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Peritubular Myoid Cells from Immature Rat Testes Secrete Activin-A and Express Activin Receptor Type II *in Vitro**

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ABSTRACT

The expression of activin type II and IIB receptors and inhibin α-, β_A-, and β_B-subunit messenger RNAs (mRNAs), and the secretion of immunoreactive and bioactive activin during culture of testicular peritubular myoid cells and peritubular myoid cell lines were studied.

Cultured peritubular myoid cells and cell lines expressed high levels of inhibin β_A-subunit mRNA and some inhibin α- and β_B-subunit mRNA. Activin receptor type II mRNA was also detected, whereas activin receptor type IIB mRNA expression was not found. Expression of the β_A-subunit mRNA was present immediately after isolation of the cells and increased during culture in Eagle's Minimum Essential Medium containing 10% fetal calf serum. β_A-Subunit mRNA expression was not regulated by the synthetic androgen R1881. Western blotting of peritubular myoid cell- and peritubular cell line-conditioned media with a polyclonal antiserum against recombinant activin-A revealed the presence of 25-kilodalton activin-A, whereas activin bioactivity was detected using the animal cap assay.

Because of the secretion of activin-A by peritubular myoid cells, the effects of recombinant activin-A on Sertoli cell inhibin and transferrin secretion were examined. Activin-A stimulated both basal and FSH-stimulated inhibin and transferrin production by Sertoli cells after 72 h of culture. These effects resemble the effects of the testicular paracrine factor PmodS on Sertoli cell function.

It is concluded that activin-A is secreted by peritubular cells *in vitro* and that activin-A shares a number of effects on Sertoli cell function with PmodS. (*Endocrinology* **135:** 759–767, 1994)

ACTIVINS, homo- or hetero-dimers of the inhibin β_A- and β_B-subunit, were originally purified from ovarian follicular fluid on the basis of their ability to stimulate the synthesis and release of FSH from pituitary cells in culture (1, 2). It is currently accepted that activins also exert many other biological functions and can be regarded as cell differentiation factors (3). Recently, activin receptors (type II and IIB) with homology to serine/threonine kinases have been cloned (4, 5). Of these receptors, activin receptor type II (ActRII) is expressed in several testicular cell types, including Sertoli cells, pachytene spermatocytes, and round spermatids (6, 7). ActRIIB is mainly expressed in Sertoli cells and A-spermatogonia (8). Therefore, activins may play an important role as para- or autocrine factors in the testis. Actually, the effects of recombinant activin-A on Sertoli cell function have been demonstrated (9, 10), and regulatory roles of activins in Leydig cell steroidogenesis (11–13) and spermatogenesis (10) have been suggested. Furthermore, the production of activin-B by Sertoli cells from testes of immature rats has been described (9), whereas the secretion of activin-A in the immature rat testis has been ascribed to Leydig cells (14).

In this study, the production of activin and the expression of activin receptors in cultured peritubular myoid cells and newly established peritubular cell lines were examined. The effects of activin-A on Sertoli cell function were also studied in more detail and compared with the effects described for the peritubular myoid cell product PmodS (15), another testicular paracrine factor, which stimulates the secretion of androgen-binding protein (ABP), transferrin, and inhibin (16, 17) and inhibits FSH-stimulated aromatase activity (18) in rat Sertoli cells.

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Materials and Methods

Isolation and culture of peritubular myoid cells

All animal experimentation described in this manuscript was conducted in accordance with the highest standards of humane animal care, as outlined in the NIH Guide for the Care and Use of Laboratory Animals. Peritubular myoid cells were isolated from testes of 21- to 23-day-old Wistar rats, as described by Blok *et al.* (19). In short, decapsulated testes were incubated for 20 min at 37 C (120 cycles/min) in PBS containing deoxyribonuclease-I (5 μg/ml; type DN25, Sigma Chemical Co., St. Louis, MO), collagenase (1 mg/ml; type CLS, Worthington Biochemical Corp., Freehold, NJ), trypsin (1 mg/ml; type TRL, Worthington), and hyaluronidase (1 mg/ml; type I-S, Sigma). Tubular fragments were separated from the peritubular cell fraction by sedimentation at unit gravity. The supernatant was filtered through a 60-μm nylon filter, and cells were precipitated by centrifugation at 250 × g for 2 min. Cells were plated at a density of approximately 5×10^4 cells/cm^2 in 175-cm^2 culture flasks (Costar, Cambridge, MA) and cultured in Eagle's Minimal Essential Medium (MEM; Gibco, Grand Island, NY) supplemented with antibiotics and nonessential amino acids containing 10% fetal calf serum (FCS; Sebak, Aidenback, Germany). After 2 days of culture, the cells were trypsinized, seeded 1:10, and cultured for another 3 days in MEM-

10% FCS. Total RNA was then isolated or the cells were cultured for a final 3-day period in serum-free MEM to obtain conditioned medium. This isolation method is referred to as method 1.

