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Activin Is Produced by Rat Sertoli Cells *in Vitro* and Can Act as an Autocrine Regulator of Sertoli Cell Function*

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ABSTRACT

The production of activin by Sertoli cells isolated from 21-day-old rats was studied using the mesoderm-inducing activity of activin on *Xenopus laevis* animal cap explants, immunoprecipitation and Western blotting. Furthermore, the effects of recombinant bovine activin-A on rat Sertoli cell aromatase activity and FSH and androgen receptor gene expression were examined.

Animal cap explants from *Xenopus laevis* blastulas elongated after culture in conditioned medium of Sertoli cells cultured with or without ovine FSH or conditioned medium of the mouse Sertoli cell-derived TM4 cell line. Animal cap explants cultured in control medium remained spherical. This elongation was also found in the more than 10-kilodalton fraction of the conditioned medium and after heating for 10 min at 95 C, indicating that heat-stable activin-like bioactivity is present in the culture medium. Immunoprecipitation of [^{35}S]methionine-labeled proteins and Western blotting of Sertoli cell-conditioned medium with polyclonal antisera against the inhibin β-subunits indicated the presence of 24- to 25-kilodalton activin-like immunoreactive material.

Sertoli cell aromatase activity was dose-dependently stimulated by ovine FSH after 72 h of culture. Recombinant bovine activin-A partly inhibited this stimulation in a dose-dependent way. This inhibition was also found after 24 h of culture. Furthermore, basal and FSH-stimulated androgen receptor mRNA expression in Sertoli cells and binding of the synthetic androgen R1881 to Sertoli cells were decreased after 24 h of culture in the presence of recombinant bovine activin-A. In the same experiments, FSH receptor mRNA expression was not significantly affected. These results indicate that activin can act as an autocrine regulator of Sertoli cell function. (*Endocrinology* **132:** 975–982, 1993)

ACTIVINS, which are homo- or heterodimers of the βA- and βB-subunits of inhibin, were originally recognized in and isolated from ovarian follicular fluid by virtue of their ability to stimulate the synthesis and release of FSH from pituitary cells in culture (1, 2). The activins are members of a family of structurally related proteins, including transforming growth factors-β (TGFβs), Müllerian-inhibiting substance, bone morphogenetic proteins, the product of the decapentaplegic gene complex of *Drosophila* and the Vg1 gene product of *Xenopus* (for review, see Ref. 3). It is now clear that activins exert many biological functions and can be regarded as cell differentiation factors (4). In the testis, activins may play a role in the regulation of both Leydig cell steroidogenesis (5–7) and spermatogenesis (8).

Shintani *et al.* (9) reported high levels of immunoreactive activin in testicular homogenates, but it is not clear by which testicular cell type activin is produced. Lee *et al.* (10) indicated that Leydig cells might be the source of activin in the testis. In contrast, de Winter *et al.* (11) demonstrated that the β-subunits of inhibin are not expressed in Leydig cells, resulting in the secretion of inhibin α-subunits only. Furthermore, Grootenhuis *et al.* (12) showed that a 25-kilodalton (kDa) fraction of Sertoli cell-conditioned medium (SCCM) could stimulate the release of FSH from pituitary cells in culture. For these reasons, we studied the presence of activin in SCCM by bioassay, immunoprecipitation, and Western blotting.

Recently, we reported that activin receptor type II mRNA is expressed in rat Sertoli cells (13). So far, effects of activin on Sertoli cells have not been described. Since the main regulators of Sertoli cell function are FSH and androgens, we investigated whether recombinant bovine activin-A can influence the actions of these hormones, using the induction of aromatase and the expression of FSH and androgen receptor genes as response parameters.

