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Stimulation of Sertoli cell inhibin secretion by the testicular paracrine factor PModS

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Summary

The testicular paracrine factor PModS is produced by peritubular myoid cells under androgen control and modulates Sertoli cell function and differentiation. The observation that luteinizing hormone (LH) stimulates inhibin production *in vivo*, but has no effect on isolated Sertoli cells *in vitro*, suggested an indirect mode of LH action, potentially mediated by PModS. The effects of the testicular paracrine factor PModS and hormones on inhibin secretion by Sertoli cells were investigated to provide insight into the endocrine control of inhibin expression. An inhibin radioimmunoassay was utilized which showed essentially parallel displacement curves with purified bovine follicular fluid inhibin, Sertoli cell conditioned medium and concentrated Sertoli cell secreted proteins. An immunoblot analysis of Sertoli cell secreted proteins with the inhibin antisera consistently detected a 32 kDa protein which is the expected size of the mature of inhibin ($\alpha\beta$) and periodically detected a 57 kDa protein which is speculated to be an incomplete processed form of the inhibin precursor ($\alpha_{43}\beta$). Follicle-stimulating hormone (FSH) was found to stimulate inhibin secretion initially between days 2 and 5 of Sertoli cell culture. Insulin and retinol alone had no significant effect on inhibin secretion; however, together they appeared to enhance the ability of FSH to stimulate inhibin secretion. Testosterone had no effect on inhibin production alone or in combination with other regulatory agents. PModS was found to stimulate inhibin secretion approximately 3-fold, but with a delayed time course of stimulation which did not occur until days 5–7 of Sertoli cell culture. Treatment with a combination of PModS and FSH resulted in an apparent maximal stimulation of inhibin secretion. Both forms of PModS, PModS (A) and PModS (B), were found to have equivalent biological activities in their ability to stimulate inhibin production with an apparent half-maximal effective concentration between 10 and 15 ng/ml. The current study provides evidence for the local testicular control of inhibin production and adds to the complexity of the endocrine control of inhibin expression. The cellular interaction is proposed in which LH acts on Leydig cells to stimulate androgen production which in turn acts on peritubular cells to regulate PModS production which subsequently can act on Sertoli cells to control inhibin production. Testicular control of inhibin production provides a potential short feedback loop for the local regulation of androgen production and an additional regulatory element for the pituitary–gonadal axis.

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Introduction

Cellular interactions between different cell types in the testis play a critical role in the maintenance and control of testicular function and the process of spermatogenesis (Skinner, 1987). Sertoli cells help form the seminiferous tubule and provide the cytoarchitectural support and microenvironment required for germinal cell development. Peritubular myoid cells surround and form the exterior wall of the tubule and are separated from the basal surface of the Sertoli cells by a complex extracellular matrix (Dym and Fawcett, 1970; Skinner et al., 1985). Leydig cells in the interstitium under luteinizing hormone (LH) control produce androgens that subsequently act on the seminiferous tubule to maintain cellular function and the process of spermatogenesis. The endocrine regulation of testicular function primarily involves the actions of pituitary gonadotropins. Although androgens may act directly on Sertoli cells (Fritz, 1978), peritubular cells also provide a site for androgen action (Verhoeven, 1979). Peritubular cells produce a paracrine factor, PModS, under androgen control which can modulate Sertoli cell function and differentiation *in vitro* (Skinner and Fritz, 1985; Skinner et al., 1988). Therefore, androgen regulation of testicular function may in part be mediated indirectly through the peritubular cells (Skinner and Fritz, 1985b). In addition to androgens and PModS, follicle-stimulating hormone (FSH) acts directly on Sertoli cells and has an important role in the maintenance and control of Sertoli cell function and differentiation (Fritz, 1978). The regulation of testicular function is postulated to require the actions of pituitary gonadotropins, steroids and locally produced paracrine factors such as PModS.

Inhibin (McCullagh, 1932) is a heterodimeric glycoprotein produced in the testis by Sertoli cells (Steinberger and Steinberger, 1976) that is postulated to provide a negative feedback on pituitary FSH secretion (review, McLachlan et al., 1988). Recent observations have also indicated that inhibin may have a role as a local paracrine factor within the testis involved in the control of steroidogenesis (Hsueh et al., 1987). The recent purification and cloning of the different forms of inhibin (Mason et al., 1985; Robertson et al.,

1985; Forage et al., 1986; Mayo et al., 1986) has established this protein as a potential endocrine and paracrine regulatory agent in both the male and female. Antibodies to purified bovine inhibin have been recently developed and utilized to quantitate inhibin levels (Robertson et al., 1988). FSH is one of the primary regulators of inhibin production both *in vivo* and *in vitro* (LeGac and de Kretser, 1982; Au et al., 1985; Bicsak et al., 1987; McLachlan et al., 1988; Morris et al., 1988). Recently, however, LH alone has also been shown to elevate inhibin levels *in vivo* (McLachlan et al., 1988). Purified human LH and human chorionic gonadotropin were found to partially restore inhibin levels in normal men whose endogenous gonadotropin levels were suppressed by testosterone administration. Since LH has no direct effects on isolated Sertoli cells, the mechanism of LH-regulated inhibin production was uncertain.

