# Regulation of Sertoli Cell Differentiation by the Testicular Paracrine Factor PModS: Analysis of Common Signal Transduction Pathways*

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## ABSTRACT

In the testis the mesenchymally derived peritubular cells produce a paracrine factor, PModS, that mediates mesenchymal-epithelial interactions and modulates Sertoli cell functions essential for the process of spermatogenesis. PModS has a more dramatic effect on Sertoli cell differentiated functions *in vitro* than any regulatory agent previously shown to influence the cells, including FSH. The current study initiates an investigation of the pharmacology of PModS through an analysis of several common signal transduction pathways. PModS was found to stimulate cGMP levels in Sertoli cells and maintain elevated levels for up to 5 days in culture. PModS had no influence on cAMP levels. In contrast, FSH stimulated cAMP, but had no influence on cGMP levels. For comparison, an agent known to influence cGMP levels, atrial naturetic factor (ANF), was used to treat Sertoli cells. ANF caused a dramatic increase in Sertoli cell cGMP levels within minutes of treatment, but did not maintain elevated cGMP levels after a 72-h treatment. Although ANF increased guanylate cyclase in whole Sertoli cell homogenates and particulate fractions, PModS did not directly influence guanylate cyclase activity. As previously shown, PModS stimulates transferrin expression as a marker of Sertoli cell differentiated function. Agents that elevate cellular cGMP, including ANF, sodium nitroprusside, and 8-bromo-cGMP, did not influence Sertoli cell transferrin expression. In addition, these agents did not influence the actions of PModS or FSH. Therefore, cGMP does not appear to directly mediate the actions of PModS. As an alternative signal transduction pathway, calcium mobilization and inositol phosphate (IP) metabolism were examined. PModS did not alter calcium uptake or intracellular calcium mobilization. PModS also did not influence the levels of inositol mono-, bis-, or trisphosphates, whereas calf serum did stimulate levels of all three IP metabolites in Sertoli cells. Therefore, PModS does not appear to act through a mobilization of calcium or increased metabolism of IP. A final signal transduction pathway involving phosphorylation was also examined. PModS treatment was found to increase tyrosine phosphorylation of specific proteins in a crude Sertoli cell cytosol preparation. Genistein is an inhibitor of tyrosine kinases and was found to reduce PModS actions at a 3.7-µM concentration of genistein and inhibit PModS actions at a 37-µM concentration of genistein. Therefore, PModS may act through a tyrosine phosphorylation event that remains to be elucidated. Combined observations indicate that PModS does not use cyclic nucleotides, calcium mobilization, or IP metabolism as a signal transduction pathway. The effects of PModS on cGMP levels appear to be indirectly related to an increase in the differentiation of Sertoli cells and not a direct second messenger. Observations are discussed with regard to the potential pharmacology of PModS and the regulation of Sertoli cell differentiation. (*Endocrinology* 134: 149–157, 1994)

THE MAINTENANCE and control of normal testicular physiology and the process of spermatogenesis require endocrine regulation. FSH initiates and maintains various Sertoli cell functions associated with a more differentiated state of the cell. In addition, LH regulates androgen production by Leydig cells that acts to maintain spermatogenesis. Both Sertoli cells (1) and peritubular myoid cells (2) express androgen receptors. Androgens have been shown to act on peritubular cells *in vitro* to stimulate the production of a paracrine factor that can regulate Sertoli cell functions (3). This paracrine factor, termed PModS, modulates Sertoli cell functions *in vitro* to a greater extent than any individual regulatory agent previously examined, including FSH (4, 5). Two isoforms of PModS have been purified, PModS(A) and PModS(B), that have slightly different mol wt and the same

apparent biological activities (4, 5). The information available implies that PModS(A) is a processed form of PModS(B) with equivalent bioactivity. Therefore, use of the term PModS will refer to the actions of PModS(A) and/or PModS(B). The only active agent in PSP found to stimulate Sertoli cell function (*e.g.* transferrin production) is PModS (4, 5). The production of PModS by peritubular cells, therefore, represents a potentially important mode of androgen action in the testis that is postulated to be vital to maintain the process of spermatogenesis.

Both FSH and PModS influence various Sertoli cell functions associated with the differentiation of the cell. One major differentiated function is the production of transferrin. Transferrin is synthesized and secreted by Sertoli cells to transport iron to the developing germinal cells sequestered by the blood-testis barrier (6). The secretion of transferrin provides a biochemical marker to assess the responsiveness of Sertoli cells to regulatory agents *in vitro*. PModS increases transferrin secretion by Sertoli cells to a greater extent than FSH alone and to the same extent as the combination of FSH, insulin, retinol, and testosterone (FIRT). The combination of PModS and FIRT increases Sertoli cell transferrin secretion in an additive fashion (5). This implies that PModS

may act through a different mechanism of action than any of the individual agents in FIRT.

The actions of FSH on Sertoli cell functions are mediated by cAMP and an increase in adenylate cyclase activity (7, 8). Analogs of cAMP mimic the actions of FSH on Sertoli functions (9, 10). In addition, FSH may influence calcium homeostasis in Sertoli cells (11). In contrast, PModS does not alter cAMP levels in Sertoli cells, but it does increase cGMP levels (5). The current study investigates the pharmacology of PModS and the potential involvement of several common signal transduction pathways.