Materials and Methods

Isolation and culture of Sertoli cells

Highly purified Sertoli cells from testes of immature (17- to 21-day-old) Wistar rats were isolated and cultured using the procedure described by Themmen *et al.* (14). In short, decapsulated testes were incubated for 20 min at 37 C in PBS, containing DNase-I (5 μg/ml; DN25, Sigma, St. Louis, MO), collagenase (1 mg/ml; CLS, Worthington, Freehold, NJ), trypsin (1 mg/ml; TRL, Worthington), and hyaluronidase (1 mg/ml; 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 previously described, and dispersed using a Dounce homogenizer. The cells were then washed four times with PBS-DNase and once with Eagle's Minimum Essential

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Medium (MEM; Gibco, Grand Island, NY) by sedimentation at 100 × *g* for 2 min. The cell preparation was plated in 75-cm^2 culture flasks (Costar, Cambridge, MA) for RNA extraction, in 6-well plates (Costar) for [^{35}S]methionine labeling of Sertoli cell-secreted proteins, in 24-well plates (Costar) for estimation of [^{3}H]R1881 binding, and in 96-well plates (Costar) for determination of aromatase activity at a density of 0.5 × 10^6 cells/cm^2. The cells were cultured for 48 h at 37 C in MEM supplemented with nonessential amino acids, glutamine, antibiotics, and 1% fetal calf serum (FCS; Sebak, Aidenback, Germany). 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, medium was replaced with MEM containing 0.1% BSA and hormones.

To obtain large amounts of SCCM, Sertoli cells isolated from testes of 21-day-old rats were cultured in 150-cm^2 flasks for 4–5 weeks in MEM with or without 500 ng ovine FSH/ml (oFSH; NIH S-16). Medium was collected every 3 or 4 days. This SCCM was used in the animal cap bioassay directly or after concentration and exchange against 20 mM Tris-HCl (pH 7.9) using a filtration unit (Amicon, Lexington, MA) with a hollow fiber (HIP 10–43, Amicon, mol wt cut-off, 10 kDa).

Culture of the Sertoli cell-derived cell line TM4 (CRL 1715)

For the preparation of conditioned medium from mouse TM4 cells (15), cells were grown to confluency in 150-cm^2 culture flasks (Costar) containing Ham's F-12 (Gibco)-Dulbecco's Modified Eagle's Medium (DMEM; Gibco; 1:1) supplemented with 2.5% FCS (Integro, Zaandam, The Netherlands) and 5% horse serum (Gibco). The cells were then cultured for 1 day in the same medium without serum and finally for 2 additional days in 30 ml serum-free medium. The latter medium was collected, filtered using a 0.22-μm filter (Costar), and stored at −20 C untill use in the animal cap or TGFβ bioassay.

Animal cap bioassay

Activin bioactivity was studied using the animal cap bioassay, in which conditioned medium from Sertoli cells, cultured with or without FSH, and conditioned medium from TM4 cells were tested for their ability to induce an elongation of animal cap explants from *Xenopus laevis* blastulas (stage 8). The assay was performed according to a modification of the method of Symes and Smith (16), as described by de Winter *et al.* (11). Briefly, stage 8 embryos were chemically dejellied using 2% cystein-hydrochloride (pH 7.8–8.1). A disc of animal pole test tissue from the center of the pigmented animal hemisphere was dissected and transferred with the blastocoel facing upward in 0.75% normal amphibian medium containing 0.1% BSA and the test factor. The animal cap explants were cultured at 22 C for 3 days.

TGFβ bioassay

The secretion of bioactive TGFβ by Sertoli cells was measured using the mink lung epithelial cell assay modified according to Cone *et al.* (17). This assay is based on growth inhibition of the Mv1L4 cell line TCC CCL64 by TGFβ. Briefly, cells were grown in DMEM supplemented with 10% FCS and 1% nonessential amino acids at 37 C. At subconfluency, cells were trypsinized and seeded at a 1:8 dilution. After 24 h, medium was replaced by DMEM containing 1% FCS and 1% nonessential amino acids, and cells were cultured for another 24 h. Then, 10^4 cells were incubated in 96-well microtiter plates with serial dilutions of test samples in DMEM-1% FCS-1% nonessential amino acids. After 48 h, [^{3}H]thymidine (0.5 μCi/well) was added, and incubation was continued for 6 h. Cell-associated counts were determined. Pure porcine TGFβ1 or porcine TGFβ2 (British Biotechnology Ltd., Abingdon, Berkshire, United Kingdom) were used as positive controls. Each sample was tested in duplicate.