Peritubular myoid cells were also isolated from testes of 19-day-old Wistar rats using an alternative method (method 2). Decapsulated testes of 20 rats were incubated for 70 min at 32 C (140 cycles/min) in 20 ml PBS containing DNase (10 μg/ml; Boehringer Mannheim, Mannheim, Germany) and trypsin (2.5 mg/ml; Boehringer Mannheim) to remove Leydig cells. Trypsinization was stopped by the addition of 0.5% soybean trypsin inhibitor (Boehringer Mannheim). The tubular fragments were washed three times with PBS and digested for 20 min at 32 C (140 cycles/min) in PBS-DNase supplemented with collagenase (1 mg/ml; Boehringer Mannheim) and hyaluronidase (1 mg/ml; Merck, Darmstadt, Germany). The supernatant was filtered through a nylon screen (Nytal, 100 μm, Swiss Silk Bolting Cloth Co., Zurich, Switzerland), and peritubular cells were seeded in two 175-cm^2 culture flasks (Costar, Cambridge, MA) and cultured for 5 h in RPMI (Seromed, Biochrom, Berlin, Germany) containing 10% FCS. Then cells were washed twice with culture medium to remove Sertoli cells, and fresh RPMI-10% FCS was added. After 3 days of culture, cells were trypsinized, seeded 1:2, and cultured for another 3 days in RPMI-10% FCS. Cells were trypsinized again, seeded 1:2, and cultured for 5 days in RPMI-10% FCS. Total RNA was isolated after this culture period.

Isolation and culture of Sertoli cells

Highly purified Sertoli cells (with <0.1% peritubular myoid cells) from testes of immature (21-day-old) Wistar rats were isolated and cultured using the procedure described by Themmen *et al.* (20). Briefly, decapsulated testes were incubated for 20 min at 37 C in PBS containing DNase-I (5 μg/ml; type DN25, Sigma), collagenase (1 mg/ml; type CLS, Worthington), trypsin (1 mg/ml; type TRL, Worthington), and hyaluronidase (1 mg/ml; type I-S, Sigma). After four successive washes by sedimentation at unit gravity in PBS-DNase, the tubular fragments were incubated in PBS-DNase containing 1 mg/ml collagenase and 1 mg/ml hyaluronidase for 20 min at 37 C. The fragments were washed four times as described above and dispersed using a Dounce homogenizer (Kontes Co., Vineland, NJ). The cells were then washed four times with PBS-DNase and once with MEM by sedimentation at 100 × *g* for 2 min. The cell preparation was plated at a density of 0.5 × 10^6 cells/cm^2 in 75-cm^2 culture flasks (Costar) for RNA extraction or in 48-well plates (Costar) for determination of the effect of recombinant activin-A on Sertoli cell inhibin and transferrin production. The cells were cultured for 48 h at 37 C in MEM supplemented with nonessential amino acids, glutamine, antibiotics, and 1% FCS. Then, they were shocked hypotonically for 2 min in 0.1-fold concentrated MEM to remove germ cells. The culture was continued for 24 h in MEM containing 0.1% BSA (fraction V, Sigma). Subsequently, total RNA was isolated or medium was replaced by MEM containing 0.1% BSA and hormones [oFSH (S-17), NIH Bethesda, MD; recombinant bovine activin-A, Innogenetics, Ghent, Belgium]. After 72 h of culture, medium was collected and frozen until measurement of immunoreactive inhibin and transferrin. Cells were lysed in 1 N NaOH, and the DNA content of the cell lysates was determined.

Alkaline phosphatase staining

Peritubular myoid cell preparations were stained for alkaline phosphatase to determine the relative amount of peritubular cells (21). Cells were fixed for 25 min in 70% ethanol and subsequently stained for approximately 20 min in 0.2 M Tris buffer (pH 9.1) containing 10 mM $MgCl_2$, 2 mM 4-aminodiphenylamine diazonium sulfate (Sigma), and 1.4 mM naphthol AS-MX phosphate (Sigma). Cells were washed with 70% ethanol and examined by microscopy.

Pregnenolone measurement

To determine the presence of Leydig cells in the preparation obtained by method 1, pregnenolone production was measured. Cells were isolated, cultured for 2 days, trypsinized, seeded 1:10, and cultured for an additional 3 days, as described for method 1. After a total culture period of 5 days, medium was replaced by MEM containing 0.1% BSA, 5 μM SU-10603 [7-chloro-3,4-dihydro-2-(3-pyridyl)1-(2H)naphthalenone; Ciba-Geigy, Basel, Switzerland], and 5 μM epostane (4α,5α-epoxy-17β-hydroxy-4β,17α-dimethyl-3-oxo-androstane-2α-carbonitrile; Sterling-Winthrop, New York, NY) as inhibitors of pregnenolone metabolism. Cells were cultured for 3 h in the presence or absence of 100 ng ovine LH (NIH oLH S23)/ml culture medium, after which medium was collected, and pregnenolone was measured by RIA (22).

Establishment of peritubular myoid cell lines

The establishment and characterization of these cell lines will be reported elsewhere in more detail (22a). Briefly, testes from 10-day-old Wistar rats were decapsulated and digested successively at 32 C with collagenase (0.5 mg/ml for 60 min), collagenase (1 mg/ml for 30 min), and hyaluronidase (1 mg/ml for 45 min). After dispersion of the remaining tubular fragments, testicular cells were seeded at 1.3 × 10^6 cells/cm^2 in 75-cm^2 culture flasks (Costar) and cultured in RPMI-10% FCS until 75% confluency was reached. Then, cells were transfected by lipotransfection [3 μg DNA-10 μl Lipofectin-1 ml Optimem (Gibco)] with pSVv-*myc* (American Type Culture Collection, Rockville, MD). This resulted in four rat testicular cell lines (RTC 8T1, RTC 8T3, RTC 8T6, and RTC 8T12). Another peritubular myoid cell line (RTC 8C) originated spontaneously. All cell lines, with the exception of RTC 8T1, were characterized as peritubular myoid-like cells based upon morphology, immunocytochemical markers, growth factor secretion, and secretion of PModS-like activity (23). The nature of RTC 8T1 is indistinct. The same procedure was used to obtain an immortalized stromal prostate cell line (RSPC 2T).

