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Evaluation of the effect of peritubular cell secretions and the testicular paracrine factor P-Mod-S on Leydig cell steroidogenesis and immunoactive inhibin production

G. P. RISBRIDGER and M. K. SKINNER* *Department of Anatomy, Monash University, Clayton, Victoria, Australia and *Department of Pharmacology, Vanderbilt University, Nashville, Tennessee, U.S.A.*

Summary

Testicular peritubular cells have been shown to produce a paracrine factor, termed P-Mod-S, under androgen control that has dramatic effects on Sertoli cell function and may provide an important mode of androgen action in the testis. Therefore, the current study was designed to investigate the possibility that peritubular cell secretory products could feedback and regulate Leydig cell function. The Leydig cell functional parameters that were examined included testosterone production and inhibin secretion. Purified forms of P-Mod-S (P-Mod-S(A) and P-Mod-S(B) shown to be biologically active on Sertoli cells) had no effect on basal or gonadotrophin-stimulated production of testosterone or inhibin by Leydig cells. A preparation of peritubular cell-secreted proteins (PSP) with molecular weights >3 kDa did not influence testosterone production by Leydig cells. PSP, however, did influence cultured Leydig cell morphology and improved cell viability. PSP also had no effect on the ability of LH to stimulate Leydig cell testosterone production. Whilst determining the effect of PSP on Leydig cell inhibin production, PSP was found to contain endogenous levels of inhibin apparently due to 2% contamination of the peritubular cell cultures with Sertoli cells. When this endogenous inhibin level was considered, PSP was found to have no influence on basal or hormone-stimulated production of inhibin by Leydig cells. Results of the current study indicate that peritubular cell secretory products, including the paracrine factor P-Mod-S, do not appear to play a major role in the regulation of Leydig cell function. Therefore, the regulation of Leydig cell function by the seminiferous tubule will primarily be due to Sertoli cell secretory products.

Keywords: inhibin, Leydig cells, peritubular cells, P-Mod-S, steroidogenesis.

Introduction

The control of testicular function results from a complex interplay between the different cellular components of the testis including Sertoli, Leydig, peritubular and germ cells. Evidence for specific interactions between Sertoli and peritubular

Correspondence: Dr G. P. Risbridger, Department of Anatomy, Monash University, Wellington Road, Clayton, Victoria 3168, Australia.

cells has been obtained from co-cultures of these two cell types. The production of transferrin, androgen binding protein, immunoactive inhibin, and the aromatase activity of Sertoli cells is influenced by peritubular cells (Verhoeven & Cailleau, 1988; Skinner *et al.*, 1989a, b). This interaction is achieved in part through the action of a peritubular cell-derived protein termed P-Mod-S (Skinner & Fritz, 1985). Purification of this paracrine factor has identified two forms of P-Mod-S, A and B, which have dramatic effects on these Sertoli cell functions (Skinner *et al.*, 1988). The production of P-Mod-S is believed to be stimulated by testosterone (Skinner & Fritz, 1985) which implicates an influence of Leydig cells on the interaction between the peritubular and Sertoli cells. The current study was designed to investigate potential interactions between peritubular and Leydig cells. The actions of crude peritubular cell secretory products and the testicular paracrine factor P-Mod-S on Leydig cell steroidogenesis were evaluated. In addition to androgen production, Leydig cells also secrete a number of proteins. Morris *et al.* (1987) have identified at least 20 proteins secreted in short-term primary cultures of Leydig cells. Recently, immunoactive inhibin has been shown to be produced by Leydig cells in culture (Risbridger *et al.*, 1989a), and P-Mod-S has been shown to influence the production of immunoactive inhibin by the Sertoli cell (Skinner *et al.*, 1989b); therefore P-Mod-S could potentially affect inhibin production by Leydig cells.

Materials and methods

Animals

Adult male Sprague Dawley rats aged 70–90 days were obtained from Sasco Inc (Mo, USA) or the Central Animal House, Monash University, Australia. Animals were given food and water *ad libitum* and maintained at 22°C on a 12 h light/12 h dark schedule.