## Materials and Methods

### Cell preparation and culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (12) with a modified procedure previously described by Tung et al. (13). Sertoli cells were isolated from 20-day-old rats to provide the highest purity cell preparation and minimize peritubular cell contamination, which increases when cells are isolated from younger rats. Decapsulated testicular fragments were digested first with trypsin (1.5 mg/ml; Gibco-BRL, Gaithersburg, MD) to remove interstitial cells and then with collagenase (1 mg/ml; type I; Sigma, St. Louis, MO) and hyaluronidase (1 mg/ml; Sigma). Sertoli cells were plated in 24-well (1 ml/well) Falcon plates (Falcon, Oxnard, CA) at $5 \times 10^5$ cells/well and in 6-well (3 ml/well) Falcon plates at $2 \times 10^6$ cells/well. Cells were maintained at 32 C in a 5% $CO_2$ atmosphere in serum-free Ham's F-12 medium (Gibco-BRL). Sertoli cell cultures were treated, as described in Results, at the time of plating and retreated after 48 h of culture when medium was replenished. Unless otherwise stated, a 72-h medium collection on day 5 of culture was obtained for analysis, and the cells were harvested for DNA or protein assay. Sertoli cell cultures were treated, as outlined in Results, with FSH (100 ng/ml; oFSH-16, NIDDK National Hormone and Pituitary Program, Baltimore, MD), insulin (5 $\mu$g/ml), retinol (0.35 $\mu$M), testosterone (1 $\mu$M), atrial natriuretic factor (ANF; 200 nM), sodium nitroprusside (1 mM), and 8-bromo-cGMP (0.1 mM). Peritubular cell-secreted proteins (PSP) and PModS were used at maximally effective concentrations to stimulate transferrin secretion by Sertoli cells. Dose-response curves were determined on each preparation of PSP and PModS. The maximal effective concentration of PSP was 5–10 $\mu$g/ml, that of PModS A was 5–10 ng/ml, and that of PModS B was 7–14 ng/ml. PSP was used at a minimum concentration of 50 $\mu$g/ml, and PModS(A) and PModS(B) at a minimum concentration of 25 ng/ml to allow for any variation in bioactivity between preparations and maintain a maximally effective concentration (4).

Peritubular cells were obtained from the collagenase-digested supernatant after tubule segments had sedimented, as described by Skinner et al. (4). Peritubular cells were plated in medium containing 10% calf serum and grown to confluence. Cells were then subcultured and plated at 25% confluence. After 3–4 days of culture, subcultured cells were confluent and were washed with serum-free medium. The cells were then cultured for up to 2 weeks in serum-free medium, with 48- to 72-h medium collections.

### PSP preparation

Freshly collected serum-free conditioned medium from peritubular cells was made 25 $\mu$M with phenylmethylsulfonylfluoride and 0.1 mM with benzamidine and then centrifuged at $1000 \times g$ for 15 min at 4 C to remove cell debris. When required, medium was frozen and stored at −20 C. Conditioned medium was concentrated 100-fold by ultrafiltration with an Amicon system (Amicon Corp., Lexington, MA) and a 3000 mol wt exclusion limit membrane. This 100-fold concentrated conditioned medium was termed PSP and had protein concentrations that ranged from 0.5–1.0 mg/ml. PSP was stored at −20 C until use.

### PModS preparation

PModS was purified from concentrated PSP, as described by Skinner et al. (4). Briefly, a 70% (wt/vol) saturated ammonium sulfate precipitate of concentrated conditioned medium was applied to a size-exclusion HPLC apparatus (Beckman Instruments, Fullerton, CA). The column was a series of TSK (Toyo-Soda) 7.5-mm × 30-cm columns taht included one SW 4000, two SW 3000, and one SW 2000. The column was equilibrated and eluted at 0.5 ml/min with 100 mM sodium sulfate, 50 mM sodium phosphate, 1 mM triethylamine, and 0.5% (vol/vol) ethylene glycol at pH 7.0. The active peak, determined by bioassay of Sertoli cell transferrin production, was collected and applied to a 1 × 15-cm heparin-Sepharose affinity column equilibrated in 50 mM Tris, pH 7.5. Proteins were eluted from the column with 1 M NaCl and applied to two successive C4 reverse phase columns (Vydac, Hesperia, CA) and eluted with a linear gradient from 25–60% acetonitrile. The purities of PModS(A) and PModS(B) were determined electrophoretically and chromatographically, as previously described (4). Purified forms of PModS were stored at −70 C before use in the presence of 1 mg/ml BSA.

### Transferrin RIA

Transferrin production by Sertoli cells was assayed by RIA, as described by Skinner and Griswold (14). An aliquot of the culture medium was incubated with rabbit antirat transferrin antibody (Cooper Biomedical, Malvern, PA) and iodinated transferrin for 1 h at 37 C, followed by a 1-h incubation with goat antirabbit immunoglobulin G antibody (Sigma). Complexed antibody was precipitated with polyethylene glycol and pelleted by centrifugation, and radioactivity in the pellets was determined. Data were normalized to Sertoli cell DNA at the time of medium collection.

### Cyclic nucleotide assay

Cyclic nucleotide assays were performed as described by Hansbrough and Garbers (15). Perchloric acid (0.5 M) that contained tracer amounts of [$^3$H]cAMP or [$^3$H]cGMP for estimation of cyclic nucleotide recoveries was added to plated cells. The samples were sonicated, centrifuged to remove cell debris, and then applied to Dowex AG 50W-X8 columns. Column fractions containing cAMP or cGMP were lyophilized and subsequently dissolved in water. An aliquot was incubated with rabbit cAMP or cGMP antibody (generously provided by Dr. D. L. Garbers, University of Texas Southwestern Medical Center, Dallas, TX) and iodinated cAMP or cGMP for 14–18 h at 4 C, followed by a 1-h precipitation at 4 C with bovine plasma (Sigma) and 12% polyethylene glycol. Complexed antibody was centrifuged, and radioactivity in the pellets was determined. Data were normalized to Sertoli cell protein at the time of cellular sonication.

### Guanylate cyclase assay

Cell fractions were prepared from Sertoli cells cultured for 2 days in control medium or from cells treated with the appropriate agents for the indicated times. Cells were suspended and sonicated in a solution containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 0.25 M sucrose, 1 mM benzamidine, 1 mM dithiotheitol, and 0.5 mM 1-methyl-3-isobutylxanthine. Membrane and cytosol fractions were obtained after centrifugation for 30 min at 400,000 × g. Forty micrograms of membrane protein or cytosol protein and 100 $\mu$g total cellular protein were incubated for 10 min at 36 C in a total volume of 0.15 ml containing 20 mM HEPES (pH 7.4), 1 mM MnCl$_2$, 0.2 mM 1-methyl-3-isobutylxanthine, and 0.1 mM GTP. Reactions were stopped by the addition of 1 vol HClO$_4$ (0.5 M). Samples were analyzed in an analogous manner as in the preceding section.