Labeling and immunoprecipitation of Sertoli cell proteins

After preculture for 3 days and hypotonic shock treatment, Sertoli cells from 21-day-old rats were incubated for 30 min in methionine-free MEM (Gibco). Subsequently, the medium was replaced with 600 μl methionine-free MEM/well containing 45 μCi [^{35}S]methionine (Amersham, Aylesbury, Buckinghamshire, United Kingdom). After 8 h of labeling, media from two wells were pooled and centrifuged at 8000 × *g* for 5 min. Supernatants were used for immunoprecipitation with polyclonal antiserum raised against a prokaryotic fusion protein containing the entire mature bovine inhibin βA-chain (AS 065, Innogenetics, Ghent, Belgium), against C-terminal amino acid residues 94–113 of the rat inhibin βB-chain, or against N-terminal amino acid residues 1–22 of the αC-subunit of 32-kDa bovine inhibin. After overnight incubation with 5 μl antiserum at 4 C, 50 μl protein-A-Sepharose CL-4B suspension (Pharmacia, Uppsala, Sweden) were added, and tubes were rotated for 30 min at 4 C. The Sepharose beads were spun down and washed three times with 1 ml 0.01 M PBS (pH 7.0) containing 1 mM EDTA, 0.05% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (vol/vol) Triton X-100 and twice with 0.001 M PBS (pH 7.0). Beads were taken up in 40 μl sample buffer (50 mM Tris, 2 mM EDTA, 10% glycerol, 2% SDS, and 0.001% bromophenol blue, pH 6.8) with or without 2% β-mercaptoethanol, boiled for 5 min, and centrifuged. The resulting supernatants were loaded on SDS-polyacrylamide gels.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE and Western blotting were carried out as described by de Winter *et al.* (11). Western blotting was performed with concentrated conditioned medium from control Sertoli cells and the antisera against inhibin βB- and α-subunits, which were also used for immunoprecipitation. Goat antirabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma), naphtol AS-MX phosphate (Sigma), and 4-aminodiphenylamine diazonium sulfate (Sigma) were used to visualize the proteins to which the antisera bound.

Aromatase assay

Sertoli cells were incubated for 24 or 72 h at 37 C under an atmosphere of 5% CO_2 in MEM containing 0.1% BSA and 0.5 μM testosterone. Incubation was performed in the presence or absence of oFSH (NIH S-17) and recombinant bovine activin-A (Innogenetics, Ghent, Belgium). After incubation, estradiol production was measured using a commercially obtained RIA (DPC Coat-a-Count, Los Angeles, CA). Cells were lysed in 1 N NaOH, and the DNA content of the cell lysates was determined by a fluorometric assay using 3,5-diaminobenzoic acid dihydrochloride (Aldrich-Chemie, Steinheim, Germany) as a fluorescent dye (11). Estradiol production was expressed per μg DNA.

RNA isolation and Northern blot analysis

Total RNA was isolated from Sertoli cells by extraction with 3 M LiCl and 6 M urea (18), followed by extraction with phenol and chloroform. Samples containing 30 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) by diffusion. To detect androgen receptor mRNA, filters were hybridized with a ^{32}P-labeled human androgen receptor cDNA probe [0.5-kilobase (kb) *Eco*RI-*Eco*RI fragment, corresponding to part of the steroid-binding domain and the 3′-untranslated region of the receptor mRNA] (19). For detection of FSH receptor mRNA, the same filters were hybridized with ^{32}P-labeled rat FSH receptor cDNA probes (20); pRK-FSHR-NH2 (0.86-kb *Eco*RI-*Eco*NI fragment, corresponding to amino acid residues 1–265 of the receptor) and pRK-FSHR-COOH (1.3-kb *Eco*NI-*Bam*HI fragment, corresponding to amino acid residues 266–692 of the receptor). Finally, filters were hybridized with a hamster actin probe. All hybridizations were performed for 48 h at 42 C in hybridization solution containing 50% formamide, 9% (wt/vol) dextran sulfate, 10 × Denhardt's [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 denaturated salmon sperm DNA. After hybridization, blots were washed to a final stringency of 0.1 × SSC-0.1% (wt/vol) 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 cDNA.

