli cell factors; their secretion in relation with the different stages of spermatogenesis is under investigation.

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