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Regulation of Sertoli Cell Function and Differentiation through the Actions of a Testicular Paracrine Factor P-Mod-S*

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ABSTRACT. The current study was designed to investigate the actions of the testicular paracrine factor P-Mod-S on Sertoli cell function and differentiation. Transferrin production by Sertoli cells was stimulated by P-Mod-S to a greater extent than any individual regulatory agent and in a manner similar to a combination of FSH, insulin, retinol, and testosterone (FIRT). P-Mod-S had an additive response in combination with FIRT. The increase in transferrin production with a combination of P-Mod-S and FIRT is the highest level of stimulation (up to 8-fold) observed. These profound effects of P-Mod-S on Sertoli cell function implied a potential unique mechanism of action for the paracrine factor. FSH and FIRT significantly stimulated cAMP levels with both 60-min and 72-h treatments. In contrast, P-Mod-S had no effect on cAMP levels with a 60-min treatment and only a small increase with a 72-h treatment. Interestingly, P-Mod-S stimulated cGMP levels that remained above basal levels up to 72 h of treatment. FSH had no effect on cGMP levels. P-Mod-S did not affect inositol phosphate hydrolysis with treatments between 15 and 60 min. The actions of P-Mod-S on cGMP levels influenced Sertoli cell function on a molecular level. Northern blot analysis indicated that P-Mod-S and FIRT both stimulated the apparent levels of the 2.6-kilobase transcript of transferrin and the 1.7-kilobase transcript of androgen-binding protein. A solution hybridization procedure was used to quantitate the influence of P-Mod-S on Sertoli cell gene expression. P-Mod-S stimulated steady state levels of both transferrin and androgen-binding protein message approximately 2-fold, similar to the effects of FIRT. Both forms of P-Mod-S had similar biological activities and mechanisms of action. P-Mod-S (A) and P-Mod-S (B) both stimulated cGMP, altered Sertoli cell gene expression, and had profound effects on transferrin production. Although slightly different biochemically, both forms of P-Mod-S appear to be functionally similar. Combined observations indicate that the paracrine factor produced by peritubular cells, P-Mod-S, acts on Sertoli cells in part through a cGMP-mediated response to influence the expression of specific genes which subsequently have profound effects on Sertoli cell function and differentiation. (*Endocrinology* **124:** 2711–2719, 1989)

THE REGULATION of Sertoli cell function and differentiation requires a number of complex cell-cell interactions as well as the actions of different regulatory agents (1). Sertoli cells are the epithelial cells that form the seminiferous tubules and provide the cytoarchitectural support and microenvironment required for germinal cell development. Regulation of Sertoli cell function and differentiation, therefore, will have a profound effect on the process of spermatogenesis and the maintenance of testis function. Peritubular cells surround the seminiferous tubules and are separated from the basal surface of the Sertoli cells by a complex extracellular matrix (2) produced cooperatively by both cell types (3). Another important cell type in the testis is the Leydig cell, which is present in the interstitium and responsible for the production of androgens. The interactions that occur among Sertoli, peritubular, Leydig, and germinal cells are critical for the maintenance and control of testicular function (1).

The endocrine regulation of testicular function primarily involves the actions of gonadotropins. FSH acts on Sertoli cells to initiate and maintain cellular differentiation. LH acts on Leydig cells to promote androgen production. Subsequently, androgens act on the seminiferous tubule to maintain the process of spermatogenesis. Although androgens may act directly on Sertoli cells (4), peritubular cells contain a high percentage of the androgen receptors (5) and provide a site of androgen action. Peritubular cells have been shown to produce a paracrine factor that can modulate Sertoli cell function, termed P-Mod-S (6). The production of P-Mod-S is stimulated by androgen actions on peritubular cells (6), and peritubular cells augment the actions of androgens on Sertoli cells (7). Therefore, the cell-cell interaction is proposed in which Leydig cells under LH control produce androgen that can act on peritubular cells to promote the production of P-Mod-S which then acts on Sertoli cells to

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influence the functions involved in the maintenance of germinal cell development (1). This cell-cell interaction is postulated to play an important role in the maintenance of the process of spermatogenesis and testicular function as well as provide an important mode of androgen action in the testis.

P-Mod-S has been purified into two apparently related forms with similar biological activities (8). P-Mod-S (A) is a 56K protein, while P-Mod-S (B) is a 59K protein. P-Mod-S was found to have a more profound effect in influencing Sertoli cell functions than any individual regulatory agent previously identified, including FSH (8, 9). These observations have led to the proposal that P-Mod-S may have a role in regulating Sertoli cell function and differentiation; therefore, P-Mod-S may indirectly regulate the process of spermatogenesis and maintain testicular function. The current study was designed to investigate further the actions of P-Mod-S on Sertoli cells. Observations are presented regarding the mechanism of action of P-Mod-S and the combined actions of P-Mod-S and hormones on Sertoli cell function.