RNA isolation and Northern blot analysis

Total RNA was isolated by extraction with 3 M LiCl and 6 M urea (24), followed by phenol-chloroform extraction. Samples containing 20 μg total RNA were separated by electrophoresis in a denaturing formaldehyde-agarose gel (1% agarose; Sigma) containing ethidium bromide and blotted onto Hybond N^+ nylon membrane filters (Amersham, Aylesbury, United Kingdom) by diffusion. Filters were hybridized with ^{32}P-labeled complementary DNA probes encoding rat inhibin α-subunit [1.25-kilobase (kb) *Eco*RI fragment, corresponding to the entire α-subunit; α7/pUC18] (25), rat inhibin β_A-subunit (1.4-kb *Eco*RI fragment, corresponding to the entire β_A-subunit; βA30/pUC18) (25), rat inhibin β_B-subunit (1.5-kb *Eco*RI fragment, corresponding to the entire mature part of the inhibin β_B-subunit and parts of the N-terminal domain and 3′-untranslated sequences; βB11/pUC18) (25), rat activin receptor type II (0.6-kb *Bam*HI/*Hin*dIII fragment, corresponding to amino acids 1–175 of activin receptor type II) (6), rat activin receptor type IIB (0.5-kb *Sac*I fragment, corresponding to subdomains II–VIII of the kinase domain of activin receptor type IIB) (Wesseling, J. G., A. Dankbar, and F. H. de Jong, unpublished results), and hamster actin. All hybridizations were performed for 48 h at 42 C in hybridization solution containing 50% formamide, 9% (wt/vol) dextran sulfate, 10 × Denhardt's solution [1 × Denhardt's contains 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinyl pyrrolidone, and 0.02% (wt/vol) BSA], 5 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 10 mM sodium phosphate (pH 6.8), and 100 μg/ml denatured salmon sperm DNA. After hybridization, blots were washed to a final stringency of 0.1 × SSC-0.1% (wt/vol) sodium dodecyl sulfate (SDS) at 42 C and autoradiographed using Hyperfilm-MP (Amersham). The hybridization signals were quantified using a Bio-Rad-1D gel scanner (model 620, Bio-Rad, Richmond, CA). Values were normalized using the signals obtained after hybridization with hamster actin complementary DNA.

SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis and Western blotting were carried out as described by de Winter *et al.* (26). Western blotting was performed with conditioned media from peritubular myoid cells and cell lines, concentrated using Centriprep 10 filters, which have a mol wt cut-off at 10 kilodaltons (kDa; Amicon, Danvers, MA). The antisera used were raised against a prokaryotic fusion protein containing the entire

mature bovine inhibin β_A-chain (AS 227, Innogenetics) or against N-terminal amino acid residues 1–22 of the α_c-subunit of 32-kDa bovine inhibin (27). The latter antiserum recognizes 30-kDa inhibin, 26-kDa pro-αc-subunit, and the 20-kDa α-subunit monomer (9). Goat antirabbit immunoglobulin G alkaline phosphatase conjugate (Sigma), naphtol AS-MX phosphate (Sigma), and 4-aminodiphenylamine diazonium sulfate (Sigma) were used to visualize immunoreactive proteins.

Rat pituitary bioassay

The amount of bioactive activin in the culture medium of peritubular myoid cells and cell lines was determined by an *in vitro* rat pituitary bioassay system, measuring the stimulation of basal FSH release (27). In this bioassay system, activin stimulates FSH release at concentrations above approximately 2.2 ng/ml. The results of the FSH assay have been expressed in terms of NIDDK rat FSH RP-2. Charcoal-treated bovine follicular fluid (bFF) was used as an inhibin standard. Inhibin bioactivity was immunoneutralized with 5 μl polyclonal antiserum against partially purified bovine inhibin (28).

Animal cap bioassay

Activin bioactivity was also studied using the animal cap bioassay, in which conditioned media from peritubular myoid cells and cell lines were tested for their ability to induce mesoderm in animal cap explants from *Xenopus laevis* blastulas (stage 8). In this bioassay, activin is active at concentrations higher than approximately 0.2 ng/ml. The assay was performed according to a modification of the method of Symes and Smith (29), as described by de Winter *et al.* (26).

Erythrodifferentiation assay

Another bioassay used to study activin bioactivity is the erythrodifferentiation assay, which is based on the activin-dependent differentiation of human erythro-leukemia cells (K652 and CCL 243) visualized by hemoglobin accumulation, as described by Yu *et al.* (30).

Inhibin RIA

Inhibin-like immunoreactivity present in the culture media of the different cell preparations was measured using an antiserum against purified 32-kDa bFF inhibin (As 1989) and iodinated 32-kDa bFF inhibin, as described by Robertson *et al.* (31). These materials were kindly donated by the NICHHD. bFF with an arbitrary potency of 1 U/μg protein (27) was used as an inhibin standard.