Androgen binding assay

The androgen binding assay was carried out as described by Blok *et al.* (21) using the synthetic androgen [^{3}H]R1881 (New England Nuclear Products, Stevenage, Hertfordshire, United Kingdom) as a specific ligand for the androgen receptor. After 3 days of preculture, Sertoli cells isolated from testes of 17-day-old rats were incubated for 24 h in MEM containing 0.1% BSA, recombinant bovine activin-A (50 ng/ml), and/or oFSH (50 ng/ml). After the incubation, Sertoli cells were washed four times with MEM containing 0.1% BSA. The cells were then incubated with triamcinolone acetonide (4 μM) to occupy progesterone receptors and [^{3}H]R1881 (7 nM) with or without excess unlabeled R1881 (10 μM) for 2 h at 37 C. Subsequently, the cells were placed on ice and washed four times with MEM to remove free [^{3}H]R1881. The cells were then lysed in 1 N NaOH, and the bound [^{3}H]R1881 was counted. Specific binding was calculated and expressed as a percentage of R1881 binding in control Sertoli cells.

Experimental animals

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.

Results

Production of activin by immature rat Sertoli cells in vitro

Animal cap explants of *Xenopus laevis* embryos were cultured in the presence of conditioned medium (nonconcentrated or concentrated and exchanged against 20 mM Tris-HCl, pH 7.9) from Sertoli cells cultured with or without FSH or in nonconcentrated conditioned medium from TM4 cells. After 1 day of culture, elongation of the explants was observed with all media tested, whereas explants cultured in control medium remained spherical (Fig. 1). After several days of culture, when control embryos were at tailbud stages, the animal caps treated with conditioned medium from the Sertoli cells were swollen by fluid intake and became vesicles several times their original volume. Some of them expressed melanophores. The control caps still remained spherical (not shown). These results indicate that mesoderm-inducing products were released by the Sertoli and TM4 cells. These products are heat stable, because elongation of the explants was also observed after heating the conditioned media from Sertoli and TM4 cells for 10 min at 95 C (not shown). Since TGFβ is heat stable and can induce mesoderm, the amount of TGFβ in the tested samples was verified using the epithelial mink lung cell bioassay. From the results presented in Table 1, it is clear that Sertoli cells do secrete some bioactive TGFβ, but not enough to cause elongation of the animal caps after the addition of nonconcentrated conditioned media from Sertoli and TM4 cells. These results indicate that activin, which is also heat stable, may be the mesoderm-inducing product secreted by Sertoli cells. To consolidate this, Sertoli cells were labeled with [^{35}S]methionine, and secreted activin was precipitated from the conditioned medium with two different rabbit polyclonal antisera against the inhibin β-subunits (Fig. 2A). Both antisera precipitated a protein of 24–25 kDa. Preincubation of the anti-βA antiserum with 50 ng recombinant bovine activin-A decreased the precipitation of labeled protein. Precipitation with preimmune serum was also performed as a control. A faint band was still present after precipitation with preimmune serum and after preincubation with activin-A. Less stringent washing of the Sepharose beads with 50 mM Tris, 0.1 M EDTA, and 1 M NaCl (pH 8.0) resulted in precipitation of equal amounts of the 24- to 25-kDa protein by preimmune and immune serum (not shown), indicating nonspecific binding of this band of radioactivity to the beads. In support of this nonspecificity of the remaining 24- to 25-kDa radioactivity, this protein was not detected after Western blotting with preimmune serum (see below). The α-antiserum precipitated 26-kDa pro-αC and 30-kDa inhibin, as described previously (11). Reduction of the samples after immunoprecipitation with the α-antiserum showed a large amount of 18-kDa free α-subunit (from inhibin and pro-αC) and some 14-kDa free β-subunit (from inhibin), whereas reduction of samples immunoprecipitated with the βB-antiserum resulted in a smaller amount of 18-kDa free α-subunit (from inhibin) and more 14-kDa free β-subunit (from inhibin and activin; Fig. 2B).

On Western blots, the antiserum against the inhibin βB-subunit detected a 24- to 25-kDa protein in SCCM, whereas preimmune serum did not. This antiserum also stained 30-kDa inhibin. Figure 3 shows the results of a representative experiment of four experiments performed. After reduction using β-mercaptoethanol, the 24- to 25-kDa protein disappeared, whereas free β-subunit appeared (Fig. 3). The α-antiserum detected 26-kDa pro-αC and 30-kDa inhibin, as described previously (11). After reduction, free α-subunit was detected.

