Mechanism of action of the factor(s) secreted by rat seminiferous tubules and inhibiting interstitial cell testosterone production in vitro

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Abstract. Rat seminiferous tubules secrete a factor which inhibits LH-dependent steroidogenesis by interstitial cells. The inhibitory activity was found to be specific for the testes, as cytosols from other rat tissues such as the kidney, heart, spleen, liver and epididymis had no significant effect on testosterone production by interstitial cells. Preliminary characterization by Ultrogel AcA 44 gel chromatography demonstrated that the active substance in SMST has a molecular weight between 40–50 kD. Spent medium from incubation of seminiferous tubules (SMST) caused a dose-dependent inhibition of LH- or cholera toxin-stimulated in vitro testosterone production by rat interstitial cells. However, SMST failed to inhibit forskolin-stimulated steroidogenesis. The effect of SMST was not altered by pre-incubating the cells with the sulphydryl reagent, N-ethylmaleimide. Considering the proposed mode of action of these modulators of adenylate cyclase activity, the present studies suggest that a high molecular weight testis specific factor acts through the guanine nucleotide-binding stimulatory regulatory protein of the adenylate cyclase complex to inhibit LH-dependent testosterone production by Leydig cells.

Much evidence has been accumulated during the last few years for the existence of local paracrine factors in the mammalian testes which modulate the function of various cell types (Sharpe 1986). There are studies indicating that macromolecular soluble factors from the seminiferous tubules can increase (Parvinen et al. 1984; Sharpe & Cooper 1984; Verhoeven & Cailleau 1985) or decrease (Papadopoulos et al. 1986) Leydig cell testosterone production.

We have previously reported the presence of factors secreted by rat seminiferous tubules which influence in vitro testosterone production by interstitial cells (Syed et al. 1985,1986). The inhibitory activity was found to be associated with a heat-stable, protease degradable, nondialysable compound. Addition of protease inhibitor to the spent medium from incubation of seminiferous tubules (SMST) did not influence its activity, suggesting that the inhibitory action was not due to the presence of proteolytic enzymes in the preparations. The active factor did not influence the binding of LH to interstitial cells and it had no effect on the cAMP-stimulated testosterone production or on the metabolism of testosterone in interstitial cells (Syed et al. 1985) suggesting that it inhibits LH action at a step later than the LH binding to its receptor but prior to the action of cAMP.

The present study was performed to investigate the specific site of action of this inhibitory activity in the interstitial cell steroidogenesis. The results indicate that it acts through the stimulatory regulatory protein of the adenylate cyclase complex.

Material and Methods

Chemicals and reagents

Tissue culture medium (Medium 199) and L-glutamine were obtained from Flow Laboratories, Irvine, Scotland. Trypsin, trypsin inhibitor and cholera toxin were purchased from Sigma Chemical Co., London. Forskolin and N-ethylmaleimide (NEM) were obtained from Calbiochem Behring Diagnostics, CA. Ovine LH (NIH-oLH-S19) was kindly provided by the NIADDK, Bethesda, MD.
Collection of seminiferous tubules
The seminiferous tubules were collected from 90-day-old Sprague-Dawley rats by enzymatic digestion of the decapsulated testes and incubated for 24 h at 32°C as previously described (Syed et al. 1986). All steps were performed using Medium 199 supplemented with L-glutamine and 0.2% BSA. The spent medium (SMST) was centrifuged at 1000 × g for 20 min and stored at −20°C.

Cytosol preparation
The cytosols from rat testes, epididymis, liver, heart, kidney and spleen were prepared as described previously (Syed et al. 1986). More than 95% of the testosterone present in the SMST or cytosols was removed by filtration through small columns of Sephadex G-25 (PD 10, Pharamcia Fine Chemicals, Uppsala, Sweden).

![Graph](https://via.placeholder.com/150)

**Fig. 1.**
Effect of SMST on oLH- and cholera toxin-stimulated testosterone production by interstitial cells. Upper panel: The SMST incubated with 1 and 4 ng of oLH caused inhibition of testosterone production by interstitial cells. The curve to the left demonstrates the dose-response curve of interstitial cells to graded doses of oLH. Lower panel: The SMST incubated with two different doses of cholera toxin showed a dose-dependent suppression of testosterone production by interstitial cells. No effect was observed on the basal testosterone production. The curve to the left demonstrates the response of cells to graded doses of cholera toxin. Each point represents the mean ± so of 6 experiments.
Effect of SMST on forskolin-stimulated testosterone production by interstitial cells. The curve to the left shows response of the cells to graded doses of forskolin. SMST had no effect on basal testosterone production, whereas it caused a slight stimulation when incubated with 62.5 µg forskolin and had no effect when incubated with a maximally stimulating dose of forskolin. Each point represents the mean ± SD of 6 experiments.