### Inositol phosphate hydrolysis

[$^3$H]Inositol phosphates were quantitated as described by Conn and Sanders-Bush (16). Sertoli cells were labeled with [$^3$H]myoinositol (American Radiolabeled Chemicals, St. Louis, MO) for 24-h. Cells were incubated for 30 min in fresh medium containing 10 mM LiCl. Cells

were then treated for the designated times. The medium was removed, and 95% (vol/vol) methanol was added. Cells were sonicated and extracted with 1 vol each of chloroform and 0.5 N HCl. The aqueous phase was applied to a Dowex-1 anion exchange resin and eluted with ammonium formate. [³H]Inositol phosphates were collected, and radioactivity was determined on a liquid scintillation counter. Data were normalized to Sertoli cell DNA.

### Calcium uptake

Radioactive calcium uptake was evaluated as described by Fukayama and Tashjian (17). Sertoli cells were cultured for 2 days and then incubated with fresh medium containing $^{45}Ca^{2+}$ (1.0 μCi/ml) in the absence or presence of regulatory agents. At designated times, the cells were washed rapidly three times with cold (4 C) $Ca^{2+}$-free buffered salt solution (Gibco-BRL) containing 0.1 mM LaCl₃. The cells were then suspended in 0.5 ml buffered salt solution and sonicated. Radioactivity was determined in a liquid scintillation counter. Net $^{45}Ca^{2+}$ uptake was calculated by subtracting the zero time value from each uptake measurement. All data were normalized to Sertoli cell DNA.

### Protein phosphorylation analysis

To examine the effects of the tyrosine kinase inhibitor genistein, Sertoli cells were plated and, after a 48-h period, incubated in the absence or presence of genistein (Sigma) for 72 h. During this 72-h incubation, cells were also incubated in the absence or presence of regulatory agents, described in Results. After the incubation, medium was collected for transferrin RIA, and cells were collected for analysis of DNA content.

To examine the direct effects of PModS on tyrosine phosphorylation, an immunoblot with a rabbit phosphotyrosine antibody (UBI, Lake Placid, NY) was performed. Sertoli cells were plated; on day 2 of culture, cells were incubated with various regulatory agents for 72 h. After the incubation, cells were collected and centrifuged at 13,000 × g for 1 h. The supernatant was collected and considered a crude Sertoli cell cytosol preparation and the pellet a crude membrane preparation. Proteins were electrophoretically separated on sodium dodecyl sulfate-polyacrylamide gels, as previously described (4). Proteins were electrophoretically blotted to a polyvinyldifluoride membrane (Immobilon, Millipore, South San Francisco, CA). The blotted membrane was blocked with 3% (w/vol) dry fat milk, incubated with a 1:4,000 dilution of phosphotyrosine antibody, treated with a secondary antibody conjugated with peroxidase at a dilution of 1:1,000, and detected by the Amersham chemiluminescence (ECL) procedure (Amersham Corp., Arlington Heights, IL) (18).

### DNA and protein assays

DNA was measured fluorometrically with ethidium bromide (19). At the end of the culture period, the medium was removed. Buffer containing 20 mM sodium chloride, 5 mM EDTA, and 10 mM Tris (pH 7.5) was added to the wells; then, the cells were sonicated. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide and 100 U/ml heparin), diluted 1:2 with buffer, and allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantitate DNA levels in the culture wells. This assay has a sensitivity of approximately 0.1 μg DNA and is linear up to 2.5 μg DNA. The total protein concentration was measured according to the method of Bradford (20).

### Statistical analysis

When designated, each data point was converted to a percentage of the control value and compared to control values, and a mean and SEM were determined from multiple experiments, as indicated in the figure legends. Data were analyzed using a SPSS statistical package (SPSS, Inc., Chicago, IL) with a one-way analysis of variance procedure and a Newman-Keuls multiple comparison procedure.

## Results

The influence of PModS and FSH on Sertoli cell cyclic nucleotide levels was examined. Sertoli cells were cultured for 2 days in the absence of regulatory agents, then treated with FSH or PModS. The concentration of FSH (100 ng/ml) used has previously been shown to be a maximal effective concentration to stimulate Sertoli cells, and dose-response curves provide an $EC_{50}$ of approximately 10 ng/ml with the current experimental conditions (5, 14). A 60-min FSH treatment increased cAMP levels dramatically in Sertoli cells. PSP and purified PModS did not alter cAMP levels in Sertoli cells (Fig. 1). In contrast, PSP and PModS(B) elevated Sertoli cell cGMP levels to a small, but significant, extent after 60 min (Fig. 1). FSH did not alter cGMP levels in Sertoli cells. The influence of PModS on Sertoli cell cGMP levels from 15 sec
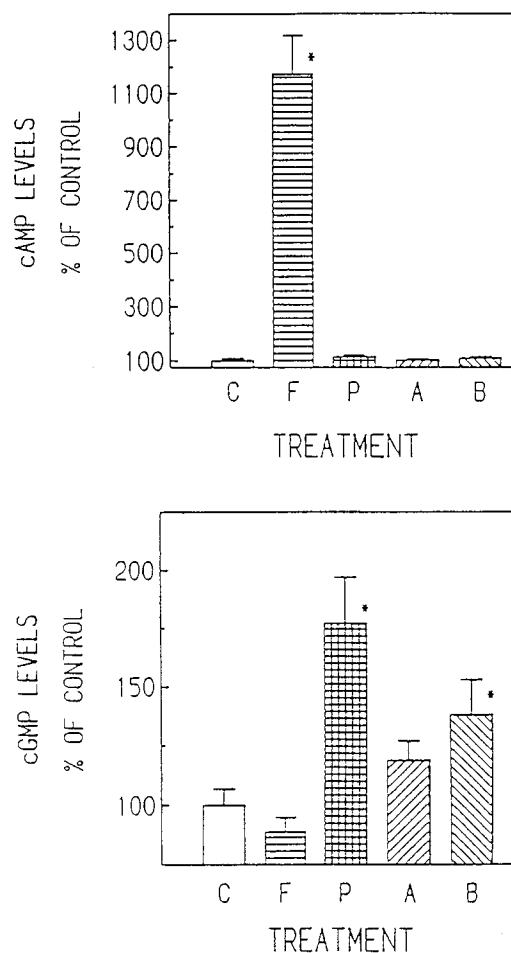