Effect of recombinant bovine activin-A on FSH-stimulated aromatase activity

FSH stimulated estradiol levels dose-dependently in culture medium of Sertoli cells obtained from 21-day-old rats after 72 h of culture (not shown). Recombinant bovine activin-A had an inhibitory effect on the aromatase activity induced by 20 ng ovine FSH/ml (Fig. 4), without a significant effect on the amount of Sertoli cell DNA. At 50 ng activin-A/ml, a significant inhibition of 25.4 ± 3.3% (mean ± SEM; n = 4 incubations; $P = 0.01$, by Tukey's test) was found. Basal aromatase activity was not affected (not shown). In two similar experiments with Sertoli cells from 17-day-old rats, 50 ng activin-A/ml also produced a significant inhibition of aromatase activity stimulated with 50 ng oFSH/ml: estradiol levels decreased to 82.4 ± 1.3% and 74.1 ± 6.6% (mean ± SEM; n = 4 incubations; $P < 0.05$, by Student's t test) of control values after 72 h of culture. Finally, in a short term (24-h) experiment with Sertoli cells obtained from 17-day-old rats, recombinant bovine activin-A suppressed FSH-stimulated (50 ng/ml) aromatase activity significantly to 54.8 ± 2.6% of control values (mean ± SEM; n = 4 incubations; $P < 0.01$, by Student's t test).

Effect of recombinant bovine activin-A on FSH and androgen receptor expression

Culture of Sertoli cells obtained from 17- to 21-day-old rats for 24 h in the presence of oFSH resulted in increased