The current study was designed to investigate the actions of regulatory agents on inhibin production by Sertoli cells. The hypothesis that the testicular paracrine factor PModS may provide a mechanism for LH and androgens to influence inhibin production by Sertoli cells was investigated. The observations presented provide insight into the endocrine and paracrine regulation of inhibin production, as well as develop a better understanding of cell-cell interactions in the testis.

Materials and methods

Cell preparation and culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (Dorrington et al., 1975) with a modified procedure previously described (Tung et al., 1984). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml) (Gibco, Grand Island, NY, U.S.A.) to remove Leydig cells, followed by a collagenase digestion (1 mg/ml type I, Sigma), 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% CO₂ atmosphere in Ham's F-12 medium (Gibco). Sertoli cultures were treated as described under Results at the time of plating and retreated under

48 h of culture when the medium was replenished. Unless otherwise stated, a 48 h medium collection on day 7 of culture was obtained for analysis and the cells harvested for a DNA assay. Sertoli cell cultures were treated as outlined under the Results section with test substances or using maximal concentrations previously determined for ovine FSH (100 ng/ml, current batch NIDDK and NHPP, Baltimore, MD, U.S.A.), insulin (5 μ g/ml, Sigma), retinol (0.35 μ M, Sigma), and testosterone (1 μ M, Sigma). Unless otherwise stated, designated cell cultures were treated with peritubular cell secreted proteins (50 μ g/ml), PModS (A) (25–50 ng/ml) or PModS (B) (25–50 ng/ml). The purity of the Sertoli cell population has been determined to be greater than 99% throughout the culture period on day 2 and day 7 with procedures previously described (Tung et al., 1984).

Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had gravity sedimented as previously described (Skinner et al., 1988). 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 or Sertoli cell serum-free conditioned medium was made 25 μ M phenylmethylsulfonyl fluoride and 0.1 mM 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 using a membrane with a 3000 molecular weight exclusion limit. This concentrated conditioned medium was referred to as Sertoli cell secreted proteins (SSP) or peritubular cell secreted proteins (PSP).

PModS preparation

PModS was purified from concentrated peritubular cell conditioned medium as previously described (Skinner et al., 1988). Briefly, an ammonium sulfate precipitate of concentrated conditioned medium was applied to a size-exclusion high-pressure liquid chromatography column. The

active peak was collected and applied to a heparin Sepharose affinity column and eluted with high salt conditions. Proteins which bound to the heparin Sepharose column were applied to two successive C4 reverse-phase columns and eluted with acetonitrile. The purity of the PModS preparations was assessed with an electrophoretic analysis and silver stain procedure as previously described (Skinner et al., 1988). Purity was confirmed with the detection of a homogenous protein preparation at 59 kDa for PModS (B) and 56 kDa for PModS (A). An additional criterion of purity utilized was the presence of a chromatographically pure 214 nm absorbance peak on the final reverse-phase column. Purified forms of PModS were stored at –70°C prior to use.

Transferrin radioimmunoassay

Transferrin production by Sertoli cells was assayed by a radioimmunoassay described previously (Skinner and Griswold, 1982). An aliquot of the culture media was incubated with rabbit anti-rat transferrin antibody (Cooper Biomedical, Melvern, PA, U.S.A.) and iodinated transferrin for 1 h at 37°C followed by a 1 h incubation with goat anti-rabbit IgG antibody (Sigma, St. Louis, MO, U.S.A.). Complexed antibody was then precipitated with polyethylene glycol (Sigma), pelleted by centrifugation, and radioactivity in the pellets determined.