Materials and Methods

Cell preparation and culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (10) with a modified procedure previously described (11). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Gibco, Grand Island, NY) to remove Leydig cells, followed by a collagenase digestion (1 mg/ml type I; Sigma, St. Louis, MO), and then a hyaluronidase digestion (1 mg/ml; Sigma). Sertoli cells were then plated in 24-well (1 ml/well) Linbro plates at approximately 5×10^5 cells/well. Cells were maintained at 32 C in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco). Sertoli cultures were treated, as described in *Results*, at the time of plating and retreated after 48 h of culture when the 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 assay or RNA isolation. Sertoli cell cultures were treated, as outlined in *Results*, with test substances or with FSH (100 ng/ml; NIDDK and National Hormone and Pituitary Program, Baltimore, MD), insulin (5 μg/ml), retinol (0.35 μM), and testosterone (1 μM). Peritubular cell-secreted proteins (PSP) were used at a concentration of 50 μg/ml, and P-Mod-S (A) and P-Mod-S (B) at a minimal concentration of 25 ng/ml.

Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had gravity sedimented as previously described (8). 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 washed for 24 h with serum-free medium. The cells were then cultured for up to 2 weeks in serum-free medium with 48-h medium collections.

Freshly collected peritubular cell serum-free conditioned medium was made 25 μM phenylmethylsulfonylfluoride and 0.1 mM benzamidine and then centrifuged at 1000 × *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) using a membrane with a 3000 mol wt exclusion limit.

P-Mod-S preparation

P-Mod-S was purified from concentrated peritubular cell conditioned medium as previously described (8). Briefly, an ammonium sulfate precipitate of concentrated conditioned medium was applied to a size exclusion HPLC column. The active peak was collected and applied to a heparin-Sepharose affinity column and eluted with high salt conditions. Proteins that bound to the heparin-Sepharose column were applied to two successive C4 reverse phase columns and eluted with acetonitrile. Purified forms of P-Mod-S were stored at −70 C before use, generally in the presence of 1 mg/ml BSA.

Transferrin RIA

Transferrin production by Sertoli cells was assayed by a RIA described previously (12). An aliquot of the culture medium was incubated with rabbit antirat transferrin antibody (Cooper Biomedical, Melvern, 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 then precipitated with polyethylene glycol (Sigma) and pelleted by centrifugation, and radioactivity in the pellets was determined. All data were normalized per μg Sertoli cell DNA at the time of medium collection and expressed as nanograms of transferrin per μg DNA.

Cyclic nucleotide assay

Cyclic nucleotide assays were performed as previously described (13). $HClO_4$ (0.5 M) was added to plated cells or collected medium samples that contained tracer amounts of [^{3}H]cAMP or [^{3}H]cGMP for estimation of cyclic nucleotide recoveries. 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, dissolved in water, and quantitated by RIA.

Phosphoinositide metabolism

Inositol phosphate hydrolysis was quantitated as previously described (14). Sertoli cells were prelabeled with [^{3}H]myo-inositol (American Radiolabeled Chemicals, St. Louis, MO) for 24 h, followed by removal of the radiolabel and incubation for 30 min in 10 mM LiCl. Cells were then treated for designated times, followed by removal of the medium and addition of 95% (vol/vol) methanol. Cells were sonicated and extracted with 1 vol each of chloroform and 0.5 M HCl, followed by low speed centrifugation. The aqueous phase was applied to a Dowex-1 anion exchange resin and eluted with ammonium formate. [^{3}H] Inositol phosphate was collected, and radioactivity was determined on a liquid scintillation counter.

RNA isolation and Northern analysis

RNA was obtained from Sertoli cells and extracted with 1% SDS, 2 mM EDTA, 0.1 M NaCl, and 10 mM Tris-HCl, pH 7.5, and passed through a 22-gauge needle, then digested with proteinase-K (50 μg/ml). Total RNA was obtained with an ethanol precipitation of the sample after a phenol-chloroform extraction. Polyadenylated RNA was isolated with a oligodeoxythymidine affinity column. Polyadenylated RNA was separated electrophoretically on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and analyzed with a Northern blot procedure previously described (15). The rat transferrin cRNA probe was obtained from a cDNA fragment that contained a 390-basepair coding region (16) (generously provided by Dr. M. D. Griswold, Washington State University, Pullman, WA). The rat androgen-binding protein (ABP) cRNA probe was obtained from a cDNA probe fragment that contained a 1400-basepair coding region (17) (generously provided by Dr. D. R. Joseph, University of North Carolina, Chapel Hill, NC). These inserts were subcloned into the plasmid SP65 in the antisense orientation with regard to the transcriptional direction of the SP6 promotor. The cRNA probes were labeled with [^{32}P]UTP, as previously described (18). A 700-basepair insert of p1B15 (19), a rat cDNA which encodes cyclophilin, was subcloned into the plasmid SP65 promotor to produce a cRNA probe. p1B15 is a gene that appears to be constitutively expressed and was used as a control probe to demonstrate intact RNA. RNA was hybridized to the various probes at 65 C for 20 h in a solution of 50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 0.1% sodium dodecyl sulfate (SDS), 0.2% Ficoll, 50 μg/ml polyadenylic acid, 0.2% BSA, 0.2% polyvinylpyrrolidone, and 400 μg/ml sonicated denatured salmon sperm DNA. Post-hybridization washes consisted of one 20-min wash at room temperature in 0.03 M sodium citrate, 0.3 M NaCl, and 0.1% SDS, then two 30-min washes at 68 C in 0.003 M sodium citrate, 0.03 M NaCl, and 0.1% SDS. Hybridized cRNA probes were detected with autoradiography.