Reagents

Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutrient mixture (F12) were obtained from Gibco, Grand Island, New York, USA. Penicillin (5000 units^{-1}) and streptomycin sulphate (5 mg l^{-1}) were purchased from C.S.L., Parkville, Australia and added to the media together with 0.1% bovine serum albumin Cohn fraction V from Sigma (St Louis, MO, USA). Percoll was purchased from Pharmacia, Uppsala, Sweden, and collagenase from Sigma (St. Louis, MO, USA).

The gonadotrophin used to stimulate the Leydig cells was rat LH (NIADDK-rLH-I-6) kindly supplied by NIADDK, NIH, Bethesda, MD, USA.

Preparation of Leydig cells

Purified Leydig cells were obtained from interstitial cell preparations following collagenase dispersion of decapsulated testes and fractionation on discontinuous Percoll density gradients as described previously (Risbridger *et al.*, 1987). Purified preparations of Leydig cells contained 90.8 ± 2.5% 3β-hydroxysteroid dehydrogenase positive cells (mean ± SD, n = 4 preparations). The Leydig cells were resuspended (5×10^5 cells ml^{-1}) in DMEM:F12 (1:1) containing 0.1% BSA and

plated out (100–200 μl well^{-1}) in 48-well culture plates (Costar, Cambridge, MA, USA). Peritubular cell contamination of the Leydig cell preparation was <0.5% as determined by staining with alkaline phosphatase using the method described by Anthony & Skinner (1989).

The Leydig cells were incubated with P-Mod-S, PSP or test substances in a volume of 50–100 μl, with or without purified gonadotrophin (LH or hCG) and the total volume adjusted with medium to 275 μl. The cells were then incubated for 20 h at 32°C in 95% 0_2/5% CO_2, the media were then collected and either stored at −20°C or freeze-dried until assayed for testosterone or inhibin by specific radioimmunoassays.

Preparation of peritubular and Sertoli cell secreted proteins

Testes from 20-day-old rats were digested sequentially by enzymes and the peritubular cells were obtained from the collagenase-digested supernatant after tubule segments had sedimented under gravity, as decribed previously (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 2–3 days of culture, subcultured cells were confluent and were 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. Sertoli cells were prepared by sequential enzymatic digestions with a modified procedure as described previously by Dorrington *et al.* (1975) and Tung *et al.* (1984).

Peritubular cell or Sertoli cell serum-free conditioned medium were collected, 25 μM phenylmethylsulfonyl fluoride and 0.1 mM benzamidine were added 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 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).

Preparation of P-Mod-S

P-Mod-S was purified from concentrated peritubular cell-conditioned medium as described previously (Skinner *et al.*, 1988). Briefly, an ammonium sulphate 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 P-Mod-S preparations was assessed by electrophoresis and silver staining as described previously (Skinner *et al.*, 1988). Purity was confirmed with the detection of a homogenous protein preparation at 59 kDa for P-Mod-S (B) and 56 kDa for P-Mod-S (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 P-Mod-S were stored at −70°C in the presence of 1 mg ml^{-1} bovine serum albumin prior to use.

Radioimmunoassays for testosterone and inhibin
Culture media were thawed or reconstituted to the original volume and assayed for testosterone using a ^{125}I-testosterone radioimmunoassay at Monash University as described previously (Kerr *et al.*, 1985); the sensitivity of the testosterone RIA was 6 pg tube^{-1}. Inhibin was measured in all samples using a specific radioimmunoassay as described previously (Robertson *et al.*, 1988). Briefly, purified 31 kDa bovine inhibin was iodinated and used as tracer with an antiserum (#1989) raised in a rabbit against bovine 31 kDA inhibin. The antiserum showed no significant (<1%) cross-reactivity with inhibin subunits produced by reduction and alkylation of inhibin or with bovine activin-A, Mullerian inhibitory substance, transforming growth factor-β or FSH suppressing protein (FSP) or follistation. A significant cross reactivity (288%) with the inhibin α-chain fragment, pro-α_c, was observed. A cytosolic extract of ovaries from PMSG-treated immature female rats (ROVE A/88) was used as the inhibin standard, and defined in terms of its *in-vitro* bioactivity using an *in-vitro* bioassay based on the suppression of FSH cell content in rat pituitary cells (Scott *et al.*, 1980), which had been calibrated against an ovine testicular lymph standard with a designated unitage of 1 U mg^{-1}. The standard was diluted in media (DMEM:F12 containing 0.1% BSA).