FIG. 1. cAMP and cGMP levels in Sertoli cells treated with PModS and FSH. Levels of cAMP (top panel) and cGMP (bottom panel) in Sertoli cells cultured for 60 min in the absence (C) or presence of 100 ng/ml FSH (F), 50 μg/ml PSP (P), 25 ng/ml PModS(A) (A), and 25 ng/ml PModS(B) (B) are shown. Maximal stimulating concentrations of the agents were used, as described in Materials and Methods. Data are presented as a percentage of the control values (mean ± SEM from seven different experiments, performed in duplicate). Control values for cAMP levels varied from 4.8–28.6 fmol/μg protein, and control values for cGMP levels varied from 1.1–16.3 fmol/μg protein. *, Statistically significant difference from the control [$P < 0.05$, determined by analysis of variance (ANOVA)].

to 5 days of treatment was then examined (Fig. 2). PSP elevated cGMP levels in Sertoli cells to approximately 200% of the control value after 15 sec. Sertoli cell cGMP levels continued to rise in response to PModS to 350% of the control value after 5 days. To verify that the elevation of cGMP levels in Sertoli cells treated with PSP was due to the actions of PModS, Sertoli cells were treated with purified PModS at selected time points. Although treatment of Sertoli cells with purified PModS did not cause an early increase in cGMP levels as did PSP (Table 1), PModS(B) elevated cGMP levels after 1- and 72-h treatments, and PModS(A) increased cGMP levels after 72 h. FSH had no influence on cGMP levels at any time point examined (Table 1). These studies suggest that the elevation in Sertoli cell cGMP levels by purified PModS occurs after a 1-h treatment, and stimulation is maintained over the time course examined.

As a comparison with PModS, the abilities of other regulatory agents to influence cGMP levels in the Sertoli cell were examined. ANF is an activator of a particulate form of

guanylate cyclase in a variety of tissues (21, 22). Treatment of cultured Sertoli cells with ANF resulted in both a dose-dependent (Fig. 3, *bottom panel*) and a time-dependent (data not shown) elevation of cGMP levels in Sertoli cells. cGMP levels were elevated to 2800% of control levels after a 10-min treatment with 1 $\mu$M ANF. Sodium nitroprusside, an activator of soluble guanylate cyclase (23), elevated Sertoli cell cGMP to levels similar to those in ANF-treated cells (data not shown). The elevation of Sertoli cell cGMP levels by ANF was compared to that by PSP (Fig. 3, *top panel*). ANF caused a dramatic elevation of cGMP levels after 3 min, whereas the increase in cGMP levels by PModS was marginal. After 72 h, PModS-treated cells had cGMP levels about 350% of the control value, whereas those in ANF-treated cells were not different from control levels.

The influence of PModS on guanylate cyclase activity was investigated as a potential cause of the increased cGMP levels. Sertoli cells were homogenized after 2 days of culture in control medium, and guanylate cyclase activity in the whole cell homogenate was determined. The linearity of the
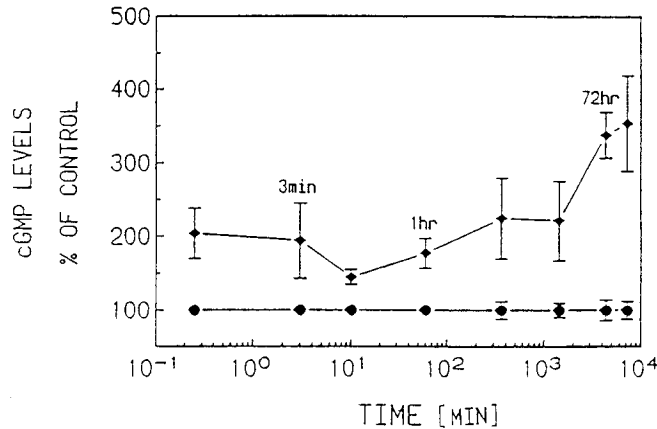


FIG. 2. Time course of cGMP levels in Sertoli cells treated with PSP. Levels of cGMP in Sertoli cells cultured for various times in the absence (●) or presence of 50 $\mu$g/ml PSP (◆). A maximal stimulating concentration of PSP was used, as described in *Materials and Methods*. Data are presented as a percentage of the control values (mean ± SEM from four different experiments, performed in duplicate). Control values varied from 1.1–28.1 fmol/$\mu$g protein. All PSP treatments were statistically significant from controls by ANOVA ($P < 0.05$).

**TABLE 1.** cGMP levels in Sertoli cells treated for various times with PMODS and FSH

| Treatment | Length of treatment | | |
|---|---|---|---|
| | 3 min | 1 h | 72 h |
| Control | 100 ± 05 | 100 ± 07 | 100 ± 05 |
| FSH | 92 ± 07 | 90 ± 06 | 108 ± 12 |
| PSP | 194 ± 51[a] | 177 ± 20[a] | 337 ± 31[a] |
| PMSA | 115 ± 13 | 119 ± 08 | 173 ± 08[a] |
| PMSB | 118 ± 24 | 138 ± 15[a] | 340 ± 78[a] |

Levels of cGMP in Sertoli cells cultured for various times in the absence (Control) or presence of 100 ng/ml FSH, 50 $\mu$g/ml PSP, 25 ng/ml PMODS A (PMSA), and 25 ng/ml PMODS B (PMSB) are shown. Maximal stimulating concentrations of the agents were used, as described in *Materials and Methods*. Data are presented as a percentage of the control values (mean ± SEM from seven different experiments performed in duplicate).