Solution hybridization

Solution hybridizations were carried out with modifications of a procedure previously described (16). Total RNA samples were dried and reconstituted with 20 μl reaction buffer [20 mM Tris (pH 7.5), 1.2 M NaCl, 10 mM EDTA, 0.2% (wt/vol) SDS, and 1 mg/ml yeast RNA from Boehringer Mannheim, Indianapolis, IN], which contained 30,000 cpm [^{32}P]UTP-radiolabeled cRNA probe. Samples were incubated for 18 h at 60 C. One milliliter of dilution buffer [10 mM Tris (pH 7.5), 0.3 M NaCl, and 5 mM EDTA], which contained RNAse T1 (100 U/ml; Sigma) and RNAse A (12.5 μg/ml; type IIA, Sigma), was added to the samples and incubated at 37 C for 30 min. After the RNAse digestion 100 μl 100% (wt/vol) trichloroacetic acid which contained 10 μg/ml yeast RNA were added to the samples and incubated on ice for 1 h. Samples were then filtered onto GF/C filters (Whatman, Hillsboro, OR) and washed with 10% (wt/vol) trichloroacetic acid at 4 C. Filters were counted in 5 ml ACS scintillation fluid (Amersham, Arlington Heights, IL). Solution hybridizations were performed on each total RNA sample with transferrin, ABP, and 1B15 cRNA probes.

DNA and protein assays

DNA was measured fluorometrically with ethidium bromide (20). At the end of the culture period, the medium was removed, ethidium bromide buffer (EBB; 20 mM sodium chloride, 5 mM EDTA, and 10 mM Tris, pH 7.5; Sigma), was added to the wells, and 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 in EBB) and diluted 1:2 with EBB 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 (21).

Statistical analysis

Transferrin data was normalized for micrograms of DNA, cyclic nucleotide data were normalized for μg protein, and solution hybridization was normalized for 1B15 hybridization. When designated, each data point was converted to fold change from control or basal treatment, then a mean and SEM were determined from multiple experiments in replicate as indicated in the figure legends. Student's t test was performed on all data, and the results are presented in the figure legends.

Results

Transferrin production by Sertoli cells is a marker of cellular function and differentiation (12, 22) and was used in the current study to examine the actions of various regulatory agents. FSH and a combination of FSH, insulin, retinol, and testosterone (FIRT) were found to stimulate transferrin production (Fig. 1). Testosterone did not have a significant effect on transferrin

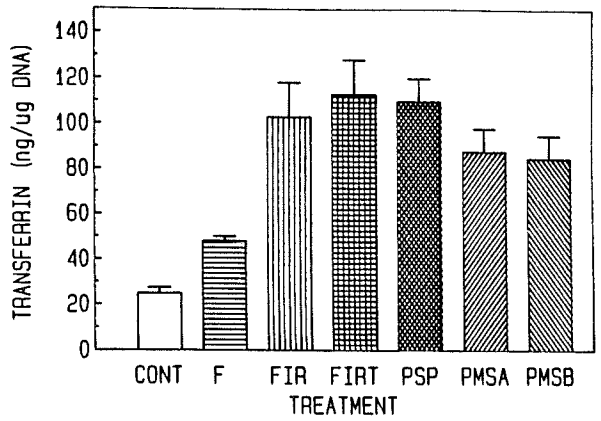

FIG. 1. Transferrin production by Sertoli cells cultured in the absence (CONT) or presence of FSH (F), FIR, FIRT, P-Mod-S (A) (PMSA), P-Mod-S (B) (PMSB), and PSP. Data are presented as nanograms of transferrin per μg Sertoli cell DNA (mean ± SEM from three different experiments performed in triplicate). All treatments were found to be statistically different from control values ($P < 0.01$).

production alone (data not shown) or in combination with FSH, insulin, and retinol (FIR). Purified forms of P-Mod-S (A) and P-Mod-S (B) were both found to stimulate transferrin production in a manner similar to a mixture of PSP (Fig. 1). The actions of P-Mod-S were greater than any individual regulatory agent, including FSH, and comparable to the effects of a mixture of FIR or FIRT. Sertoli cells were treated with P-Mod-S in the absence or presence of FSH or FIRT to investigate the effects of regulatory agents on P-Mod-S actions (Fig. 2). FSH increased basal levels of transferrin production approximately 2-fold and resulted in an additive response with P-Mod-S (A), P-Mod-S (B), or PSP (data not shown). Interestingly, FIRT stimulated transferrin production approximately 4-fold and in combination with P-Mod-S (A), P-Mod-S (B), or PSP resulted in approximately an 8-fold stimulation (Fig. 2). The response to the combination of FIRT and P-Mod-S was an additive effect and showed no statistically significant synergism (Fig. 2). The magnitude of the stimulation obtained with the combination of P-Mod-S and FIRT was a higher level of stimulation than that caused by any other mixture of regulatory agents examined.