Transferrin RIA

Sertoli cell transferrin production was measured by RIA using a rabbit antiserum against rat transferrin, as described by Swinnen *et al.* (32).

Estimation of DNA

The DNA content of the cell lysates was determined by a fluorometric assay using 3,5-diaminobenzoic acid dihydrochloride as a fluorescent dye (Aldrich-Chemie, Steinheim, Germany). Samples were neutralized with 1 N HCl. A 50-μl portion of the neutralized sample was mixed with 50 μl 1.5 M 3,5-diaminobenzoic acid dihydrochloride and incubated for 45 min at 60 C. Subsequently, 2 ml 1 N HCl was added, and the fluorescence of the samples was measured using a Perkin-Elmer fluorimeter at wavelengths of 415 nm (excitation) and 500 nm (emission). Calf thymus DNA was used as a standard.

Results

Expression of messenger RNA (mRNA) for the inhibin subunits and activin receptors

Peritubular myoid cells were isolated by method 1 and stained for alkaline phosphatase after 1, 2, and 5 days of culture in MEM-10% FCS (Fig. 1). After 1 day of culture, approximately 40% of the cells were alkaline phosphatase positive. This increased to approximately 70% after 2 days of culture. After trypsinization on day 5 of culture, alkaline phosphatase staining was weaker, but approximately 90% of the cells were positive. In this final culture (5 days after isolation), pregnenolone was detected in the culture medium (mean ± SEM, 5.4 ± 0.43 pmol/3 h; n = 6), but its level was not stimulated by the addition of LH (5.0 ± 0.39 pmol/3 h; n = 6). These data indicate that some Leydig cells might be present in this preparation, but Leydig cell contamination is below 10%.

High expression of inhibin β_A-subunit mRNA was found in peritubular myoid cells isolated by method 1, but not in Sertoli cells (Fig. 2). Cultured peritubular myoid cells showed low expression of inhibin α- and β_B-subunit mRNA compared to that in Sertoli cells (Fig. 2). Furthermore, two ActRII transcripts (6 and 4 kb) were expressed, whereas no ActRIIB mRNA was detected in the peritubular myoid cells. ActRIIB expression was low in Sertoli cells.

In freshly isolated peritubular myoid cell preparations, expression of the inhibin β_A-subunit mRNA was also found (Fig. 3). The expression of this messenger increased after culture in MEM containing 10% fetal calf serum.

In the four different rat peritubular myoid cell lines, expression of ActRII and inhibin α-, β_A-, and β_B-subunit mRNA was found, as in cultured peritubular cells isolated by method 1 and 2 (Fig. 4). In the cell line RTC 8T6, the expression of inhibin β_A-subunit mRNA was low, whereas expression of β_B-subunit mRNA was not detected. Immortalized rat stromal prostate cells also expressed ActRII and inhibin α-, β_A-, and β_B-subunit mRNA.

Activin production by peritubular myoid cells

By Western blotting, a 25-kDa protein, comigrating with recombinant activin-A, was detected in peritubular myoid cell-conditioned medium, using an antiserum raised against recombinant activin-A (cells prepared by method 1, Fig. 5; by method 2, not shown). Using normal rabbit serum, this protein was not stained. Upon reduction, an immunoreactive protein of 14 kDa appeared, again indicating the presence of immunoreactive activin-A. The same protein was secreted by the immortalized peritubular and prostate cells (Fig. 6). The amount of activin-A was relatively high in those lines, where high expression of β_A-subunit mRNA was found (RTC 8T1, 8T3, and 8T12), whereas low amounts of protein were found in media from RTC 8T6 and RSPC 2T. In the latter cell lines, the expression of β_A-subunit was also low. The secretion of immunoreactive activin by the peritubular cell line RTC 8C was not investigated.

Secretion of bioactive activin

In an *in vitro* rat pituitary bioassay system we found no significant increase in FSH release with media from different peritubular cultures and peritubular cell lines. To exclude that this was due to the presence of bioactive inhibin interfering with activin, inhibin bioactivity was immunoneutral-