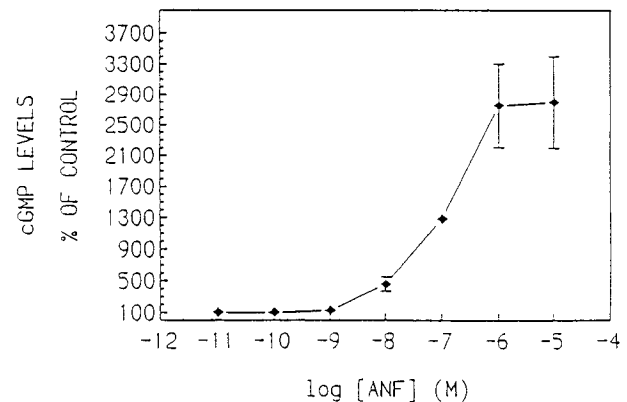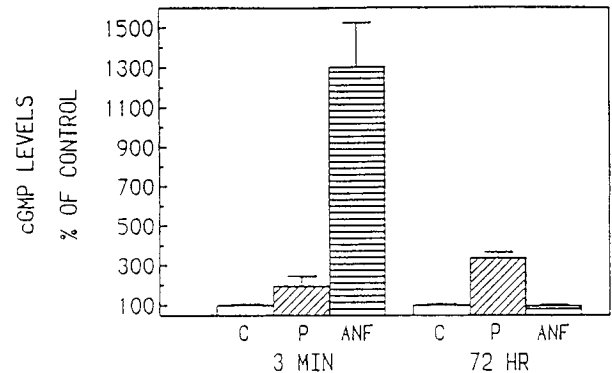[a] Statistically significant difference from the control ($P < 0.05$, determined by ANOVA).



FIG. 3. cGMP levels in Sertoli cells treated with PSP and ANF. Levels of cGMP were determined in Sertoli cells cultured for 3 min or 72 h in the absence (C) or presence of 50 $\mu$g/ml PSP (P) and 200 nM ANF (*top panel*). Data are presented as a percentage of control values (mean ± SEM from seven experiments, performed in duplicate). Levels of cGMP were determined in Sertoli cells cultured for 10 min in the presence of increasing concentrations of ANF (*bottom panel*). Data are presented as a percentage of control values (mean ± SEM from four experiments, performed in duplicate).

assay was determined with respect to time and quantity of enzyme. Neither PSP nor the purified forms of PModS increased guanylate cyclase activity (Fig. 4). In contrast, both ANF and sodium nitroprusside increased guanylate cyclase activity. A particulate fraction of Sertoli cells was prepared, as described in *Materials and Methods*. Guanylate cyclase activity in the particulate fraction represented about 30% of the activity in the total homogenate. PModS did not increase the activity of particulate guanylate cyclase, whereas ANF did increase the activity of particulate guanylate cyclase (data not shown). In addition, Sertoli cells were cultured in the presence of PModS for 3-min, 1-h, or 72-h treatments, and guanylate cyclase activity was determined in whole cell homogenates. PModS did not increase guanylate cyclase activity in these homogenates (data not shown). These observations suggest that elevation of Sertoli cell cGMP levels by PModS does not occur though detectable direct activation of either the particulate or soluble forms of guanylate cyclase under the experimental conditions used. PModS also did not influence the level of cGMP-dependent phosphodiesterase (data not shown). Therefore, the hydrolysis of cGMP did not appear to be influenced by PModS.

The ability of increased levels of cGMP to stimulate a physiological response in Sertoli cells was investigated. Sertoli cells were cultured with agents that elevate cGMP levels to examine effects on transferrin secretion. Neither ANF, sodium nitroprusside, nor 8-bromo-cGMP alone influenced transferrin secretion by cultured Sertoli cells (Fig. 5). Under the same conditions, PSP and FIRT increased transferrin secretion about 330% and 380%, respectively. ANF also did not alter the effects of PSP or FIRT on transferrin secretion (Fig. 5). Similar results were obtained with sodium nitroprusside and 8-bromo-cGMP in combination with PSP or FIRT (data not shown). These observations imply that the increase in cGMP levels associated with PSP or PModS
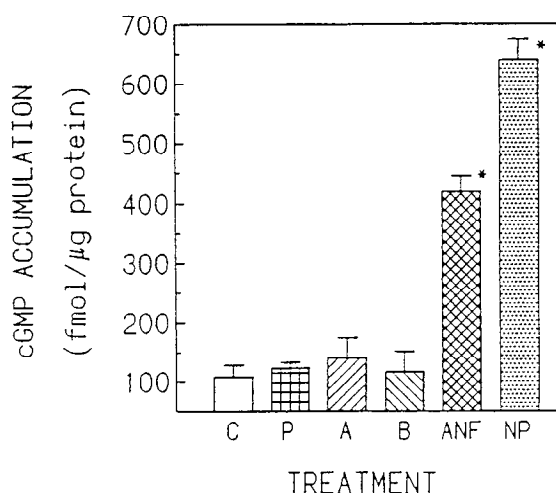