Due to the significant effect of P-Mod-S on Sertoli cell function and the additive effects with other regulatory agents the mechanism of P-Mod-S action on Sertoli cells was investigated. Sertoli cells were cultured for 2 days in the absence of regulatory agents and then treated with various agents for 60 min or 72 h, followed by analysis of cyclic nucleotide levels. Basal cAMP levels were variable, with an average of 12 fmol/μg protein. After a 60-min treatment with FSH or a mixture of FIRT both cellular and extracellular (excreted) cAMP levels were stimulated approximately 5- to 12-fold (Fig. 3). In contrast, P-Mod-S or PSP had no effect after a 60-min treatment on cAMP levels. With a 72-h treatment both FSH and FIRT maintained a stimulation of cellular (Fig. 4) and extracellular cAMP (data not shown). P-Mod-S and PSP treatment for 72 h did result in a small increase in cAMP levels, which was less than the effects of FSH. In contrast to FSH, the actions of P-Mod-S on Sertoli cell function do not appear to initially involve a cAMP-mediated response.

Alternatively, the effects of regulatory agents on cGMP levels were analyzed. The basal levels of cGMP were approximately one fifth those of cAMP and were variable, with an average of 3 fmol/μg protein in the cell and 2 fmol/μg protein excreted. Treatment of Sertoli cells for 60 min with FSH or FIRT resulted in a decrease in cellular cGMP levels with a 60-min treatment (Fig. 5). Interestingly, P-Mod-S (A), P-Mod-S (B), and PSP all stimulated cellular cGMP levels and extracellular cGMP levels (Fig. 5). FIRT increased extracellular cGMP levels with a 60-min treatment. Treatment of Sertoli cells for 72 h with FSH or FIRT showed no effect on cGMP levels (Fig. 6). As shown for 60 min, P-Mod-S and PSP also stimulated cellular cGMP after the pro-

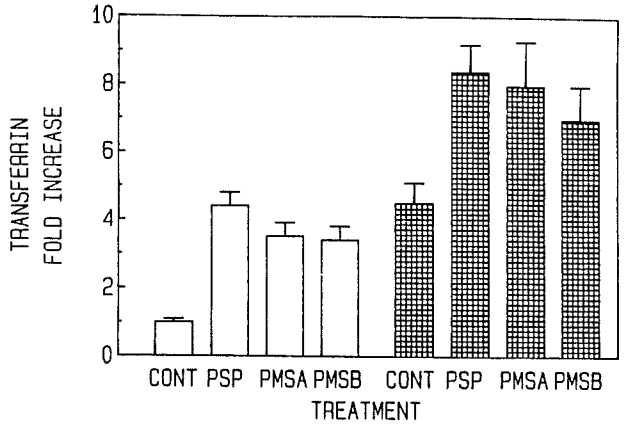

FIG. 2. Fold increase in transferrin production by Sertoli cells cultured in the absence [□; control (CONT)] or presence of FIRT (▦) when cells were treated with P-Mod-S (A) (PMSA), P-Mod-S (B) (PMSB), or PSP. Data are presented as the fold increase above values in control nontreated cells (mean ± SEM from three different experiments performed in triplicate). All treatments were statistically different from controls ($P < 0.01$).

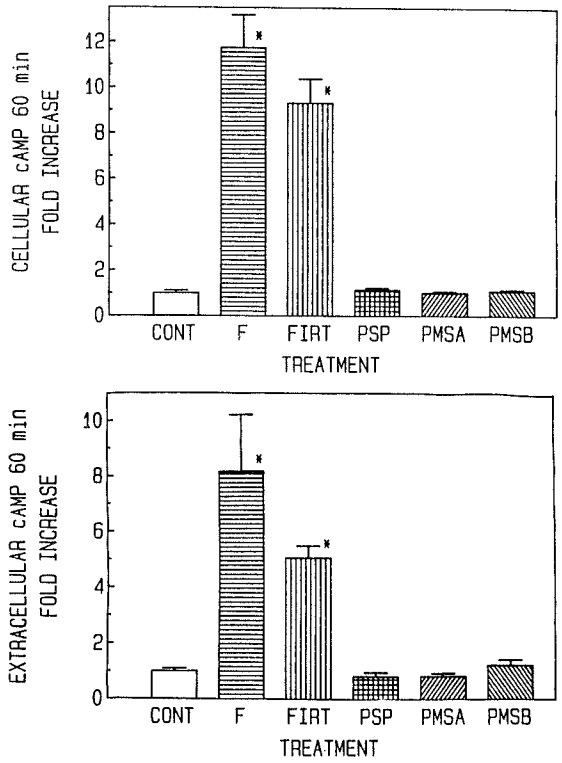

FIG. 3. Cellular and extracellular levels of cAMP in Sertoli cells cultured for 60 min in the absence (CONT) or presence of FSH (F), FIRT, P-Mod-S (A) (PMSA), P-Mod-S (B) (PMSB), and PSP. Data are presented as the fold increase above levels in control nontreated cells (mean ± SEM from a minimum of seven different experiments performed in replicate). *, Statistical difference from the control ($P < 0.01$, determined with Student's t test).