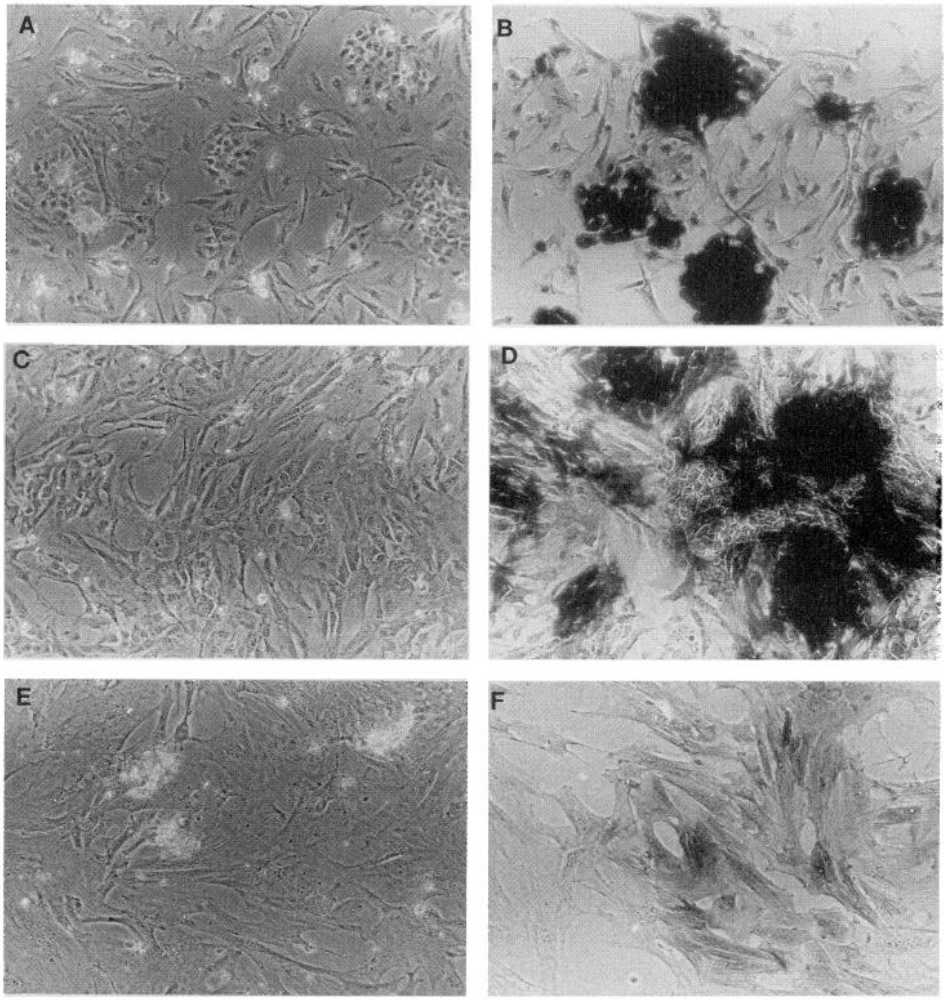

FIG. 1. Alkaline phosphatase staining of peritubular myoid cell preparations cultured in MEM-10% FCS. Peritubular myoid cells were isolated by method 1. Cells were stained after 1 (B), 2 (D), and 5 (F) days of culture. Unstained cells are shown in A (day 1), C (day 2), and E (day 5).

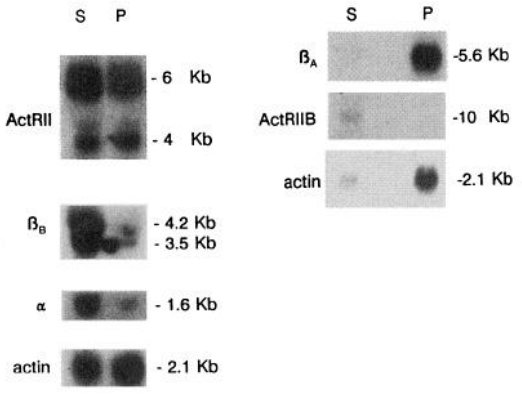

FIG. 2. Expression of mRNA for the inhibin α-, β_A-, and β_B-subunits; ActRII; and ActRIIB in cultured Sertoli cells (S) and peritubular myoid cells (P). Peritubular myoid cells were isolated by method 1, cultured for 2 days, split 1:10, and than cultured for an additional 3 days. Sertoli cells were cultured for 2 days, hypotonically shocked, and cultured for an extra day. The results of two separate blots are shown.

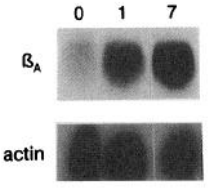

FIG. 3. Expression of inhibin β_A-subunit mRNA in peritubular myoid cells immediately after isolation (0) and after 1 day (1) and 7 days (7) of culture in MEM containing 10% FCS. Cells were isolated by method 1.

ized. In these experiments, again, no increase in FSH release was found with the media tested, although in the same experiment the inhibiting effect of follicular fluid on FSH release was blocked. This leads to the conclusion that the activin concentration in these media is below the detection limit of the assay.

In the animal cap bioassay, activin-like material was demonstrated to be present in media of peritubular cells and cell lines. The strongest mesoderm induction was found with medium from the RTC 8T1 cell line. This medium also stimulated hemoglobin production in K562 cells before and after heat treatment (5 min; 95 C). The results of these bioassays have been summarized in Table 1.

Secretion of other inhibin-like proteins

As shown in Fig. 4, peritubular cells expressed inhibin α-subunit mRNA after culture. Therefore, the amount of im-

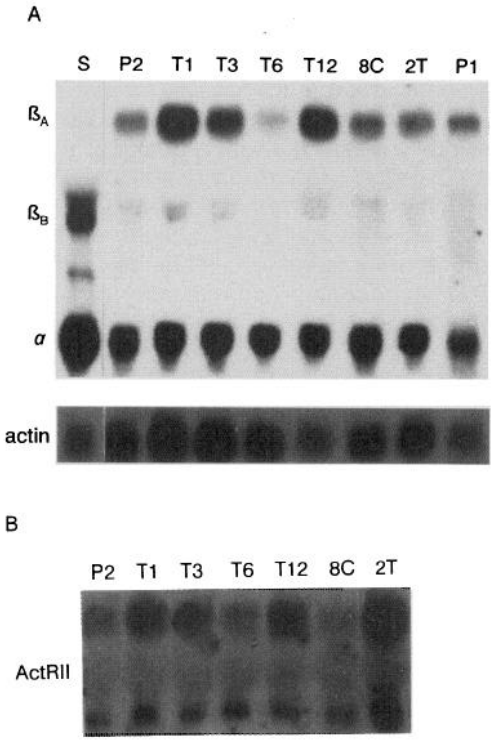

FIG. 4. A, Expression of mRNA for the inhibin α-, β_A-, and β_B-subunit in Sertoli cells (S), peritubular myoid cells isolated following method 2 (P2), immortalized peritubular cells (T1, T3, T6, T12, and 8C), immortalized stromal prostate cells (2T), and peritubular myoid cells isolated following method 1 (P1). B, Expression of ActRII in P2, T1, T3, T6, T12, 8C, and 2T. Both the 4- and 6-kb activin receptor type II transcripts are present.