Results

Effect of P-Mod-S on Sertoli cells
To confirm the biological activity of P-Mod-S, Sertoli cells were treated with both P-Mod-S(A) and P-Mod-S(B). The ability of P-Mod-S to stimulate transferrin

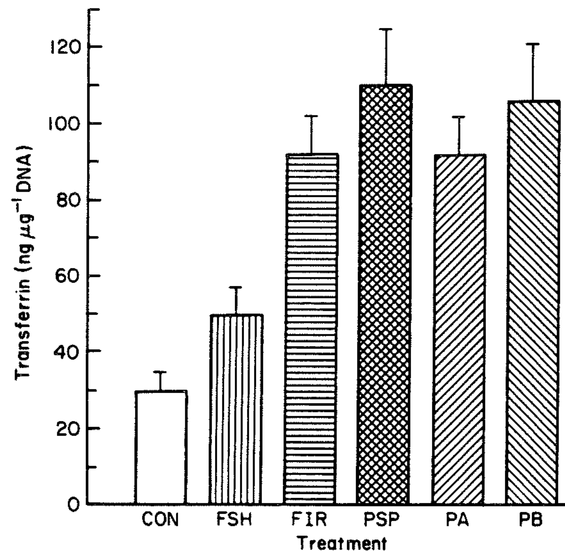

Fig. 1. Assay for P-Mod-S bioactivity based on Sertoli cell transferrin production. Sertoli cells were cultured in the absence (CON) or presence of 25 ng ml^{-1} FSH (FSH); FSH, 5 μg ml^{-1} insulin, and 0.35 μM retinol (FIR); 50 μg ml^{-1} PSP (PSP); 25 ng ml^{-1} P-Mod-S(A) (PA); 25 ng ml^{-1} P-Mod-S(B) (PB). A 72-hour medium collection on day 5 of culture was analysed for transferrin levels with an RIA and is expressed as ng transferrin ug^{-1} Sertoli DNA. The mean ± SD from a representative of three experiments is presented.

production by cultured Sertoli cells is used as a bioassay for P-Mod-S. Both forms of P-Mod-S and the crude preparation of peritubular cell secreted proteins (PSP) were found to stimulate transferrin production (Fig. 1). The level of stimulation was greater than that seen for FSH and equivalent to the combined actions of FSH, insulin and retinol. These data confirm previous observations and indicate that the P-Mod-S preparations used in the current study were bioactive.

Effects of P-Mod-S or PSP on Leydig cells

Leydig cells were isolated and cultured to examine the actions of P-Mod-S on testosterone production. Neither PSP or the purified forms of P-Mod-S, P-Mod-S(A) or P-Mod-S(B) were found to influence Leydig cell testosterone production (Fig. 2). Similar results were also found in the absence or presence of hCG. Therefore, neither P-Mod-S nor PSP were found to significantly affect testosterone production by Leydig cells.