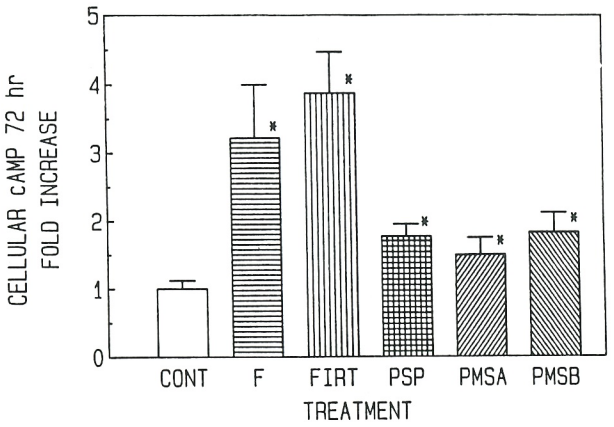

FIG. 4. Cellular levels of cAMP in Sertoli cells cultured for 72 h with regulatory agents, as discussed in Fig. 3. Data are presented as the fold increase above levels in control untreated cells (mean ± SEM from a minimum of four different experiments performed in replicate). *, Statistical difference from the control ($P < 0.01$, determined with Student's t test).

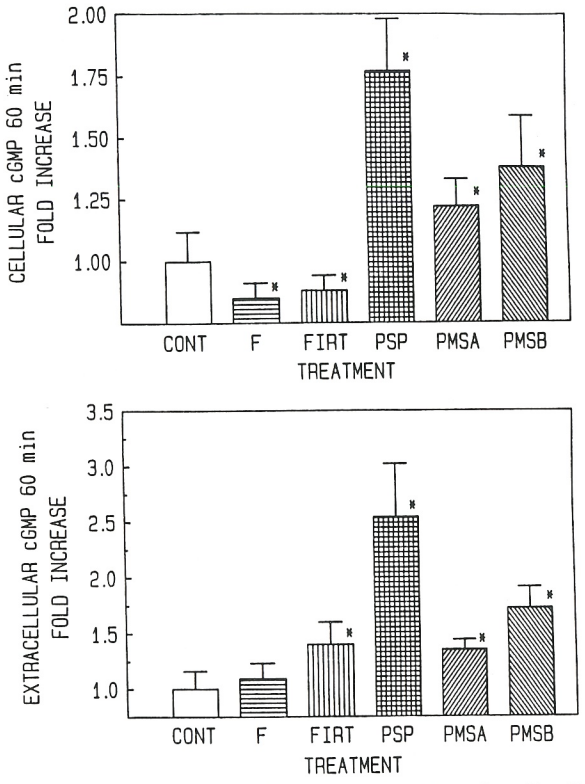

FIG. 5. Cellular and extracellular levels of cGMP in Sertoli cells cultured for 60 min in the absence (CONT) or presence of FSH (F), FIRT, P-Mod-S (A) (PMSA), P-Mod-S (B) (PMSB), and PSP. Data are presented as the fold increase above levels in control nontreated cells (mean ± SEM from a minimum of seven different experiments performed in replicate). *, Statistical difference from the control ($P < 0.01$, determined with Student's t test).

longed 72-h treatment. Observations imply that P-Mod-S (A) and P-Mod-S (B) both may act in part through a cGMP-mediated response.

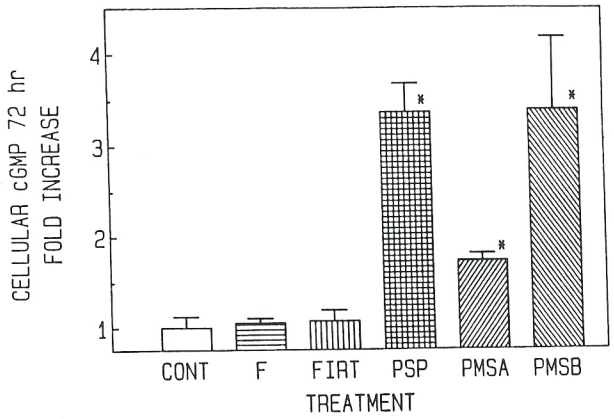

FIG. 6. Cellular levels of cGMP in Sertoli cells cultured for 72 h with regulatory agents, as discussed in Fig. 5. Data are presented as the fold increase above levels in control nontreated cells as the mean ± SEM from a minimum of four different experiments performed in replicate. *, Statistical difference from the control ($P < 0.01$, determined with Student's t test).