**Interstitial cell preparation and incubation**

Interstitial cells were prepared from 60-day-old rat testes by collagenase treatment of the decapsulated testes (Syed et al. 1985). The suspension of interstitial cells contained about 30% 3β-hydroxysteroid dehydrogenase (3β-HSD) positive cells and about 10% phagocytic cells. After preculture for 1 h at 34°C, 100 µl of cell suspension (approximately 85,000 cells) was incubated with graded doses of either oLH, forskolin or cholera toxin. The ability of the inhibitory factor to block testosterone production was then studied by incubation of cells with different doses of SMST in the presence or absence of half maximal and maximal doses of the above agonists.

For one set of experiments the interstitial cells were first incubated for 30 min with NEM (0.01 mmol/l). Cells treated with higher doses of NEM failed to respond to LH. After washing, the cells were incubated with or without oLH in the presence of SMST for 3 h. The testosterone concentration in the incubation media was determined by radioimmunoassay (Van Damme et al. 1974)

**Monolayer cultures of interstitial cells**

Approximately 8 × 10⁵ interstitial cells prepared by collagenase treatment or 6.5 × 10⁵ Percoll purified Leydig cells (Syed et al. 1985) in 2 ml of Medium 199 (containing 20 mmol/l L-glutamine, 1% fetal bovine serum (FBS), 10⁵ IU/l penicillin, and 50mg/l streptomycin) were added to plastic cell culture Petri dishes (diameter 35 mm). The cell suspension was incubated at 34°C for 1 h under 5% CO₂ and 95% air. During this period of time, most of the viable nucleated somatic cells attach to the plastic surface, whereas germ cells, erythrocytes, and dead cells remain floating (Rommerts et al. 1985). The latter cells were removed by washing three times with medium. The suspension of interstitial cells showed about 30% 3β-HSD positive cells and 10% macrophages. After plating, about 30% of the attached cells were positive for 3β-HSD, and about 10% were macrophages as demonstrated by in vivo phagocytosis of trypan blue. The Percoll purified cell suspension contained about 50% 3β-HSD positive cells and about 40% macrophages. After plating, the percentage of 3β-HSD positive cells was between 45–50%, but that of macrophages had decreased to about 20–25%. The cells were then incubated with oLH (0.5 µg/l) in a total volume of 2 ml for 3 h. The SMST was added at different doses in the absence or presence of oLH (0.5 µg/l). In order to examine the recovery of the Leydig cells after exposure to SMST, medium was aspirated after 3 h of incubation, the cells were washed three times, and incubation was continued for another 3 h with or without the addition of oLH, but in the absence of SMST. Testosterone was measured in the incubation medium as described above.

**Gel chromatography of the inhibitory activity**

SMST, 40 ml, was precipitated in 80% saturated ammonium sulphate solution overnight at 4°C. After washing with 80% saturated ammonium sulphate solution, the precipitates were dissolved in 0.1 mol/l Tris HCl buffer (pH 7.4) and dialysed for 24 h at 4°C against the same buffer. The precipitated SMST proteins were fractionated by
Ultragel AcA 44 (LKB produkter, Bromma, Sweden) gel chromatography (column dimensions 1.6 × 80 cm). Elution was performed at a speed of 6 ml/h using 0.1 mol/l Tris HCl buffer. The eluent was collected in 4-ml fractions and stored at −20°C until assayed for interstitial cell inhibitory activity. The fractions causing inhibition of testosterone production by interstitial cells were pooled and rechromatographed by the above procedure.

Results

Effect of SMST on cholera toxin- and forskolin-stimulated testosterone production by interstitial cells

As seen previously (Syed et al. 1985), SMST had no effect on the basal testosterone production by the interstitial cells. However, the LH, or cholera toxin-stimulated testosterone production was inhibited by SMST in a dose-dependent manner (Fig. 1). In contrast, the forskolin-stimulated testosterone production could not be blocked by the SMST (Fig. 2).