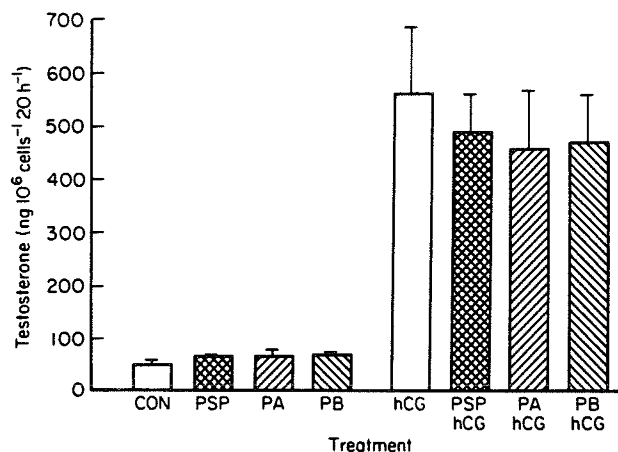

Fig. 2. Effects of PSP and P-Mod-S on Leydig cell testosterone production. Leydig cells were cultured in the absence (CON) or presence of PSP (PSP) (50 μg ml^{-1}), P-Mod-S(A) (PA) or P-Mod-S(B) (PB) (25 ng ml^{-1}) with or without human chorionic gonadotrophin, 1 μg ml^{-1} (hCG). Testosterone was measured in media collected after 20 h incubation and expressed as ng testosterone 10^6 cell^{-1} 20 h^{-1}. The mean ± SD is presented from 5 replicate experiments.

An additional experiment was performed to assess the effects of peritubular cell secretory products on the ability of Leydig cells to respond to LH. Purified rat LH significantly stimulated testosterone production by Leydig cells in a dose-dependent manner. The addition of PSP caused variable effects on steroidogenesis, being slightly stimulatory under basal conditions but tending to be inhibitory with increasing doses of LH, although these changes were not significant except with 0.5 ng ml^{-1} LH (Fig. 3). Therefore, peritubular cell products appear to have negligible effects on Leydig cell steroidogenesis or hormone responsiveness.

Although peritubular cell secretory proteins had no major effect on Leydig cell steroidogenesis, an effect on cell morphology was clearly observed. The addition of PSP to the Leydig cell cultures altered the light microscopic appearance of the

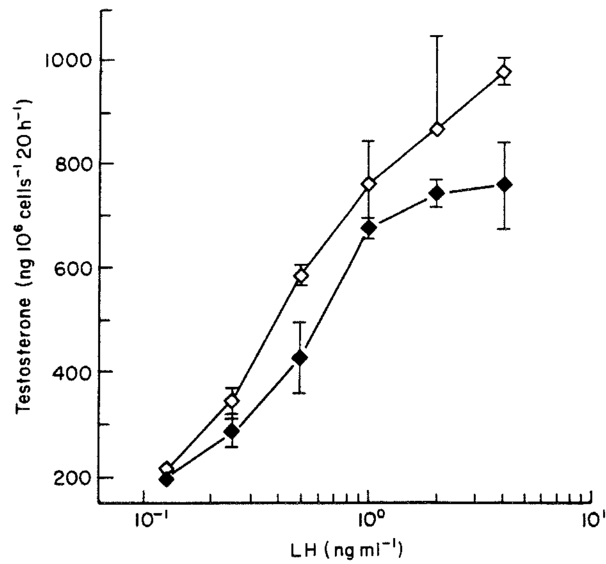

Fig. 3. LH dose response curve for Leydig cell testosterone production. Leydig cells were cultured in the absence (open diamond) or presence (closed diamond) of PSP with increasing concentrations of LH. Data are expressed as ng testosterone 10^6 cell^{-1} 20 h^{-1} and the mean ± SD from 4 replicate wells are presented. Basal levels of testosterone production (i.e. in the absence of LH) were 40.7 ± 9.8 (mean ± SD, n = 4.)