FIG. 4. Guanylate cyclase activity in whole homogenates of Sertoli cells. cGMP levels were determined in whole Sertoli cell homogenates after 10 min in the absence (C) or presence of 50 μg/ml PSP (P), 25 ng/ml PModS(A) (A), 25 ng/ml PModS(B) (B), 1 μM ANF, and 1 mM sodium nitroprusside (NP). Data are the mean ± SEM from four experiments, performed in triplicate. *, Statistically significant difference from the control (P < 0.05, determined by ANOVA).
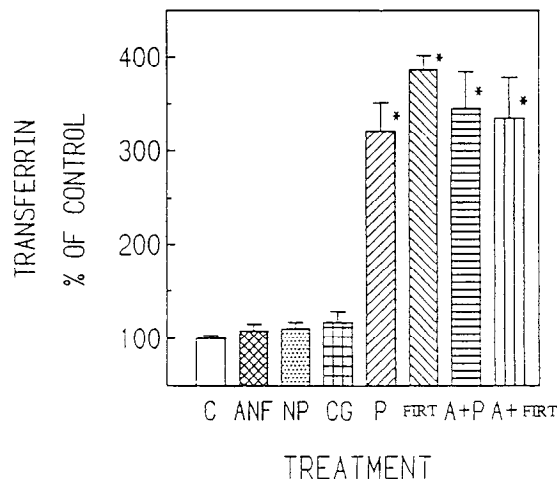


FIG. 5. Transferrin secretion by Sertoli cells in the presence of agents that elevate cGMP levels. Transferrin secretion by Sertoli cells cultured in the absence (C) or presence of 200 nM ANF, 1 mM sodium nitroprusside (NP), 0.1 mM 8-bromo-cGMP (CG), 50 μg/ml PSP (P), FIRT, ANF and PSP (A+P), and ANF and FIRT (A+FIRT) is shown. Data are presented as a percentage of the control values (mean ± SEM from four different experiments, performed in duplicate). Control values varied from 14.6–24.7 ng/μg DNA. *, Statistically significant difference from the control (P < 0.05, determined by ANOVA).

treatment of Sertoli cells does not appear to directly mediate the actions of PModS to increase transferrin secretion. As previously discussed, the only agent found in PSP to stimulate transferrin production is PModS (4).

To examine the influence of PModS on PI hydrolysis, Sertoli cells were cultured for 2 days in the absence of regulatory agents and then treated with PSP or fetal calf serum for a minimum of 15 sec and a maximum of 5 min. PSP did not increase the levels of inositol trisphosphate ($IP_3$), inositol bisphosphate ($IP_2$), or inositol monophosphate ($IP_1$) in Sertoli cells at any time point examined (Fig. 6). Fetal calf serum, which served as a positive control, increased the levels of all three inositol phosphates. To confirm the results obtained with PSP, purified PModS was used to treat Sertoli cells at selected time points and was found not to influence PI hydrolysis (data not shown). Longer term treatments with PModS at 30 and 60 min also had no influence on PI metabolism (data not shown).

The ability of PModS to influence calcium uptake by Sertoli cells was examined. Sertoli cells were cultured in the absence of regulatory agents for 2 days, then were treated with either FSH or PSP in medium containing $^{45}Ca^{2+}$. Neither FSH nor PSP altered calcium uptake by Sertoli cells after 10 min (Fig. 7). A calcium ionophore, ionomycin, was used as a positive control and increased $^{45}Ca^{2+}$ uptake after 10 min. Purified PModS was used to confirm the results obtained with PSP and was not found to influence $^{45}Ca^{2+}$ uptake (data not shown). Preliminary experiments to investigate intracellular calcium mobilization used fura-2, as previously described with Sertoli cells (24). These studies were generously performed by Dr. David Handelsman, University of Sydney (Sydney, Australia). PModS had no effect on calcium mobilization, as measured by alterations in intracellular fura-2 fluorescence (data not shown). These results indicate that
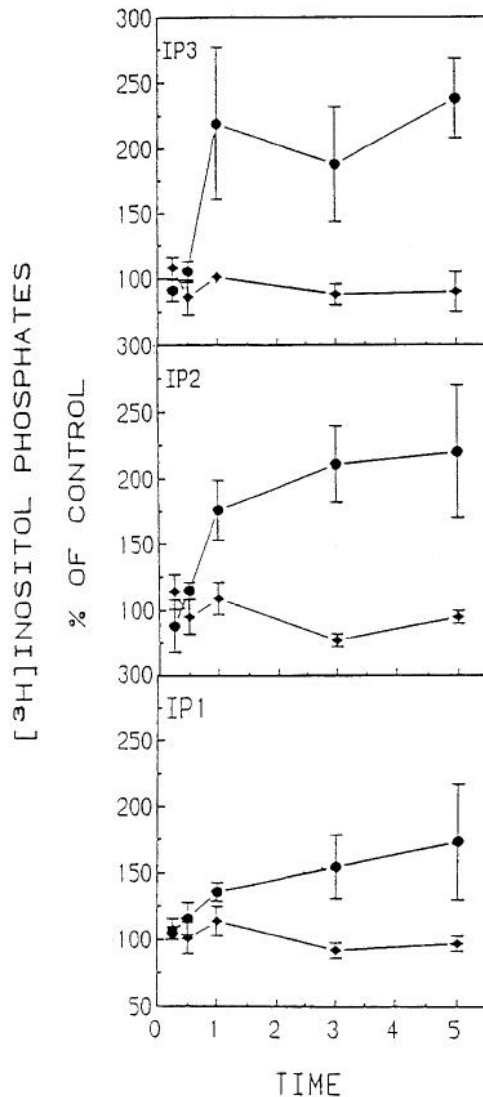
FIG. 6. Time course of $IP_3$, $IP_2$, and $IP_1$ levels in Sertoli cells treated with PSP and fetal calf serum. Levels of $IP_3$, $IP_2$, and $IP_1$ were determined in Sertoli cells cultured for various times in the presence of 50 $\mu g$/ml PSP (♦) or 10% fetal calf serum (●). A maximal stimulating concentration of PSP was used, as described in *Materials and Methods*. Data are presented as a percentage of the control values (mean ± SEM from four different experiments, performed in duplicate). Control values varied from 3.8–17.6 cpm/$\mu g$ DNA.