Inhibin radioimmunoassay (RIA)

The inhibin RIA was performed using a rabbit anti-inhibin antiserum (As 1989) raised to bovine follicular fluid (bFF) inhibin (Robertson et al., 1988). Purified 31 kDa bFF inhibin was used as an initial standard and as ¹²⁵I-radioiodination material in the assay. Previously it has been demonstrated that bovine Müllerian inhibitory substance, human transforming growth factor- β , bovine activin A and free inhibin α - and β -subunits, obtained following reduction and alkylation of purified 31 kDa bFF inhibin, show less than 1% cross-reactivity in the RIA (Robertson et al., 1988). A standardized preparation of concentrated SSP showed parallel displacement curves to purified bovine inhibin in the RIA. The SSP material was quantitated against purified bovine inhibin and was used as a standard. Standard and samples

(200 μ l in phosphate buffer) and inhibin antiserum (100 μ l, 1:3000 containing 1:200 normal rabbit serum) were incubated overnight at room temperature then iodinated inhibin (100 μ l, 10 000 cpm in assay buffer containing 0.1% Triton X-100) added and the incubation continued for a further 24 h at room temperature. Goat anti-rabbit IgG was then added and the incubation continued overnight at 4°C, then 2 ml of cold normal saline was added, the tube centrifuged (2500 \times *g* for 60 min), decanted and counted. Samples were assayed at three dilutions in duplicate and values read between 80 and 20% displacement. At 100 μ l/tube the sensitivity was 0.14 ng/ml and the half-maximal displacement concentration was 0.44 ng/ml. The intra-assay coefficient of variation (cv) in the upper, middle and lower regions of the standard curve were 8.8, 4.6 and 3.7%, respectively and the inter-assay cv was 8.8% from 11 assays.

Electrophoresis and immunoblot procedures

Concentrated Sertoli cell conditioned medium (5–10 ml; 100-fold concentrated) was applied to a C18 reverse-phase Sep-Pak column (Waters, Milford, MA, U.S.A.) and eluted with 75% acetonitrile in 0.1% trifluoroacetic acid. Proteins were electrophoretically separated on sodium dodecyl sulfate (SDS) 7.5–15% polyacrylamide gradient slab gels with the Laemmli (Laemmli, 1970) buffer system under non-reducing conditions. The gel was then blotted to nitrocellulose, and specific lanes were immunoblotted as previously described (Skinner et al., 1987) with the inhibin antiserum (the generous gift of Dr. David de Kretser, Monash University, Melbourne, Australia). A 1:100 dilution of primary antisera was used and detected with an ¹²⁵I-radiolabeled goat anti-rabbit immunoglobulin second antibody (Skinner et al., 1987). The immunoblot was then autoradiographed.

DNA and protein assays

DNA was measured fluorometrically with ethidium bromide (Karsten and Wollenberger, 1977). At the end of the culture period, the medium was removed, ethidium bromide buffer (EBB) (20 mM sodium chloride, 5 mM EDTA, 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, 100 units/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 μ g of DNA and is linear up to 2.5 μ g of DNA. Total protein concentration was measured according to the method of Bradford (Bradford, 1976).

Data normalization and statistical analysis

All data were normalized per μ g of Sertoli cell DNA at a time of medium collection and expressed as pg inhibin or ng of transferrin per μ g of DNA. Data was generally presented as the mean \pm standard error of the mean (SEM) and statistical analysis of the data utilized a Student's *t*-test. Unless otherwise designated the level of significance for statistical differences was $P < 0.01$.

Results

Inhibin radioimmunoassay and antibody specificity

The inhibin RIA was initially utilized to determine the immunological similarity of reactive components in Sertoli cell conditioned medium with purified 31 kDa bovine inhibin. Purified inhibin, Sertoli cell conditioned medium and concentrated Sertoli cell secreted proteins (SSP) all showed parallel displacement curves in the RIA, Fig. 1. The sensitivity of the RIA was such that the inhibin levels in Sertoli cell conditioned medium could be determined in 3–100 μ l of sample. Analysis of the specificity of the inhibin antibody has previously been reported (Robertson et al., 1988). None of the regulatory agents or PSP alone was found to be reactive in the inhibin RIA (data not shown). To address the specificity of the inhibin antisera for reactive components in Sertoli cell conditioned medium an immunoblot procedure was performed. Sertoli cell secreted proteins were electrophoretically separated under non-reducing conditions and blotted to nitrocellulose. The low levels of inhibin present required the analysis of large quantities of Sertoli cell secreted proteins which resulted in some band distortion.