Although pretreatment of the interstitial cells with NEM reduced the testosterone production to some extent, yet it did not influence the inhibitory effects of SMST (Fig. 3).

Effect of SMST on LH-dependent testosterone production by Leydig cells in monolayer culture

Monolayers of freshly isolated interstitial cells were incubated for 3 h with 0.25, 0.5 and 1 ml of SMST in the presence or absence of oLH (0.5 µg/l). A marked suppression of LH-dependent testosterone production was observed (Fig. 4A, open columns), similar to that observed with interstitial cells in suspension. Similar results were obtained when interstitial cells were purified on Percoll gradient and then plated in Petri dishes (Fig. 4B). The inhibition was reversible; during a second 3-h incubation period in the absence of SMST, the monolayers of interstitial cells produced the same amounts of testosterone as control cells (Fig. 4, hatched columns). However, both control and experimental cultures showed reduced testosterone production during

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**Fig. 3.** Rat interstitial cells with (right) or without (left) previous treatment with NEM were incubated with SMST in the absence or presence of two doses of oLH for 3 h. A dose-dependent inhibition of LH-stimulated testosterone production was observed. Inhibition was similar to that with untreated cells, although the response of NEM-treated cells to LH was lower. Each point represents the mean ± SD of 5 experiments.
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Previously the presence of the representative giving the 45—50% of the 3ß-HSD the test material (+) and hatched columns the succeeding 3-h incubations in the absence of test material (—). The experiments were performed three times. A representative experiment is shown. Note significant inhibition of testosterone production with SMST in the presence of oLH. After removal of the inhibitor, the previously suppressed cells show the same testosterone production as the controls.

Fig. 4.

Influence of SMST on interstitial cell cultures (A) or Percoll purified Leydig cells (B) in the presence or absence of oLH. The columns to the left represent basal and LH-stimulated testosterone levels in the absence of SMST. The open columns represent the first 3-h incubation with the test material (+) and hatched columns the succeeding 3-h incubations in the absence of test material (—). The experiments were performed three times. A representative experiment is shown. Note significant inhibition of testosterone production with SMST in the presence of oLH. After removal of the inhibitor, the previously suppressed cells show the same testosterone production as the controls.

eluted at an apparent molecular weight of 40—50 kD (Fig. 5). Active fractions were pooled, concentrated and dialysed for 24 h in 0.1 mol/l Tris HCl and then re-chromatographed on the same column. An identical elution profile was obtained (results not shown).

In order to study possible aggregation or degradation of LH caused by SMST during the 3-h incubation, 1 ng 125I-hLH (100 000 cpm) was incubated for this period at 34°C with or without SMST. The radioactivity from both incubation media showed an identical elution pattern when chromatographed on an AcA 44 Ultrogel column (not shown).

Tissue specificity of inhibitor

When interstitial cells were incubated with the same amount of cytosol proteins (6-5 g/l) obtained from liver, kidney, heart, spleen, epididymis and testis, in the presence or absence of 1 ng of oLH, a dose-dependent inhibition of LH-stimulated testosterone production was observed with testicular cytosol only. Cytosols from other tissues had no effect on LH-dependent testosterone production. The basal testosterone production was not affected by any of the cytosols tested (data not shown). Moreover, the inhibitory activity of testicular cytosol was eluted in the same fractions as found after ACA 44 chromatography of SMST proteins (data not shown).

Discussion

In previous studies it was established that the inhibition of LH-dependent testosterone production caused by testicular SMST or cytosol is due to a heat-stable, trypsin-sensitive and nondialysable protein factor (Syed et al. 1985, 1986). The present gel filtration experiments indicate that the inhibitory compound has a molecular weight of about 40—50 kD. A similar factor (mol wt 40—50kD) was also found to be present in testicular cytosol proteins, but not in cytosols prepared from other tissues. In principle, inhibitory effects could be due to a reduction in LH binding to its receptors. Although the presence of LH receptor binding inhibitors in gonadal preparations have been previously demonstrated (Yang et al. 1979; Rojas et al. 1981), the LH binding inhibitor found in rat testes had a molecular weight of less than 12 000 (Rojas et al. 1981). Furthermore, we previously found no in-
fluence of SMST on $^{125}$I-hCG binding to interstitial cells (Syed et al. 1985).