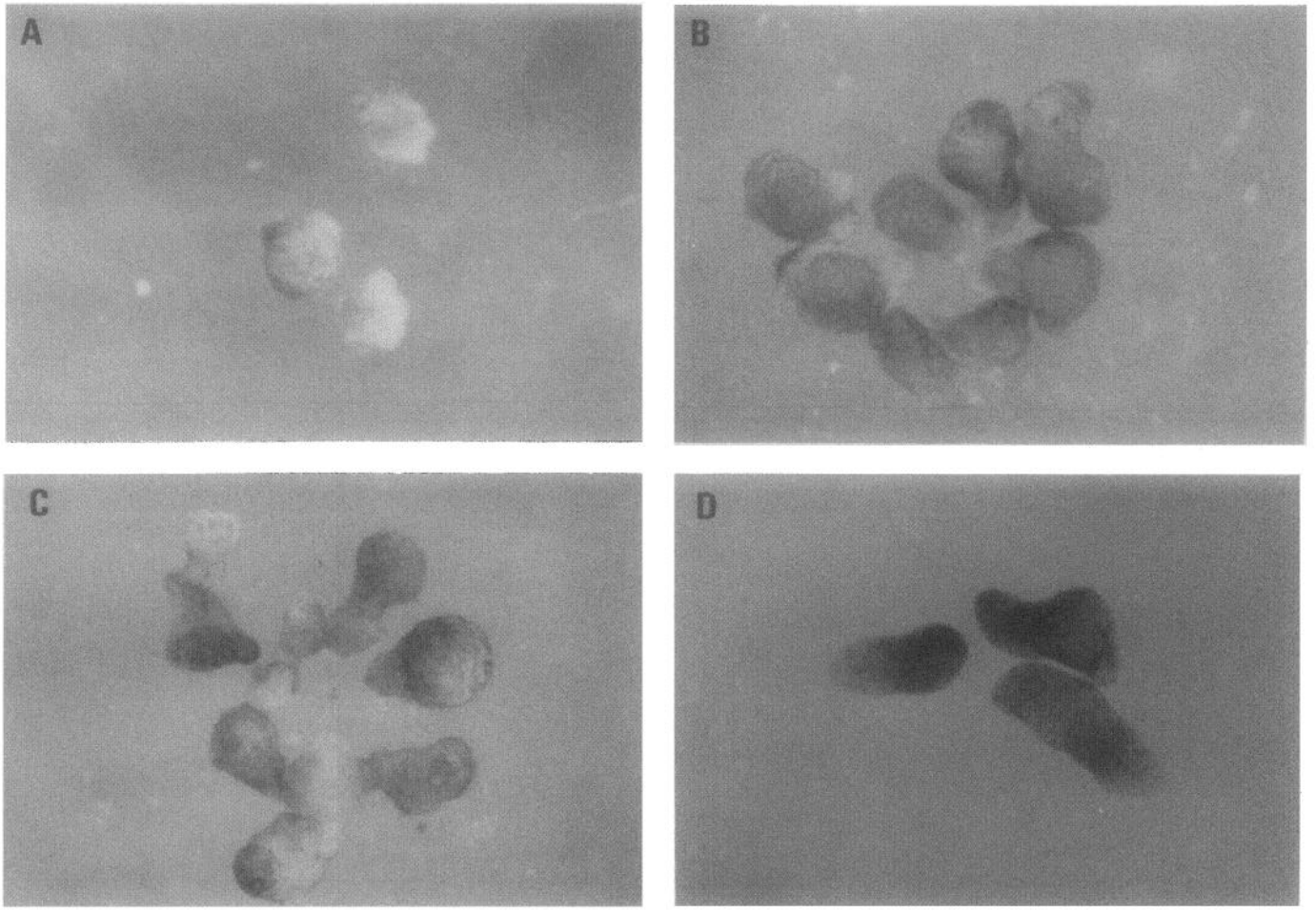

FIG. 1. Effect of conditioned media from Sertoli cells and TM4 cells on elongation of animal cap explants isolated from stage 8 *Xenopus laevis* embryos. Explants were cultured for 1 day in 0.75% normal amphibian medium (NAM) containing 0.1% BSA (A), in the same medium diluted 1:2 with concentrated conditioned medium from control Sertoli cells (B) or oFSH-stimulated Sertoli cells (C), and with a 1:6 dilution of nonconcentrated medium of TM4 cells (D). The experiments were performed three or four times with animal caps from different embryos. The figure is a representative sample from one experiment.

TABLE 1. Concentration of bioactive TGFβ present in conditioned medium used in the animal cap bioassay

Conditioned medium	TGFβ (ng/ml)
Control Sertoli cell (nonconcentrated)	0.83
FSH-stimulated Sertoli cell (nonconcentrated)	0.66
Control Sertoli cell (50-fold concentrated)	87.8
FSH-stimulated Sertoli cell (25-fold concentrated)	20.0
TM4 cell (nonconcentrated)	1.20

Mink lung epithelial cells were cultured in 2-fold serial dilutions of conditioned medium or TGFβ standard (TGFβ1 or TGFβ2), as described in *Materials and Methods*. Each sample was tested in duplicate. The concentration of TGFβ in the conditioned medium was calculated by comparing the growth inhibition induced by the conditioned medium with the inhibition obtained using the TGFβ standard.

androgen receptor mRNA expression (182.3 ± 36.0% of control values; mean ± SEM; n = 3 experiments) and decreased FSH receptor mRNA expression (67.9 ± 12.6% of control values; n = 4 experiments), as reported previously (14, 21). Recombinant bovine activin-A reduced both basal and FSH-stimulated androgen receptor mRNA expression to 53.1 ± 6.3% (n = 3 experiments) and 73.2 ± 7.5% (n = 3 experiments) of values without activin, respectively. The addition of activin-A had no significant effect on basal or FSH-inhibited FSH receptor mRNA expression, reaching 81.4 ± 16.6% (n = 4 experiments) and 86.8 ± 15.9% (n = 4 experiments) of the value obtained without activin. The results of one representative experiment are displayed in Fig. 5A. The decrease in androgen receptor mRNA expression was reflected in a significant decrease in specific [^{3}H]R1881 binding (Table 2).

In short term experiments (4–5 h), FSH has been shown to inhibit androgen receptor mRNA expression (21a). In a single experiment, we also studied whether recombinant bovine activin-A might have an effect on this short term down-regulation and found that after 4 h of culture, basal and FSH-inhibited androgen receptor mRNA expressions were reduced to 62% and 68% (corrected for actin hybridization) of values without activin, respectively (Fig. 5B).