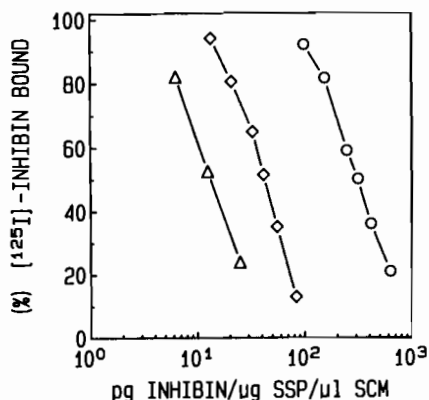


Fig. 1. Inhibin radioimmunoassay displacement curves with bovine follicular fluid inhibin (\diamond), Sertoli cell conditioned medium (Δ) and Sertoli cell secreted proteins (\circ). The percent bound [125 I]inhibin to the inhibin antisera versus concentrations of test substances is presented. Data is representative of three different experiments.

Reactive components were detected with the inhibin antisera and an 125 I-radiolabeled second antibody, Fig. 2. A 32 kDa protein corresponding to the mature form of inhibin ($\alpha\beta$) was identified in all six immunoblots analyzed. In three blots, an additional 57 kDa protein was also identified, Fig.

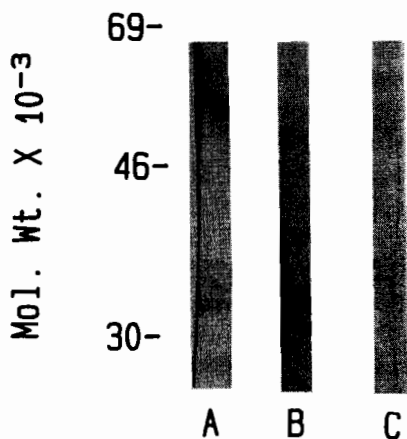


Fig. 2. Immunoblot of Sertoli cell secreted proteins with the inhibin antisera. Proteins were electrophoretically separated on an SDS gel and blotted to nitrocellulose with molecular weight markers as designated. Representative secreted protein preparations from two experiments (A, B) are presented. Incubation with the absence of inhibin antibody was performed as a control (C). Inhibin antibody binding was detected with 125 I-radiolabeled second antibody and autoradiographed.

2. The presence of the 57 kDa protein was found to be independent of the hormonal stimulation of the Sertoli cells (data not shown) and corresponds to the size of an incompletely processed form of inhibin ($\alpha_{43}\beta$) with a partially processed α -subunit (Robertson et al., 1985; McLachlan et al., 1988).

Regulation of inhibin secretion

The time course of the ability of regulatory agents to stimulate inhibin secretion by Sertoli cells was investigated to determine the optimal period to quantitate inhibin production. Sertoli cells were cultured and medium collections made on days 2, 5, 7 and 9 for analysis. A combination of FSH, insulin, retinol and testosterone (FIRT) has previously been shown to stimulate Sertoli cell function (Skinner and Griswold, 1982; Skinner et al., 1988). The concentrations of individual agents used have previously been shown to be optimal to stimulate a number of Sertoli cell functions (Skinner and Griswold, 1982; Skinner et al., 1988).

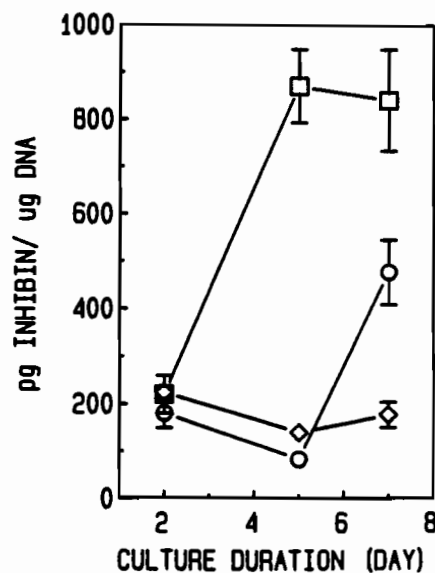


Fig. 3. Time course of the effects of a combination of FSH, insulin, retinol and testosterone (FIRT) (\square) and peritubular cell secreted proteins (PSP) (\circ) as compared to control (no treatment) (\diamond) on inhibin production by cultured Sertoli cells. Medium collections were made on days 2, 5 and 7 of culture and analyzed for the presence of inhibin expressed as pg inhibin/ μ g Sertoli cell DNA. Data presented is representative of three separate experiments.