FIG. 7. Effects of FSH and PSP on $^{45}Ca^{2+}$ uptake by Sertoli cells. $^{45}Ca^{2+}$ uptake was determined in Sertoli cells cultured for 10 min in the absence (C) or presence of 100 ng/ml FSH (F), 50 $\mu g$/ml PSP (P), and 1 $\mu M$ ionomycin (I). Maximal stimulating concentrations of the agents were used, as described in *Materials and Methods*. Data are presented as a percentage of the control values (mean ± SEM from four different experiments, performed in duplicate). Control values varied from 12.6–37.9 cpm/$\mu g$ DNA. *, Statistically significant difference from the control ($P < 0.05$, determined by ANOVA).



FIG. 8. Phosphotyrosine immunoblot of Sertoli cell proteins from a crude cytosol preparation. Cells were cultured in the absence (CON) or presence of PSP or PModS-B (PMS) for 72 h before collection and electrophoresis. Migrations of protein markers (kilodaltons) are listed in the *left margin*, and the *arrow* identifies the 55-kilodalton protein. Data are representative of three different experiments with separate cell preparations.

PModS does not appear to influence calcium flux or mobilization.

The final signal transduction pathway examined involved protein phosphorylation. Sertoli cells were treated with PModS, FSH, and $^{32}P$ to analyze alterations in abundant phosphorylated proteins. These preliminary studies were generously performed by Dr. Mary Hunzicker-Dunn, Northwestern University (Evanston, IL), as previously described (25). Although FSH was found to both induce and eliminate the phosphorylation of various cellular proteins in Sertoli cells, PModS did not alter the phosphorylation of detectable phosphorylated proteins (data not shown). However, PModS influenced phosphotyrosine in proteins from a crude Sertoli cell cytosol preparation, as determined with an immunoblot
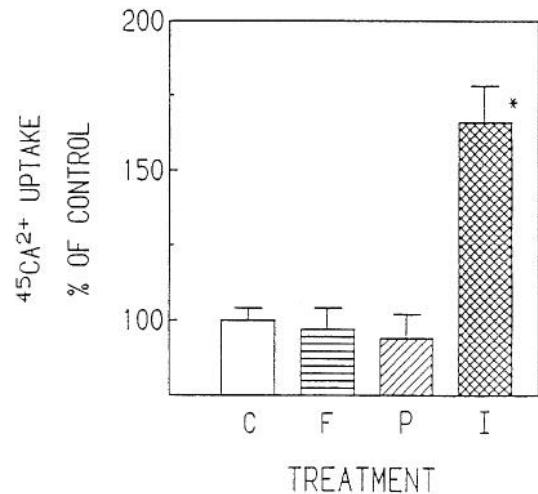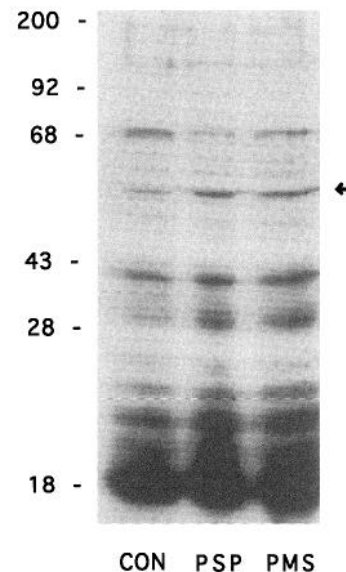
using phosphotyrosine antibodies (Fig. 8). PModS was found to consistently increase and/or induce the levels of a 55-kilodalton protein in the cytosol preparation. Other proteins were also periodically influenced by PModS, but not reproducibly between experiments. The phosphotyrosine-containing proteins were not affected by PModS in the crude Sertoli cell membrane preparation. To extend these studies, Sertoli

cells were incubated in the absence or presence of the tyrosine kinase inhibitor genistein (26). Genistein reduced the effect of PModS on Sertoli cells at a concentration of 3.5 $\mu$M (Fig. 9). This same concentration of genistein inhibited the actions of FSH. At higher genistein concentrations of 17.5 $\mu$M (data not shown) and 35 $\mu$M, the ability of PModS to stimulate transferrin production was completely inhibited (Fig. 9). Similar results were obtained with PSP (data not shown). Calf serum (10%, vol/vol) was used as a positive control and was not affected by the presence of genistein at any concentration used. Therefore, the Sertoli cells exposed to a 35-$\mu$M genistein concentration maintained viability and responsiveness to calf serum. These results imply that PModS can influence tyrosine phosphorylation of Sertoli cell proteins, and inhibition of tyrosine phosphorylation abolishes the activity of PModS on Sertoli cells.

## Discussion

Sertoli cell functions are influenced by a variety of regulatory agents. The gonadotropin FSH and the testicular paracrine factor PModS stimulate functions associated with a more differentiated state of the Sertoli cell. These functions include the secretion of androgen-binding protein and transferrin (4, 5). Previous studies demonstrated that FSH increases transferrin secretion by Sertoli cells to about 200% of control levels, whereas PModS elevates transferrin secretion to about 400% of control levels. In addition, the combination of maximally effective concentrations of FIRT and PModS stimulates transferrin secretion by Sertoli cells to a higher level than achieved by either treatment alone (5). The observations that PModS has such dramatic effects on an important Sertoli cell function and that the combination of PModS and FIRT results in an additive response suggest that PModS
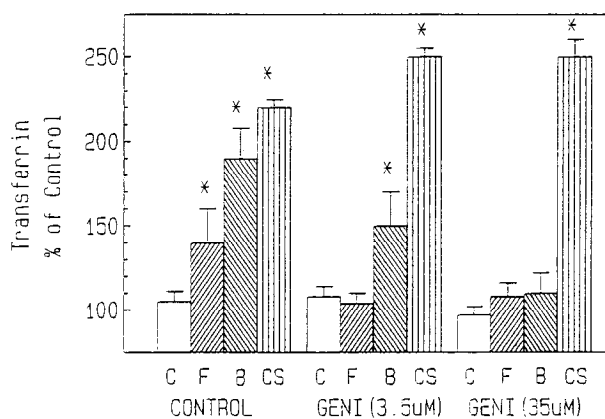


FIG. 9. Effects of a tyrosine kinase inhibitor, genistein, on PModS-stimulated Sertoli cell transferrin secretion. Cells were cultured in the absence (control) or presence (geni) of genistein at the concentrations indicated and incubated in the absence (C) or presence of PModS-B (B), FSH (F), or 10% (vol/vol) calf serum (CS) for 72 h. Medium was collected, and transferrin levels were determined and normalized for micrograms of DNA (transferrin, nanograms per $\mu$g DNA), then expressed as a percentage of the control. The control transferrin level was 25 ng/$\mu$g DNA. Data are presented as the mean ± SEM from seven different experiments, performed in replicate. *, Statistically significant difference from control ($P < 0.05$).

is a major regulator of Sertoli cell function and differentiation. In addition, PModS appears to have a mechanism of action different from that of the individual regulatory agents in FIRT.