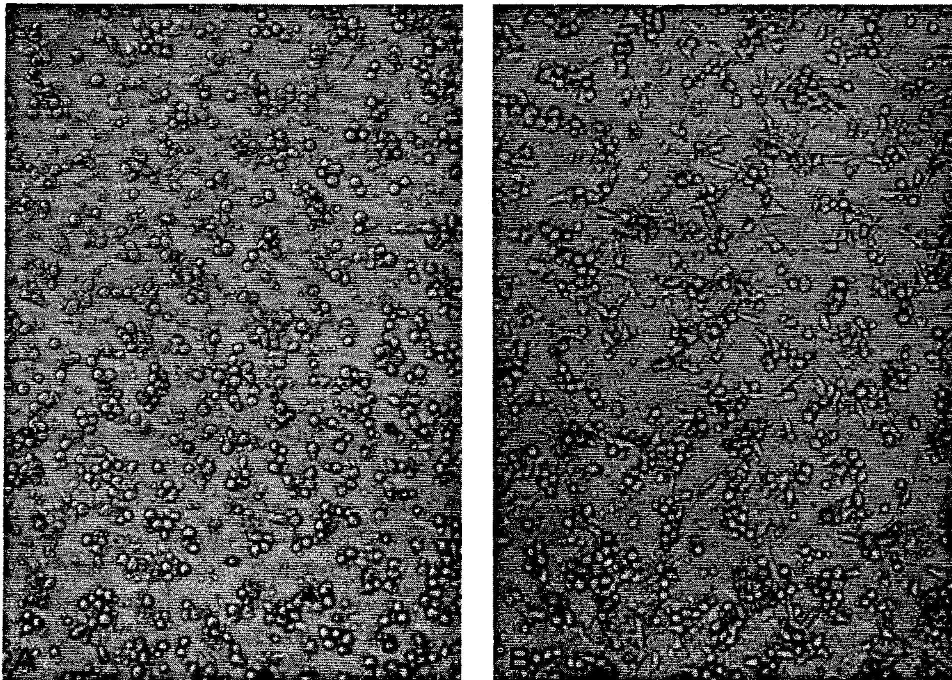

Fig. 4. Light microscope appearance of Leydig cells cultured in the absence (A) or presence (B) of PSP (50 μg ml^{-1}). Final magnification × 236.

Leydig cells so that they flattened out on the culture dish and the projections of the cells were more pronounced (Fig. 4).

As an alternative parameter of Leydig cell function, inhibin production was measured by radioimmunoassay. Addition of hCG stimulated I-inhibin production significantly by the Leydig cells as observed previously (Risbridger *et al.*, 1989a). PSP increased I-inhibin ($P<0.05$) under both basal and gonadotrophin-stimulated conditions, whilst P-Mod-S-A and -B were without any significant effect (Fig. 5).

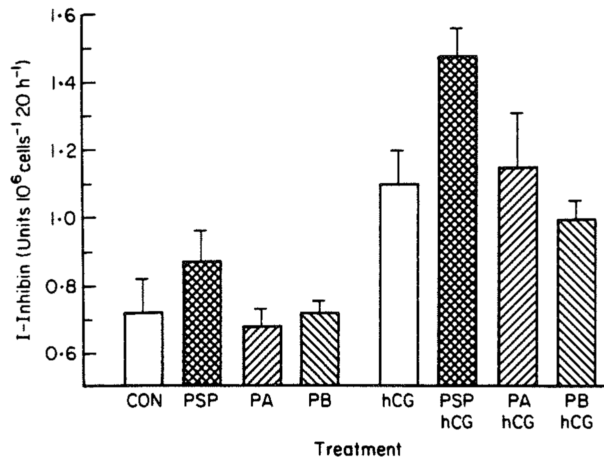

Fig. 5. Effects of PSP and P-Mod-S on Leydig cell production of immunoactive inhibin (I-inhibin). Leydig cells were cultured and treated as outlined in the legend to Figure 2. Immunoactive inhibin was determined in media collected after 20 h incubation and expressed as units inhibin 10^6 cell^{-1} 20 h^{-1} (mean ± SD, n = 3).

Previously, the levels of I-inhibin in PSP had been reported as being non-detectable (Skinner *et al.*, 1989b). However there was detectable levels of I-inhibin in the two PSP preparations that were used in these studies. The log-logit plots of the diluted doses of PSP were linear, and parallel to the ROVE A/88 standard. The estimated relative potencies of I-inhibin in the two preparations were 9.61 U ml^{-1} and 10.47 U ml^{-1}. The inhibin activity in PSP was also bioactive and was determined for PSP29 to be 16.34 U ml^{-1} (Table 1). The bioactive/immunoactive inhibin ratio was 1.70, which is similar to that reported for Sertoli cell inhibin (Risbridger *et al.*, 1989) suggesting that the activity in PSP was due to Sertoli cell contamination of the peritubular cell cultures of the order of $<2\%$. This low level of Sertoli cell contamination in the peritubular cell cultures has been reported previously (Anthony & Skinner, 1989) and is presumed to be the source of the I-inhibin detected in the PSP preparations. When the endogenous level of inhibin present in PSP is considered, PSP was not found to significantly influence inhibin production by Leydig cells.