FIRT stimulated inhibin production initially between days 2 and 5 of culture then declined slightly by day 9 of culture, but was not statistically different from data presented on day 5, Fig. 3. Interestingly, a crude preparation of PModS designated peritubular cell secreted proteins (PSP) demonstrated a delayed stimulation of inhibin production. PSP had no detectable effect on day 5 of culture, but did stimulate inhibin levels between days 5 and 7 of culture, Fig. 3. Due to a limited amount of purified PModS available the experiments were initially performed with PSP and were subsequently confirmed with purified PModS (A) which gave essentially the same results as PSP (data not shown). Results indicate that a maximum dose of PModS stimulates inhibin secretion with a delayed response in comparison with the combination of regulatory agents in FIRT. Further investigation of the actions of regulatory agents on inhibin secretion utilized a medium collection on day 7 of culture for analysis.

The effects of various regulatory agents on inhibin secretion are shown in Fig. 4. FSH was the only individual hormone which had a significant effect ($P < 0.01$) on inhibin production by Sertoli cells. Insulin and retinol treatment both resulted in small increases which were not statistically dif-

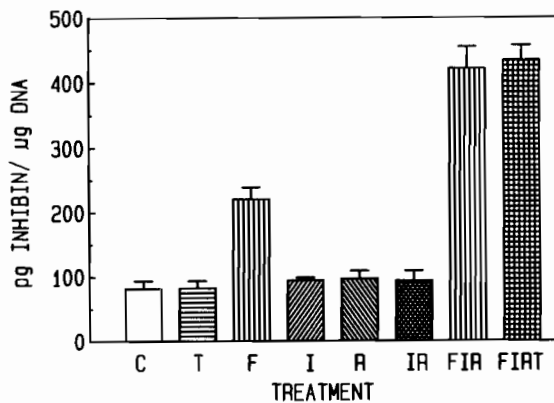


Fig. 4. The effects of regulatory agents on inhibin production by Sertoli cells. Cells were cultured in the absence (C) or presence of FSH (F), insulin (I), retinol (R), testosterone (T), insulin and retinol (IR), FSH, insulin and retinol (FIR) and a combination of FIRT. The effects on inhibin production on day 7 of culture were assessed and expressed as pg inhibin/ μ g Sertoli cell DNA. Data is presented as the mean \pm SEM from four different experiments done in replicate.

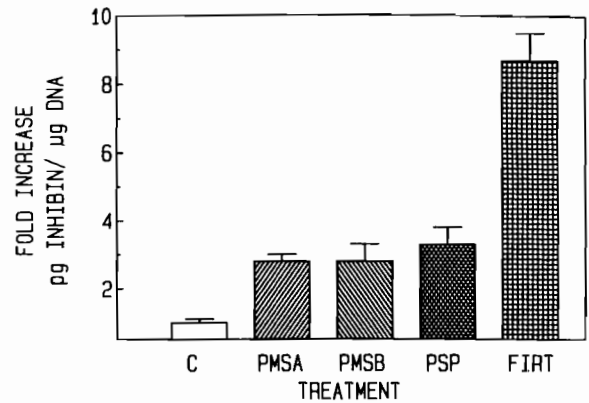


Fig. 5. The effects of PModS on inhibin production by Sertoli cells. Cells were cultured in the absence (C) or presence of FIRT (FIRT), peritubular cell secreted proteins (PSP), PModS (A) (PMSA) and PModS (B) (PMSB). Effects were determined on day 7 of culture and expressed as fold increase above control in pg inhibin/ μ g Sertoli cell DNA. Data is presented as the mean \pm SEM from three different experiments done in replicate.

ferent from control levels. In contrast, analysis of transferrin levels in these same medium samples demonstrated that retinol and insulin both stimulated transferrin secretion significantly (data not shown). Testosterone had no effect on inhibin production alone or in combination with other regulatory agents, Fig. 4. A combination of FSH, insulin and retinol (FIR) had a greater effect than FSH alone. Observations imply that insulin and retinol act synergistically with FSH to elevate inhibin production.

Investigation of the effects of the testicular paracrine factor PModS initially utilized a crude preparation of PModS termed peritubular cell secreted proteins (PSP). PSP was found to stimulate inhibin secretion approximately 3-fold, Fig. 5. Purified forms of PModS (A) and PModS (B), which have slightly different molecular weights but similar biological activities (Skinner et al., 1988), were both found to stimulate inhibin secretion approximately 2.8-fold. The magnitude of the stimulation by PModS, however, was less than that observed for FIRT, Fig. 5. In contrast, analysis of transferrin levels in the same medium samples indicated that PSP, PModS (A) and PModS (B) all stimulated transferrin production to a similar level as FIRT, Fig. 6. This observation con-