TABLE 1. Phosphoinositide turnover as measured by the release of IP and normalized for the total accumulation of PIP and represented as a ratio of IP release/PIP accumulation

Treatment	IP hydrolysis (IP release/PIP accumulation)
Control	0.15 ± 0.07
FIRT	0.13 ± 0.02
PSP	0.13 ± 0.07

Sertoli cells were cultured in the absence (control) or presence of FIRT or PSP for 30 min before assessment of IP release. Data are presented as the mean ± SEM from five different experiments in replicate. No statistical difference was detected between treatments with Student's t test.

The ability of regulatory agents to influence inositol phosphate (IP) hydrolysis was investigated as an alternate signal transduction pathway. None of the individual agents (data not shown) or the combination of FIRT influenced IP hydrolysis (Table 1). Sertoli cells were cultured in the absence of regulatory agents for 2 days, then treated with agents for 30 min followed by the determination of IP metabolism in cells prelabeled with [^{3}H]myoinositol. PSP is a crude preparation of P-Mod-S and also did not influence IP metabolism (Table 1). Similar results were obtained with treatments of 15 or 60 min (data not shown). P-Mod-S does not appear to influence IP hydrolysis as a signal transduction system under the conditions used.

The actions of P-Mod-S on Sertoli cell gene expression were investigated to confirm that P-Mod-S acts on a molecular level. Molecular probes were obtained to transferrin and ABP, as outlined in *Materials and Methods*. These cRNA probes were used initially in a Northern blot procedure on polyadenylated Sertoli cell RNA. Sertoli cells were cultured and treated with various agents for 5 days, followed by the isolation of RNA from the

cells. A single 2.6-kilobase transcript was observed for transferrin and a 1.7-kilobase transcript for ABP (Fig. 7). The amount of hybridization observed appeared to increase in cells treated with FIRT or PSP. Scanning densitometry of transferrin hybridization on the Northern blots indicated a 2.0 ± 0.3-fold increase with FIRT treatment and a 1.8 ± 0.1-fold increase with PSP treatment. Analysis of these Northern blots with a 1B15 probe to cyclophilin, a constitutively produced protein (19), revealed a 1-kilobase transcript with no detectable difference in the hybridization observed between the different treatments (data not shown). Both FIRT and PSP increase the apparent gene expression of transferrin and ABP.

A solution hybridization was used to more quantitatively determine the effects of regulatory agents on gene expression. Sertoli cells were cultured for 5 days in the presence of various agents, followed by the isolation of total RNA. Each of the total RNA samples was analyzed with a solution hybridization assay for transferrin, ABP, and 1B15. A linear assay was established with cRNA probes for transferrin between 10–50 μg total RNA, for ABP between 10–100 μg total RNA, and for 1B15 between 1–25 μg total RNA (data not shown). All hybridization data obtained with transferrin or ABP were normalized with the hybridization obtained on the same sample with the constitutive probe 1B15. This normalization procedure corrects for variable RNA integrity during the isolation procedure and for alterations in cell number. Similar results were obtained with normalization with Sertoli cell protein or DNA (data not shown). Therefore, data are presented as fold increase above values in control nontreated cells, as represented by a ratio of transferrin or ABP hybridization to 1B15 hybridization on each individual sample. The data presented in Fig. 8A indicate that both FIR and FIRT stimulate steady state levels of transferrin message approximately 1.8-fold. P-Mod-S (A), P-Mod-S (B), and PSP stimulated transferrin gene expression 1.9-, 2.1-, and 2.2-fold, respectively (Fig. 8A). FIR and FIRT also stimulated ABP message levels approximately 2-fold, similar to the effect of FSH alone (Fig. 8B). P-Mod-S (A), P-Mod-S (B), and PSP all stimulated steady state levels of ABP message 1.8-, 2.0-, and 1.9-fold, respectively (Fig. 8B).

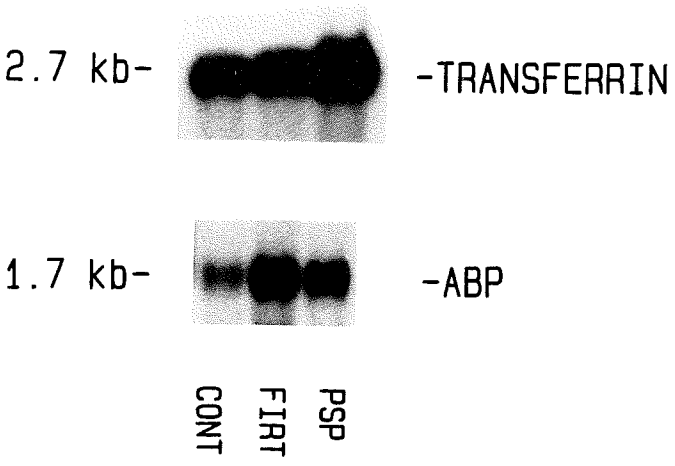

FIG. 7. Northern blot analysis of polyadenylated RNA from Sertoli cells cultured in the absence (CONT) or presence of FIRT or PSP. Transferrin hybridization is shown for a 2.6-kilobase (Kb) transcript, while ABP hybridization is shown with a 1.7-kilobase transcript. Data are representative of three different experiments.