The inhibition of testosterone production proved to be reversible. When monolayer cells were washed free from SMST, there was no difference between the previously exposed and control cells (Fig. 4).

Others have shown that the inhibitory G protein ($G_i$) can be inactivated by treatment of intact cells with the sulfhydryl reagent N-ethylmaleimide (NEM), whereas the stimulatory regulatory protein ($G_s$) has been shown to be resistant to this reagent (Jakobs et al. 1982). The functionally active $G_s$ (44 kD) and $G_i$ (39 kD) have been shown to be involved in the regulation of steroidogenesis in rat Leydig cells (Themmen 1986; Adashi et al. 1984).

In the present study, the SMST-derived inhibitor of Leydig cell function reduced LH- and cholera toxin-stimulated testosterone production. However, the SMST did not suppress the effects of forskolin on Leydig cell testosterone production. Moreover, our results show inhibition of LH-dependent testosterone production even after NEM treatment of the cells, indicating that the inhibitor does not involve $G_i$ to express its action. Accepting the proposed mechanisms of action of cholera toxin, forskolin and NEM discussed above, the present results indicate that the inhibitor may act at $G_s$ to execute its effect.

Cholera toxin induces a persistently activated state of adenylate cyclase and inhibits the hormone-stimulated GTP hydrolysis at $G_s$, apparently by ADP-ribosylation of the $\alpha$ subunit of the coupling component (Birnbaumer & Iyengar 1982; Cassel & Pfeuffer 1978; Pfeuffer et al. 1983). It has been reported that the hormones which cause adenylate cyclase inhibition stimulate GTPase (Aktories & Jakobs 1981; Aktories et al. 1982, 1983; Koski & Klee 1981). In contrast to $G_s$ associated GTPase activity. GTPase activation by inhibitory hormones is not blocked by cholera toxin, which does not affect hormonal inhibition of adenylate cyclase either (Aktories et al. 1982). Taking the above findings together with our results it seems that the SMST might activate GTPase activity at $G_s$. These conclusions are supported by the recent findings of Vihto & Huhtaniemi (1988) who confirmed the inhibitory action of SMST on Leydig cell (crude and purified) testosterone production and, furthermore, showed that this is paralleled by an inhibition of cAMP production.

SMST could not block forskolin action on the Leydig cells. The moderate potentiation of the forskolin effects by the SMST remains unexplained, as the full mechanism of the action of forskolin is still obscure.

When the Percoll purified Leydig cells were used in a monolayer culture, the inhibition caused
by SMST was similar to that observed using unpurified interstitial cells. In a previous study, however, we found that the SMST stimulated testosterone production by Percoll purified Leydig cells in suspension (Syed et al. 1985). The contrasting results obtained in these studies could be due to several factors. Cells adhering to the supporting surfaces may behave differently from those in suspension. Adhesion and subsequent washing of the monolayer is an effective way of eliminating damaged cells. It has been reported that Percoll purified cell suspensions contain broken cells (Laws et al. 1985), and Percoll may also affect the membrane properties of the cells (Wakefield et al. 1982). The membrane damage caused by Percoll could possibly be repaired during the initial phase of incubation during attachment of the cells to the plastic surface. In a separate series of experiments (to be published elsewhere), we found that when Leydig cells were purified on a Percoll gradient after prior treatment of animals with trypan blue, the phagocytic cells appear in the same fraction as Leydig cells (density, 1.07 kg/l). Actually, the relative proportion of macrophages increased when the Leydig cells were purified. After adhesion of cells to plastic, the number of macrophages coincides with that present in the crude cell preparations. Possibly, the varying proportions between macrophages and Leydig cells may explain the different effects of SMST on Percoll fractionated and crude interstitial cells. The present study, therefore, indicates that the inhibitor may require intact cells for its action. Furthermore, the inhibitor does not seem to exert long-lasting non-specific toxic effects upon cells, since the inhibitory influence is reversed when culture is continued in the absence of SMST.

On the basis of the results obtained in the present study it is concluded that the inhibitory factor is specific for testis, has a mol wt of 40–50 kD and probably involves guanine nucleotide-binding stimulatory regulatory protein (G_{s}) to express its action on Leydig cell steroidogenesis.

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