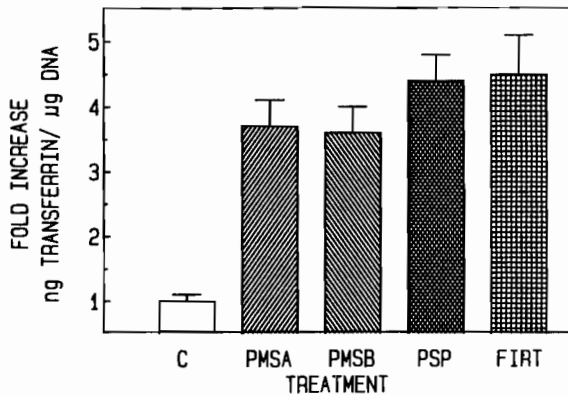


Fig. 6. The effects of PModS on transferrin production by Sertoli cells. Cells were cultured in the absence (C) or presence of FIRT (FIRT), peritubular cell secreted proteins (PSP), PModS (A) (PMSA) and PModS (B) (PMSB). Effects were determined on day 7 of culture and expressed as fold increase above control in ng transferrin/ μ g Sertoli cell DNA. Data is presented as the mean \pm SEM from three different experiments done in replicate.

firm's previous reports of the effects of PModS on transferrin production (Skinner et al., 1988) and implies that the decreased effects of PModS on inhibin secretion are not due to a decrease in bioactivity. Results indicate that PModS can stimulate inhibin production, but to a lesser degree than FIRT. The level of stimulation achieved with FIRT could not be increased with the addition of PModS (data not shown).

Dose-response curves for the effects of purified PModS on inhibin production are shown in Fig. 7. The concentrations required to obtain a maximal response were similar for PModS (A) and PModS (B). The level of stimulation achieved with 25 ng/ml PModS was the same as that found with 50 ng/ml PModS and represents the concentration required for maximal stimulation for PModS (A) and PModS (B). The minimum concentration of PModS required to stimulate inhibin secretion was approximately 3–5 ng/ml PModS (A) or PModS (B). The levels required for an apparent half-maximal response were between 10 and 15 ng/ml. Results shown in Fig. 7 indicate that PModS (A) and PModS (B) have the same apparent biological activities in the ability to stimulate inhibin secretion.

The effects of PModS in combination with FSH and/or various regulatory agents on inhibin

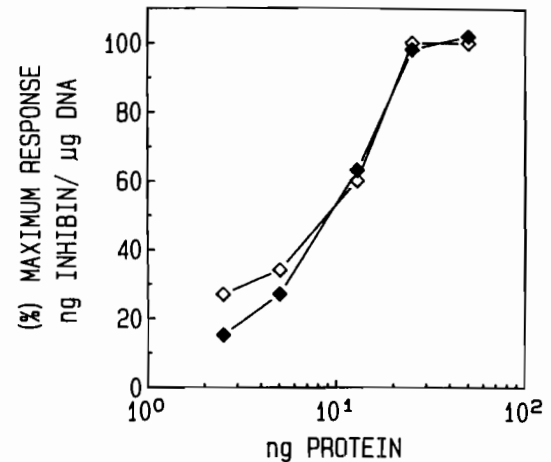


Fig. 7. Dose-response curves for PModS (A) (\diamond) and PModS (B) (\blacklozenge) on inhibin production by Sertoli cells. The effects of various concentrations of PModS on inhibin production were expressed as (% maximum response in pg inhibin/ μ g Sertoli cell DNA. Data presented are representative of three different experiments.

production are shown in Fig. 8. The level of stimulation achieved with FIRT could not be increased with additional PModS treatment. Interestingly, PModS and FSH were found to act synergistically with a degree of stimulation similar to

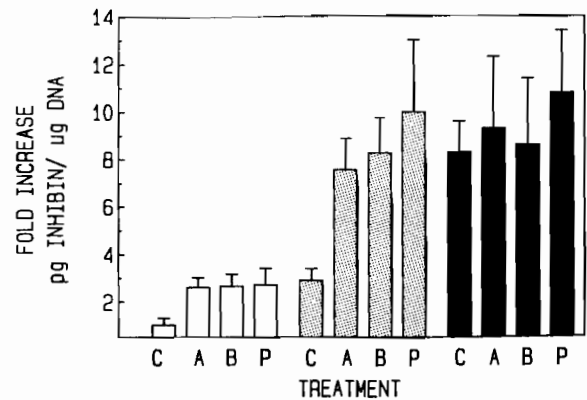


Fig. 8. The combined actions of PModS and hormones on inhibin production by Sertoli cells. Cells were cultured in the absence (C) or presence of PmodS (A) (A), PModS (B) (B) or peritubular cell secreted proteins (P) in the absence (open bar) or presence of FSH (hatched bar) or FSH, insulin, retinol and testosterone (closed bar). Effects on inhibin production are expressed as fold increase in pg inhibin/ μ g Sertoli cell DNA and are presented as the mean \pm SEM from three different experiments.

that of FIRT. Similar results were obtained for both PModS (A) and PModS (B), Fig. 8. Results indicate that an apparent maximal level of stimulation of inhibin production is obtained with the combined actions of PModS and FSH or the combined actions of FIR.