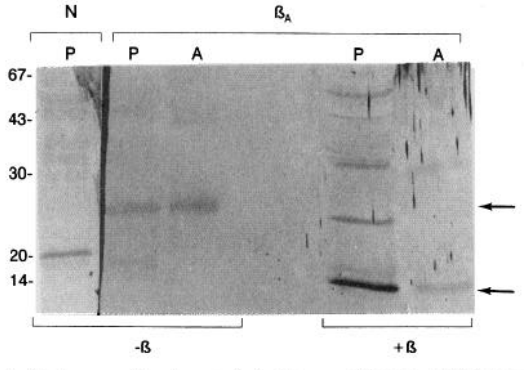

FIG. 5. Activin secretion by peritubular myoid cells. A Western blot of peritubular myoid cell-conditioned medium (P) and recombinant activin-A (A) was performed with an antiserum against recombinant activin-A (β_A) or normal rabbit serum (N). Samples were not reduced ($-\beta$) or reduced with 2% β-mercaptoethanol ($+\beta$). Cells were isolated by method 1. *Arrows* indicate activin (*upper arrow*) and inhibin β_A-subunit (*lower arrow*).

munoreactive inhibin secreted by the cells was measured. No immunoreactive inhibin was detected in the culture medium. Western blotting with an antibody against N-terminal amino acid residues 1–22 of the αc-subunit of 32-kDa bovine inhibin revealed the presence of a 20-kDa protein in culture medium of RTC 8T1 both before and after reduction with 2% β-mercaptoethanol (Fig. 7). This 20-kDa protein was not

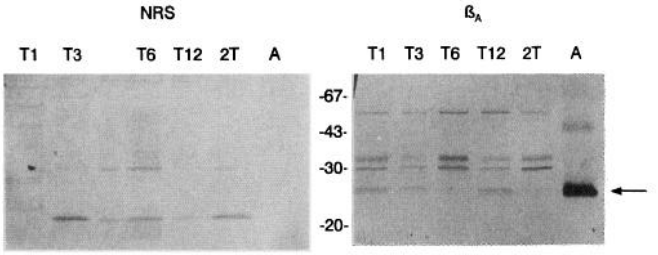

FIG. 6. Activin secretion by peritubular myoid cell lines. A Western blot of conditioned media from immortalized peritubular cells (T1, T3, T6, and T12), immortalized stromal prostate cells (2T), and recombinant activin-A (A) was performed with an antiserum against recombinant activin-A (β_A) or normal rabbit serum (NRS). The *arrow* indicates activin.

TABLE 1. Secretion of biologically active activin by peritubular myoid cells and peritubular cell lines determined by different bioassays

	P1	P2	8T1	8T3	8T6	8T12	8C
Pit −	−	−	−	−	−	−	−
+	−	−	−	−	−	−	−
Cap	+	+	++	+	+	+	ND
K562	ND	ND	+	ND	ND	ND	ND

Culture media of peritubular myoid cells isolated by method 1 (P1) or method 2 (P2) and peritubular myoid cell lines were tested for the presence of bioactive activin in a rat pituitary bioassay (Pit), without (−) or with (+) inhibin immunoneutralization; in the animal cap bioassay (Cap); or in the K562 cell assay (K562). −, No response; +, response; ++, strong response; ND, not determined. Data from two or three separate experiments, which all yielded similar results, have been summarized.

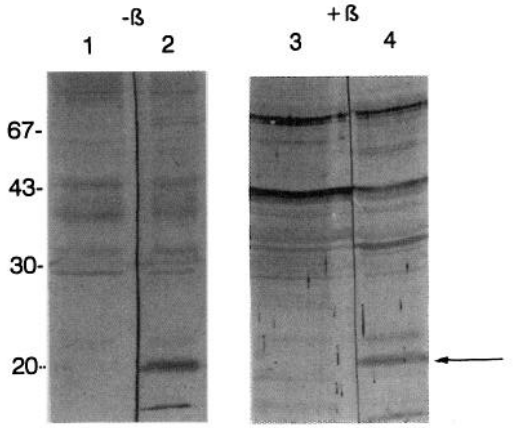

FIG. 7. Secretion of inhibin α-subunit by peritubular cells. A Western blot of conditioned medium from RTC 8T1, before ($-\beta$) and after reduction ($+\beta$) with 2% β-mercaptoethanol, is shown. Lanes 2 and 4 were stained with antiserum against N-terminal amino acid residues 1–22 of the αc-subunit of 32-kDa bovine inhibin; lanes 1 and 3 were stained with serum from the same rabbit obtained before immunization (preimmune serum). The *arrow* indicates the 20-kDa αc-subunit.

detected by preimmune serum from the rabbit in which the antiserum was raised. The same result was obtained with media from RTC 8T3 and RTC 8T6, whereas in medium from RTC 8T12, a small amount of the 20-kDa protein was detected after reduction only (not shown).