Discussion

The testicular paracrine factor P-Mod-S has previously been shown to have profound effects on a number of Sertoli cell functions (6–9). FIRT stimulates functions, such as transferrin and ABP production, approximately 4-fold, which was previously thought to be a maximal stimulation of Sertoli cell function (12, 23). The concentration of FSH used alone or in combination with FIRT was 100 ng/ml, which is approximately 2 orders of

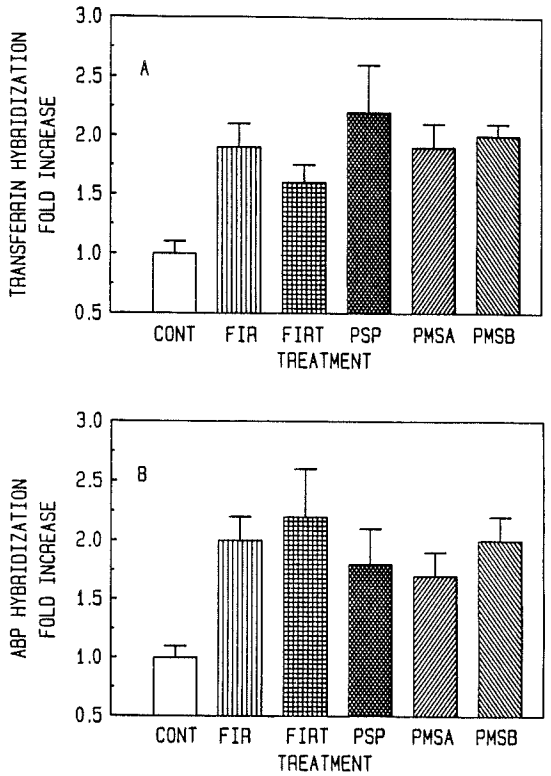

FIG. 8. Solution hybridizations for transferrin (A) and ABP (B) gene expression in Sertoli cells cultured in the absence (CONT) or presence of FIR, FIRT, PSP, P-Mod-S (A) (PMSA), or P-Mod-S (PMSB). Data are presented as the fold increase in hybridization with the respective [^{32}P]cRNA probes above that in control nontreated cells and are represented as the mean ± SEM from three different experiments, performed in triplicate. All treatment values were statistically different from control values ($P < 0.01$).

magnitude higher than physiological levels. These concentrations of FSH were used to reduce variability in bioactivity; however, previous studies have shown that physiological concentrations of FSH give similar results (12). The high concentration of insulin used is required and has previously been postulated to have its primary response through cross-reactivity with the insulin-like growth factor receptor (12). P-Mod-S alone stimulated Sertoli cell functions in a manner similar to the mixture of FIRT, which confirms previous observations (8). Data presented in the current study indicate that a combination of FIRT and P-Mod-S increases transferrin production approximately 8-fold, which was the highest level of stimulation observed. This level of stimulation, however, is dependent on the cell culture conditions used, which may be altered by variables such as the presence of extracellular matrix. The level of stimulation observed was an additive response to both FIRT and P-Mod-S, with no apparent synergism of the combination of regulatory agents. These observations imply that the combined actions of hormones such as FSH and the paracrine factor P-Mod-S will be required to promote Sertoli cell differentiation and maintain optimal cellular function. Therefore, data presented support the hypothesis that the testicular paracrine factor P-Mod-S may have a role in the maintenance and control of testicular function and the process of spermatogenesis.

The ability of P-Mod-S to have a greater effect on Sertoli cell function than any other individual regulatory agent and the ability to have an additive response in combination with FSH or FIRT implies that P-Mod-S may have a unique mechanism of action. For these reasons, several potential signal transduction systems were investigated. The initial signal transduction event identified in Sertoli cells was the effects of FSH on adenylate cyclase and cAMP (24). Data presented confirm the ability of FSH and the combination FIRT to increase cAMP levels (25). The effects of FSH on cAMP were maintained for up to 3 days and influenced both cellular and excreted cAMP levels. The function(s) of the excreted cyclic nucleotides is unknown, and it is presumed that alterations in cellular cyclic nucleotide levels will be more physiologically important. P-Mod-S or total PSP had no influence on cAMP levels after a 60-min treatment. This observation implies that P-Mod-S does not appear to initially act through a cAMP-mediated response. Interestingly, after a 72-h treatment of Sertoli cells with P-Mod-S a small increase in cAMP was observed. Whether this delayed effect of P-Mod-S on cAMP is required for the long term effects of P-Mod-S on Sertoli cell differentiation remains to be investigated. These observations provide a potential explanation for the ability of P-Mod-S to have a greater effect on Sertoli cell functions alone and an additive response in combination with FSH. If the same signal transduction system was used by both P-Mod-S and FSH, then both regulatory agents would not necessarily have similar effects on Sertoli cells nor provide an additive response in combination.