Discussion

The observation that the testicular paracrine factor PModS stimulates inhibin secretion by Sertoli cells provides a potential mode of action for LH and androgens to regulate inhibin levels *in vivo*. This potential testicular control of inhibin production was proposed from the previous observations that LH stimulates inhibin levels *in vivo* in men whose endogenous gonadotropin levels were suppressed by testosterone administration (McLachlan et al., 1988). The inability of LH and androgens to directly influence inhibin production by isolated Sertoli cells *in vitro* supports a potential indirect mode of regulation. Observations presented add to the complexity of the endocrine regulation of inhibin production and support the importance of local cell-cell interactions in the regulation of testis function.

The specificity of the inhibin antibody utilized has previously been shown to primarily be directed at the heterodimeric form of inhibin ($\alpha\beta$) (Robertson et al., 1988). In the current study it was found that displacement curves of purified bovine inhibin, Sertoli cell conditioned medium and Sertoli cell secreted proteins were all essentially parallel. This observation indicates that inhibin and the reactive component in Sertoli cell conditioned medium are immunologically similar. An immunoblot of Sertoli cell secreted proteins with the inhibin antisera consistently detected a 32 kDa protein which is the expected size of the mature processed form of inhibin (McLachlan et al., 1988). A 57 kDa protein was also detected in half of the inhibin immunoblots examined. An incomplete processed form of the inhibin precursor with an extended NH_2 -terminal α -subunit ($\alpha_{43}\beta$) corresponds to this molecular weight (Robertson et al., 1985; McLachlan et al., 1988). The presence of this 57 kDa protein was independent of the hormonal stimulation of the Sertoli cells.

The speculation is made that this 57 kDa protein detected by the inhibin antisera is an incomplete processed form of the inhibin precursor. Whether the presence of this high molecular weight component in some preparations is due to variable concentrations of extracellular proteases in the Sertoli cell culture remains to be investigated. It should be noted that no 20 or 35–40 kDa material was detected which would have suggested free α -subunit or an $\alpha\alpha$ -dimer. The inhibin RIA specificity and sensitivity were judged to be sufficient to assess the ability of regulatory agents to influence inhibin production by Sertoli cells.

FSH was found to markedly stimulate inhibin secretion by Sertoli cells as previously reported (LeGac and de Kretser, 1982; Bicsak et al., 1987; Morris et al., 1988). Other regulatory agents previously shown to influence Sertoli cells including insulin, retinol and testosterone were not found to have a significant effect on inhibin secretion. The concentrations of these regulatory agents used have previously been shown to be optimal for the stimulation of a number of Sertoli cell functions (Skinner and Griswold, 1982; Skinner et al., 1988). Demonstration that transferrin secretion was stimulated in the same medium samples as in those in which inhibin secretion was not altered provides support that these individual agents do not influence inhibin production. Interestingly, insulin and retinol were found to enhance the ability of FSH to stimulate inhibin production. Whether this synergism between insulin, retinol and FSH is due to an increase in the number of FSH receptors and/or an enhanced ability for FSH to cause a signal transduction remains to be investigated. Previous reports of the regulation of transferrin production by Sertoli cells indicate that FSH, insulin and retinol each stimulates transferrin production while a combination of FIR results in an additive response of the three regulatory agents (Skinner and Griswold, 1982). Transferrin production by Sertoli cells appears to be regulated by multiple signal transduction pathways. In contrast, inhibin production appears to be primarily regulated by an FSH-induced cAMP-dependent mechanism (Bicsak et al., 1987). The potential differential regulation of various Sertoli cell functions has previously been investigated (Perez-Infante et al., 1986; Skinner et al., 1989).