Regulation of β_A-subunit mRNA expression in peritubular cells

Figure 8 shows a representative experiment in which the peritubular myoid cells were cultured for 24 h in the presence of Sertoli cell-conditioned medium (SCCM), the synthetic androgen R1881, or $(Bu)_2$cAMP, starting 2 days after isolation. Compared to untreated peritubular myoid cells, SCCM stimulated the expression of β_A-subunit mRNA (mean ± SD, 200 ± 38.9% of control values; n = 2). Despite the presence of androgen receptors (not shown), R1881 had no significant effect (76.3 ± 21.7% of control values; n = 3), whereas $(Bu)_2$cAMP inhibited the expression of β_A-subunit mRNA (46.0 ± 35.8% of control values; n = 3).

Effects of recombinant activin-A on Sertoli function

Recombinant activin-A significantly stimulated basal and FSH-stimulated secretion of immunoreactive inhibin and transferrin by Sertoli cells obtained from testes of 21-day-old rats. The results of one representative experiment are shown in Table 2. Similar results were obtained in two independent experiments. Combination of the data from these three experiments gave rise to the same significant differences, as indicated in Table 2, although for transferrin, the differences were not always significant in the separate comparisons. Overall, inhibin secretion was stimulated to 157 ± 14% of control values (mean ± SEM; $P < 0.01$, by Student's t test), whereas transferrin secretion was stimulated to 113.8 ± 3.8% of control values (mean ± SEM; $0.02 < P < 0.05$, by Student's t test). There was no effect of activin-A on the amount of Sertoli cell DNA (not shown).

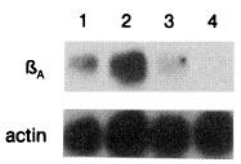

FIG. 8. Effects of various factors on inhibin β_A-subunit mRNA expression in peritubular myoid cells. Two days after isolation by method 1, peritubular myoid cells were cultured for 24 h in MEM (lane 1) or MEM supplemented with SCCM (lane 2), 1×10^{-8} M R1881 (lane 3), or 5×10^{-4} M $(Bu)_2$cAMP (lane 4).

TABLE 2. Effects of recombinant activin-A on secretion of immunoreactive inhibin and transferrin by rat Sertoli cells after 72 h of culture

Addition	Inhibin secretion (U/μg Sertoli cell DNA)	Transferrin secretion (ng/μg Sertoli cell DNA)
Control	17.0 ± 1.3	184.2 ± 3.6
Activin (50 ng/ml)	27.8 ± 1.2[a]	209.2 ± 7.9[b]
FSH (50 ng/ml)	63.9 ± 5.1	274.1 ± 14.2
FSH (50 ng/ml) + activin (50 ng/ml)	85.7 ± 3.8[a]	319.6 ± 8.9[b]

Sertoli cells were isolated from 21-day-old rats and incubated with the indicated hormones for 72 h. Subsequently, immunoreactive inhibin and transferrin were measured in the culture medium. Results represent the mean ± SEM of one experiment with quintuplate incubations.

[a] Significantly different from values without activin ($P < 0.01$, by Student's t test).

[b] Significantly different from values without activin ($0.02 < P < 0.05$, by Student's t test).

Discussion

Cultured peritubular myoid cells and peritubular cell lines express inhibin β_A-subunit mRNA and secrete immunoreactive and bioactive activin-A. Since peritubular myoid cells and stromal prostate cells show morphological and functional similarities (32–34), it is interesting to note that activin is also secreted by immortalized stromal prostate cells, especially as activin-A has been reported as an inhibitor of the growth of the human prostatic carcinoma cell line LNCaP (35).

Expression of inhibin β_A-subunit mRNA was found in peritubular cell preparations immediately after isolation. Nevertheless, it remains unclear whether peritubular myoid cells *in vivo* do secrete activin. Several researchers investigated the expression of inhibin subunit mRNAs (7, 36) and inhibin subunit proteins (36–38) in rat testes using *in situ* hybridization and immunocytochemistry, but no one reported the presence of β_A-subunits in peritubular myoid cells. This could be due to the flat nature of these cells and the small amount of cytoplasm in which the mRNA and protein should be localized. The low initial expression of β_A-subunit mRNA increased during culture in MEM-10% FCS. This suggests that activin production *in vitro* may partially be a culture artifact. This raises the interesting question of whether purification of candidate testicular paracrine factors, such as PmodS, from culture medium of testicular cells provides a good model system for the interactions between testicular cells *in vivo*. As for activin, the secretion of PmodS by peritibular myoid cells *in vivo* has not been demonstrated. Expression of the inhibin β_A-subunit is inhibited by $(Bu)_2$cAMP, which could indicate that activation of this second messenger system *in vivo* is responsible for the low initial β_A-subunit expression. A factor that could increase expression of the inhibin β_A-subunit in peritubular myoid cells is transforming growth factor-β (TGFβ), as it has been shown in vascular endothelial cells (39) and differentiated cell lines derived from P19 embryonal carcinoma cells (40). TGFβ is present in serum (41), fetal calf serum (Vanderstichele, H. M. J., unpublished results), and Sertoli cell culture medium (42), of which the latter two increase expression of inhibin β_A-subunit. Moreover, TGFβ1 increases the production of a number of radiolabeled proteins secreted by peritubular myoid cells (42).