These observations were extended to examine the potential influence of PSP on Leydig cell hormone responsiveness. As 50 µl of PSP was added to the culture wells the mean level of I-inhibin in the wells was approximately 0.5 U I-inhibin. The data

Table 1. Inhibin levels in PSP and SSP preparations

Sample and code name	Immunoactive (U ml^{-1})	Bioactive (U ml^{-1})	Transferrin (μg ml^{-1})
PSP 29	9.61	16.34	0.12
PSP 007	10.47	Not determined	0.11
SSP	629.2	Not determined	9.7

PSP and SSP samples were assayed at multiple dilutions and are linear and parallel to the standard Rove A/88.

presented in Fig. 6 are calculated and expressed as I-inhibin/10^6 cell^{-1}, so that if 2 $\times$ 10^5 cells are cultured in the well then at least 2.5 U I-inhibin produced by the Leydig cells is due to the inhibin content of the exogenously added PSP; therefore this amount was subtracted from all incubations containing PSP. Although a significant ($P<0.05$) stimulation of I-inhibin production by Leydig cells was observed with the addition of rLH, no significant effect of PSP was observed (Fig. 6).

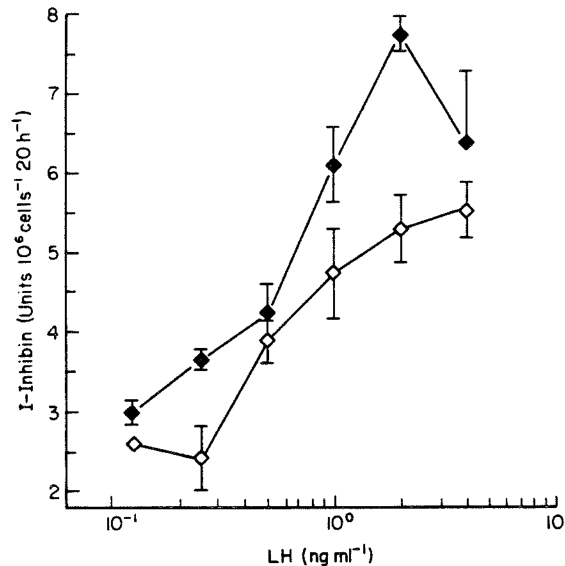

Fig. 6. LH dose response curve for Leydig cell production of immunoactive inhibin (I-inhibin). Leydig cells were cultured in the absence (◇) or presence (◆) of PSP and treated as outlined in the legend to Figure 3. Data are expressed as units Inhibin 10^6 cell^{-1} 20 h^{-1} and the mean ± SD from replicate experiments are presented.

Discussion

Testosterone has been shown to modulate the production of P-Mod-S by peritubular cells in culture (Skinner & Fritz, 1985). The present data demonstrates that purified P-Mod-S A or B does not appear to act on the Leydig cells themselves to regulate their secretions as determined by the measurement of testosterone or immunoactive Inhibin under basal or LH stimulation *in vitro*.

The peritubular cell secreted protein preparations which contain P-Mod-S activity also have detectable levels of immunoactive Inhibin (approximately 10.04 U ml^{-1}). If the endogenous levels of immunoactive inhibin are subtracted from the PSP then Leydig cell production of inhibin did not change significantly when cultured with PSP. Testosterone levels did not alter either in response to the addition of PSP, following LH stimulation, although there was a variable increase in basal production. We have reported previously that the level of LH in interstitial fluid ranges from 0.53–0.86 ng equiv. I_6 ml^{-1} ($n = 6$ animals; Simpson *et al.*, 1990), which is sufficient to stimulate the Leydig cell, but it is unlikely that P-Mod-S modulates steroidogenesis by the Leydig cells. The effect of PSP on basal testosterone production by the Leydig cells in culture is probably due to the addition of other peritubular products such as the extracellular matrix proteins which promote the integrity of the cells in culture, in the absence of gonadotrophin. This is supported by the effects PSP had on cultured Leydig cell morphology.