Peritubular cell secreted proteins and purified PModS were both found to stimulate inhibin secretion. The degree of stimulation, however, was less than that observed for FIRT. While PModS stimulated inhibin production to a lesser degree than FIRT, examination of PModS effects on transferrin production in the same cultures indicated a significant effect of PModS alone which was similar to that of FIRT. This data confirms previous observations regarding the actions of PModS on transferrin production (Skinner et al., 1988) and indicated that the biological activity of the PModS preparation was optimal. Both forms of PModS, PModS (A) and PModS (B), were found to affect inhibin secretion in a similar manner and at similar effective concentrations. The minimum effective concentration was 3–5 ng/ml and maximal effective concentration approximately 25 ng/ml. This observation supports the previous data indicating that PModS (A) and PModS (B) have similar biological activities (Skinner et al., 1988). Although PModS was found to stimulate inhibin secretion, the effects were delayed until days 5–7 of Sertoli cell culture. This is in contrast to the effects observed for FSH which initially occurred between days 2 and 5 of culture. This delayed effect of PModS on Sertoli cell inhibin production may reflect its mechanism of action. PModS appears to primarily act via a cGMP-mediated response and not cAMP (Norton and Skinner, 1989). However, after several days of treatment with PModS, cAMP levels rise, but not to the same extent as cAMP levels rise in response to FSH (Norton and Skinner, 1989). Therefore, the effects of PModS on inhibin production may correspond to the long-term delayed effects of PModS on cAMP.

The only individual regulatory agents found to stimulate inhibin production *in vitro* were PModS and FSH. Interestingly, a combination of FSH and PModS was found to act synergistically and result in a stimulation of inhibin secretion similar to that found with FIR treatment. Inhibin production was not increased with the additional treatment of PModS and FIR. Observations imply that an apparent maximal level of inhibin secretion is achieved with the combined treatments of PModS and FSH or insulin, retinol and FSH. An 8- to 10-fold stimulation of inhibin production was ob-

served with these these combined treatments of Sertoli cells. The pharmacologic mechanisms involved in the synergistic actions of PModS or insulin and retinol with FSH remain to be investigated. Results presented in the current study, however, imply that regulatory agents which can directly or indirectly influence cAMP levels will likely have an important role in regulating inhibin production by Sertoli cells.

The endocrine regulation of inhibin production has previously been thought to primarily be mediated through the actions of FSH (LeGac and de Kretser, 1982; Au et al., 1985; Bicsak et al., 1987; McLachlan et al., 1988a, b; Morris et al., 1988). This correlates with the proposal that inhibin provides a negative feedback on FSH secretion by the pituitary (McLachlan et al., 1988b). Recently, LH has also been found to elevate inhibin levels *in vivo* (McLachlan et al., 1988a). Since LH only acts on Leydig cells to increase androgen production and does not act directly on Sertoli cells, the ability of androgens to regulate inhibin production was postulated (McLachlan et al., 1988a). The current study confirms previous studies (Bicsak et al., 1987; Morris et al., 1988) in that testosterone has no direct effect on inhibin production by Sertoli cells *in vitro* either alone or in combination with FSH, insulin and retinol. The ability of testosterone to directly regulate inhibin production *in vivo* remains to be determined. The testicular paracrine factor PModS has profound effects on Sertoli cell function (Skinner et al., 1988) and is produced under the apparent control of androgens (Skinner and Fritz, 1985). Indirect effects of androgens on Sertoli cells mediated through the peritubular cells via PModS have previously been suggested (Skinner and Fritz, 1985b). Therefore, the ability of PModS to stimulate inhibin production provides a potential mechanism for LH to elevate inhibin levels. The cellular interaction is postulated in which LH acts on Leydig cells to influence androgen production which in turn acts on peritubular cells and elevates PModS production which subsequently acts on Sertoli cells to stimulate inhibin production. This additional mechanism for the endocrine regulation of inhibin production adds complexity to the overall control of inhibin expression by Sertoli cells. In addition, observations contribute to the hypothesis that

PModS may have an important role in the regulation of testicular function. Although these *in vitro* experiments demonstrate the potential for PModS regulation of inhibin secretion, physiological experiments are now required to determine the presence and/or importance of such an interaction *in vivo*.

Further investigations are required to understand the physiological significance of inhibin to the endocrine system and in the regulation of testis function. Recently, inhibin has been shown to suppress the ability of LH to stimulate androgen production by Leydig cells in the testis and theca cells in the ovary (Hsueh et al., 1987). The inhibin β -subunit dimer activin has been shown to augment LH actions on Leydig cells and may also have a role in the regulation of steroidogenesis (Hsueh et al., 1987). This potential local cell-cell interaction of inhibin to regulate gonadal steroidogenesis may be an important additional function for inhibin. The ability of PModS to stimulate inhibin secretion, therefore, may provide a local control mechanism and feedback loop for the regulation of androgen production in the testis.

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