An alternate second messenger for the actions of regulatory agents is cGMP. Both FSH and FIRT initially reduced cellular cGMP levels. FIRT did increase extracellular cGMP levels after a 60-min treatment. Whether this increase was due to the corresponding decrease in cellular cGMP remains to be investigated. Neither FSH nor FIRT had any effect on cGMP for a 72-h treatment. In contrast, P-Mod-S and PSP both stimulated cGMP levels with both 60-min and 72-h treatments. This observation implies that P-Mod-S actions on Sertoli cells are mediated in part through effects of cGMP levels. The ability of P-Mod-S to maintain high levels of cGMP for 3 days of treatment implies that the longer term effects of P-Mod-S on Sertoli cell differentiation and function will probably involve continued effects on cGMP levels. Whether cGMP is the initial second messenger of P-Mod-S actions is under investigation with short term (<60 min) treatments. Effects of P-Mod-S on guanylate cyclase or phosphodiesterase is also under investigation. P-Mod-S (B) generally had greater effects on cGMP levels than P-Mod-S (A). Whether this is due to differences in the biological activities of the two forms of P-Mod-S or differences in the signal transduction systems used remains to be investigated. The observations presented, however, indicate that cGMP is involved in the actions of P-Mod-S on Sertoli cells. This is the first demonstration that a cGMP-mediated response may influence Sertoli cell function and differentiation. This mechanism may explain in part how P-Mod-S can have an additive effect in combination with FIRT as well as have such profound effects alone on Sertoli cell functions.

Another signal transduction system investigated was IP hydrolysis. FSH alone or in combination with FIRT had no effect on IP hydrolysis, which confirms previous reports (26). A crude preparation of P-Mod-S was also not found to influence IP hydrolysis between 15–60 min of treatment. Therefore, IP metabolism does not appear to be involved in the long term effects of P-Mod-S on Sertoli cell function. However, many IP-responsive systems increase within seconds and return to basal conditions within minutes (14, 27). Whether IP hydrolysis may play a role in the initial signal transduction event of P-Mod-S remains to be investigated with short term treatments. Recent observations regarding the actions of FSH and IP hydrolysis indicate that cAMP-mediated responses are antagonistic to IP hydrolysis (28). This implies that regulatory agents that act via IP hydrolysis may be antagonized by the actions of FSH. The current

study indicates that the combined actions of FSH and P-Mod-S are additive and not antagonistic. Therefore, these previous observations regarding cAMP and IP hydrolysis (28) support the suggestion that P-Mod-S actions on Sertoli cells do not appear to involve phosphoinositide hydrolysis.

In addition to providing insight into the potential signal transduction event(s) of P-Mod-S, the current study provides evidence for the ability of P-Mod-S to influence Sertoli cell gene expression. P-Mod-S and PSP both stimulated transferrin and ABP gene expression, as measured with Northern blot and solution hybridization procedures. The solution hybridization procedure provides the more quantitative determination of changes in gene expression, and data have been normalized to the expression of a constitutively produced protein. P-Mod-S stimulation of gene expression was similar to that caused by FIRT. The effects observed on gene expression correlate with responses of both transferrin and ABP production (8). Similar effects of FIRT on transferrin gene expression have previously been reported (16). Observations provide direct evidence for the ability of P-Mod-S to influence Sertoli cell functions on a molecular level. Whether the effects of P-Mod-S on gene expression are an influence on transcription and/or translation remains to be investigated and cannot be distinguished in the current study. Transferrin and ABP are markers of Sertoli cell function that correlate with the induction and maintenance of Sertoli cell differentiation. The ability of P-Mod-S to directly influence Sertoli cell gene expression supports the postulate that P-Mod-S may play a role in the initiation and maintenance of Sertoli cell differentiation.

Two forms of P-Mod-S have previously been identified and found to be different in mol wt and hydrophobicity (8). P-Mod-S (A) is a 56K protein that is less hydrophobic than P-Mod-S (B), which is a 59K protein. Both P-Mod-S (A) and P-Mod-S (B) stimulate ABP (8) and transferrin production in a similar manner and have an additive response in combination with FSH or FIRT. The two forms of P-Mod-S both stimulate cGMP and initially have no effect on cAMP levels. Neither form affects IP hydrolysis. In addition, P-Mod-S (A) and P-Mod-S (B) stimulate transferrin and ABP gene expression in a similar manner. The data presented indicate that both forms of P-Mod-S have similar biological activities, use a similar signal transduction system, and similarly influence Sertoli cells on a molecular level. Observations imply that P-Mod-S (A) and P-Mod-S (B) are functionally similar, and the speculation is made that both may be derived from the same parent molecule.

The current study demonstrates that P-Mod-S has profound effects on Sertoli cell functions and in combination with other regulatory agents will stimulate specific cellular functions to the highest level achieved to date. The ability of P-Mod-S to have such dramatic effects on Sertoli cell function may be due to its unique signal transduction system, which is in part mediated through alterations in cGMP levels. These effects on Sertoli cells are mediated on a molecular level, with modulation of the expression of specific genes. The actions of P-Mod-S on Sertoli cells, therefore, may have an important role in the promotion of Sertoli cell differentiation and the maintenance of cellular functions. Peritubular cell-Sertoli cell interactions mediated via P-Mod-S are postulated to be a regulatory interaction (1) required for the control and maintenance of testicular function and the process of spermatogenesis.

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