The detection of inhibin immunoactivity and bioactivity in the peritubular cell secreted protein preparations was due to contamination of the cultures with Sertoli cells. Whilst this may have been minimal, it can be calculated from the levels of inhibin in SSP (629 U ml^{-1}) that contamination of the peritubular cell preparations with only 1.6% Sertoli cells, would result in 10.04 U ml I-inhibin^{-1} in a PSP sample which had been concentrated 100-fold. The fact that the bioactive: immunoactive inhibin in PSP was approximately 1.7, (which is similar to the B/I ratio of unfractionated, unconcentrated Sertoli cell culture media; Risbridger *et al.*, 1989b) would support this concept. Finally, in contrast to the PSP, the purified forms of P-Mod-S A and B did not increase media inhibin levels, presumably because inhibin had been removed during the procedure for purification. The absence of any effect of P-Mod-S on Leydig cell function in the presence of LH *in vitro* suggests that there is no direct short loop feedback between the Leydig and peritubular cells, but that Sertoli cell function and differentiation is regulated by P-Mod-S which is produced by the peritubular cells and regulated in turn by Leydig cell testosterone.

An intratesticular feedback loop between Sertoli, peritubular and Leydig cells could operate through the action of Sertoli cell products (stimulated by P-Mod-S) on Leydig cell steroidogenesis. A number of hormones and factors are produced by the Sertoli cell, but an obvious example that comes to mind is immunoactive inhibin. P-Mod-S stimulates Sertoli cell inhibin production (Skinner *et al.*, 1989b), and inhibin has been reported to regulate interstitial cell testosterone (Hsueh *et al.*, 1987). Thus it is possible that P-Mod-S, in the presence of testosterone, could stimulate inhibin secretion by the Sertoli cell which, in turn, stimulated testosterone production further. However, this hypothesis requires further evaluation as inhibin does not alter adult rat Leydig cell steroidogenesis *in vitro* (Liu *et al.*, 1989; Grootenhuis *et al.*, 1990). Other Sertoli factors have been reported to regulate steroidogenesis in a paracrine manner, including growth factors (TGF, EGF, IGF) and a number of novel factors in culture media or interstitial fluid which either inhibit or stimulate Leydig cell steroidogenesis (Verhoeven & Cailleau, 1990; Vihko & Huhtaniemi *et al.*, 1990; Hedger *et al.*, 1990). Whether the production of these factors is regulated by P-Mod-S remains to be shown.

The results of the current study indicate that peritubular cell secretory products,

including the testicular paracrine factor P-Mod-S, do not appear to play a major role in the regulation of Leydig cell function. Therefore, the regulation of Leydig cell function by the seminiferous tubule will probably be due to Sertoli cell secretory or derived products. The specific paracrine factor(s) that mediate Sertoli cell-Leydig cell interactions remain to be elucidated.

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Announcement

27–30 March 1992
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Serono lecture: The formation of the cytoskeleton of mammalian spermatozoa, Yves Clermont
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Paracrine interactions in the in-vitro and in-vivo testis, Guido Verhoeven
Workshop on adolescent varicicele, Stuart Howards
Symposium 1 — Pediatric andrology: Abnormalities of testicular determination, Claude Migeon
Diagnosis and treatment of ambiguous genitalia, Pat Donahoe
Neuroendocrinology of puberty, Mel Grumbach
Congenital adrenal hyperplasia, Maria New
Symposium 2 — Celular interactions: Connective tissue in health and disease: molecular implications in genitourinary tumour metastasis, Leo Furcht
Androgen regulation of programmed death of normal and neoplastic prostatic cells, John Isaacs
Mesenchymal-epithelial interactions in the regulation of normal and neoplastic tissues, Gerald Cunha
Role of nerve growth factor-like protein in the paracrine regulation of prostatic growth, Daniel Djakiew

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For registration information please contact:

Robert Schmidt
ASA
309 West Clark Street
Champaign, IL. 61820
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