FSH is known to influence Sertoli cell functions though an elevation of cAMP levels, whereas previous studies demonstrated that PModS increases cGMP levels (5). For this reason, further analysis of the influence of PModS on cyclic nucleotide levels in Sertoli cells was initiated. Although PModS did not influence Sertoli cell cAMP levels, PModS did elevate cGMP levels. Previous reports have identified agents that elevate cGMP levels in whole testicular preparations or Leydig cells (27-29). However, this is the first report of an alteration in Sertoli cell cGMP levels by any agent. PSP rapidly increased and maintained cGMP levels in Sertoli cells over an extended period. In contrast, purified PModS only mimicked PSP actions after treatment of Sertoli cells for 1 h and longer. The difference between the effects of PSP and PModS at 3 min is unclear. PSP may contain an alternate substance not associated with PModS that increases short term cGMP levels in Sertoli cells. The elevation in cGMP levels observed after 3-5 days of PModS treatment may be due to the long term effects of PModS on Sertoli cell differentiation.

Both ANF and sodium nitroprusside also increased cGMP levels in cultured Sertoli cells. ANF stimulates the activity of the particulate fraction of guanylate cyclase from Sertoli cells, whereas sodium nitroprusside stimulates the cytosolic form of the enzyme. In contrast, neither PSP nor purified PModS stimulated guanylate cyclase activity under the experimental conditions used. It is possible that PModS does stimulate guanylate cyclase activity in Sertoli cells, but that the response is dependent on a particular set of experimental conditions not present in the current study. The physiological significance of the effects of ANF and sodium nitroprusside on cGMP levels in Sertoli cells is unclear. Neither agent nor a cGMP analog altered transferrin secretion. Although PModS can stimulate cGMP levels in Sertoli cells, cGMP does not appear to directly mediate the actions of PModS. The increase in cGMP may be indirectly related to an induction of Sertoli cell differentiation by PModS.

Another second messenger reported to be influenced by FSH in Sertoli cells is calcium. Increased cellular calcium levels could provide a mechanism for the PModS-induced elevation of cGMP levels in Sertoli cells. Nitric oxide synthetase is a calcium-calmodulin-dependent enzyme that catalyses the oxidation of L-arginine to nitric oxide and citrulline (30). Thus, an elevation of cellular calcium levels could lead to the formation of nitric oxide with subsequent activation of the soluble form of guanylate cyclase and an elevation of cGMP levels. FSH has been shown to alter calcium homeostasis in Sertoli cells by increased uptake of extracellular calcium (31, 32). The inability of FSH to alter calcium uptake in the current study could be due to the sensitivity of the assays or the use of 20-day-old rat Sertoli cells, which are less responsive to FSH than younger 10- to 15-day-old rat Sertoli cells. PSP does stimulate 20-day-old Sertoli cells, but did not alter calcium uptake by Sertoli cells. In addition,

preliminary studies using fluorescent procedures indicate that PModS does not influence intracellular calcium mobilization. Therefore, the actions of PModS do not appear to be mediated by calcium mobilization. Calcium homeostasis also may be influenced by elevation of $IP_3$ levels by PI hydrolysis and release of calcium from intracellular stores by $IP_3$. In choroid plexus cells of the brain, serotonin activates PI hydrolysis and increases transferrin secretion. However, receptor antagonists that block PI hydrolysis in choroid plexus cells do not block transferrin production (33). PModS did not alter the levels of inositol phosphates in Sertoli cells. In addition, FSH inhibits PI hydrolysis in Sertoli cells (34), whereas the actions of FSH and PModS on transferrin secretion are additive. These observations suggest that inositol phosphates do not mediate the actions of PModS on Sertoli cells.

The current study demonstrates that PModS elevates Sertoli cell cGMP levels, but cGMP does not directly mediate the actions of PModS. Other Sertoli cell signaling pathways, such as cAMP levels, calcium uptake, and PI hydrolysis, were not influenced by PModS. These observations imply that PModS may influence an important phosphorylation event. PModS was found to influence the tyrosine phosphorylation of a Sertoli cell protein(s) after a 72-h treatment. Whether these tyrosine phosphorylations are associated with the increased differentiation of the cell and/or mediate the actions of PModS will require a detailed time-course study and characterization of these phosphorylated proteins. The tyrosine kinase inhibitor studies indicate that genistein inhibits the actions of PModS and support a potential role of tyrosine phosphorylation in the actions of PModS. A limitation to this study is that it is difficult to eliminate potential nonspecific effects of the kinase inhibitor on cell viability and hormone responsiveness. Therefore, a more thorough investigation of the actions of PModS on protein phosphorylation is required to elucidate the pharmacology of PModS.

Recent findings suggest that PModS increases the expression of nuclear transcription factors (e.g. c-fos). This is further supported by the ability of an antisense oligonucleotide to c-fos mRNA to partially inhibit the actions of PModS on Sertoli cell transferrin secretion (35). The ability of PModS to induce Sertoli cell differentiation is postulated to involve an alteration in the expression of unique transcription factors. Understanding the signal transduction pathway used by PModS to induce the expression of these transcription factors will provide insight into the molecular mechanisms that control Sertoli cell differentiation.

## Acknowledgments

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