Activin secretion by Leydig cells from immature rat testis has also been reported (14), but seems to be restricted to the first 19 days postnatally (43). In Leydig cells from 21-day-old and adult rat testes, no activin was detected (26). Therefore, it is unlikely that the activin found in the peritubular myoid cell cultures obtained from testes of 21- to 23-day-old rats using method 1 is of Leydig cell origin, although some Leydig cells might be present. Moreover, Leydig cells were removed by trypsin treatment in method 2, which was demonstrated by the absence of C_{19} and C_{21} steroids in the culture medium of these cells and their insensitivity to LH (44). In these cells and in the peritubular myoid cell lines, β_A-subunit expression and activin-A secretion were also found.

Despite the expression of inhibin α-subunit mRNA in peritubular myoid cells and cell lines, neither inhibin immunoreactivity nor inhibin bioactivity was detected in the

culture medium. This surprising result can be explained on the basis of the results of Western blotting of peritubular myoid cell line culture medium with an antibody against amino acid residues 1–22 of the αc-subunit of bovine inhibin, in which the 20-kDa inhibin α-subunit monomer was detected. This inhibin α-subunit monomer is not recognized by the RIA used (31). In culture medium of Sertoli cells, the 20-kDa monomer is only found after reduction of the secreted proteins and is derived from 32-kDa inhibin and 29-kDa pro-αc-subunit (45). This indicates that the processing of the inhibin α-subunit in peritubular myoid cells is different from that in Sertoli and Leydig cells, as in the latter cells, pro-αc-subunit is also found (26).

Peritubular myoid cells and peritubular myoid cell lines also express activin receptor type II. Therefore, auto- and paracrine effects of activin on these cells can be expected. The expression of activin receptor type II in peritubular cells is comparable to that in Sertoli cells, but is much higher than the expression found in Leydig cell preparations (6).

Mesenchyme-derived peritubular myoid cells surround the seminiferous tubules and form a basement membrane in cooperation with epithelium-derived Sertoli cells (46). The close proximity between peritubular myoid cells and Sertoli cells suggests a mutual effect on the function of both cell types. In fact, conditioned medium from Sertoli cells of 20-day-old rats contains heparinoids that inhibit the incorporation of [^{3}H]thymidine and the proliferation of peritubular myoid cells (47). On the other hand, several researchers reported the effects of peritubular myoid cells on Sertoli cell function. Coculture of peritubular myoid cells and Sertoli cells causes the formation of seminiferous tubule-like structures (48, 49). Activin secretion by peritubular myoid cells might be an explanation for this phenomenon, because Sertoli cell monolayers reaggregate into tubule-like structures upon activin treatment (10). Furthermore, the secretion of ABP (33, 50–53), transferrin (33, 51, 52, 54), α_2-macroglobulin, and clusterin (55) from Sertoli cells is stimulated by coculture with or conditioned medium from peritubular myoid cells, whereas Sertoli cell aromatase activity (33, 44) and production of plasminogen activator (56) are inhibited. Most of these effects are attributed to the androgen-regulated peritubular myoid cell product, PmodS (15), which stimulates the secretion of ABP, transferrin, and inhibin by Sertoli cells (16, 17) and inhibits FSH-stimulated aromatase activity in Sertoli cells from 20-day-old rats (18). PmodS has no effect on plasminogen activator activity (16), whereas effects on α_2-macroglobulin and clusterin have not been documented.

Recently, we demonstrated that activin inhibits FSH-stimulated aromatase activity in Sertoli cells (9). Here we show that activin-A stimulates Sertoli cell production of immunoreactive inhibin and transferrin. Stimulation of inhibin secretion by activin-A has also been demonstrated in cultured granulosa cells (57) and may be an autocrine protection mechanism against overexposure to activin.

Comparing activin-A and PmodS, it is likely that we deal with different factors. The mol wt of PmodS A (56 kDa, reducing conditions) (15) and PmodS B (59 kDa, reducing conditions) (15) are higher than the mol wt of activin-A (14 kDa, reducing conditions). Another difference is the effect on Leydig cells; activin can inhibit Leydig cell steroidogenesis (11–13), whereas PmodS has no effect (58). Furthermore, the secretion of PmodS is stimulated by androgens (52), whereas we found no effect of the synthetic androgen R1881 on peritubular expression of the inhibin β_A-subunit mRNA. Finally, the effects of activin-A on Sertoli cell transferrin production are less pronounced than the effects of PmodS. However, the present results indicate that a number of effects of peritubular myoid cell-conditioned medium that have been ascribed to PmodS may also be partially due to the presence of activin-A in this medium. From this point of view, it would be interesting to study the effects of activin-A on the secretion of ABP, plasminogen activator, α_2-macroglobulin, and clusterin by Sertoli cells, as peritubular myoid cells affect these parameters.

In conclusion, activin-A secretion by peritubular myoid cells and immortalized peritubular and stromal prostate cells *in vitro* was demonstrated in this study. Activin-A shares some effects on Sertoli cell function with the testicular paracrine factor PmodS: it inhibits FSH-stimulated aromatase activity and stimulates inhibin and transferrin secretion. However, activin secretion does not seem to be regulated by androgens, and the characteristics of both substances are dissimilar. We, therefore, conclude that peritubular myoid cell-derived activin-A has PmodS-like effects on Sertoli cells, but is not identical to PmodS.

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