Discussion

An early event in the embryogenesis of vertebrates is the induction of mesodermal tissue from cells of the animal pole

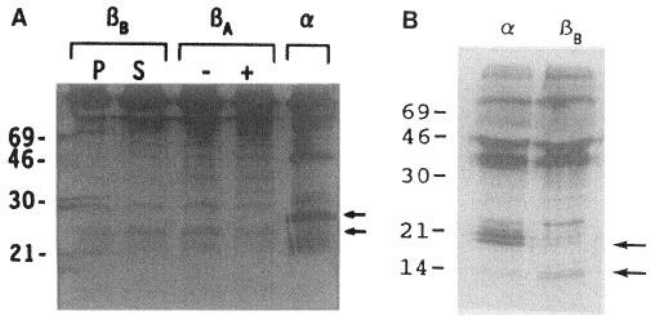

FIG. 2. A, Autoradiogram of immunoprecipitated [^{35}S]methionine-labeled proteins secreted by Sertoli cells. Proteins secreted by Sertoli cells obtained from 21-day-old rats were precipitated with polyclonal antisera against the inhibin βB-subunit (P, preimmune serum; S, antiserum), the inhibin βA-subunit (−, without competition with recombinant bovine activin-A; +, with competition), and the inhibin α-subunit and separated by SDS-PAGE. For description of the antisera, see *Materials and Methods*. *Arrows* indicate 26-kDa pro-αC (*upper arrow*) and 25-kDa activin (*lower arrow*). B, Autoradiogram of immunoprecipitated and reduced [^{35}S]methionine-labeled proteins secreted by Sertoli cells. Proteins were first precipitated with polyclonal antisera against the inhibin α- and βB-subunits and then reduced using 2% β-mercaptoethanol. *Arrows* indicate 18-kDa free α-subunit (*upper arrow*) and 14-kDa free β-subunit (*lower arrow*).

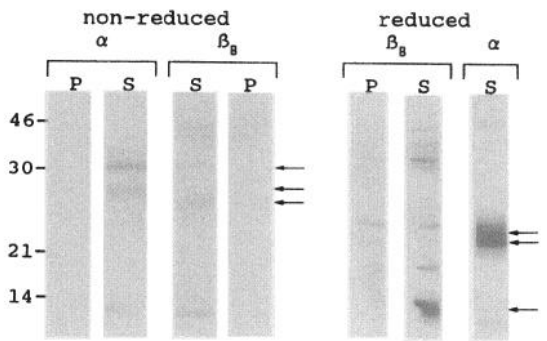

FIG. 3. Western blot of SCCM. Proteins secreted by Sertoli cells obtained from 21-day-old rats were not reduced (*left panel*) or were reduced with 2% β-mercaptoethanol (*right panel*) and separated by SDS-PAGE. Polyclonal antisera against the inhibin βB-subunit and the inhibin α-subunit (P, preimmune serum; S, antiserum) were used to detect inhibin-like proteins. *Arrows in the left panel* indicate 30-kDa inhibin (*upper arrow*), 26-kDa pro-αC (*middle arrow*), and 24- to 25-kDa activin (*lower arrow*). *Arrows in the right panel* indicate free inhibin α-subunit doublet (*upper and middle arrows*) and free inhibin β-subunit (*lower arrow*).

by signals coming from the vegetal pole. This process can be mimicked *in vitro* by members of the heparin-binding growth factor family, acidic and basic fibroblast growth factor (22, 23) and the products of the protooncogenes *int*-2 and *hst* (kfgf) (24), and by members of the TGFβ family, TGFβ2 (25), TGFβ3 (26), activin-A (27–29), and activin-B (30). Recently, the product of protooncogene *int*-1 was found to be involved in mesoderm induction (31). The usual test for mesoderm-inducing activity involves the culture of isolated animal pole explants in the presence of the test factor. In the absence of a mesoderm-inducing component, the test tissue differentiates as epidermis and remains spherical. Induced tissue elongates and mesodermal cell types (mesenchyme, muscle, notochord, and neural tissue) arise. This change in shape can be used as a reliable marker for mesoderm induction (16). In

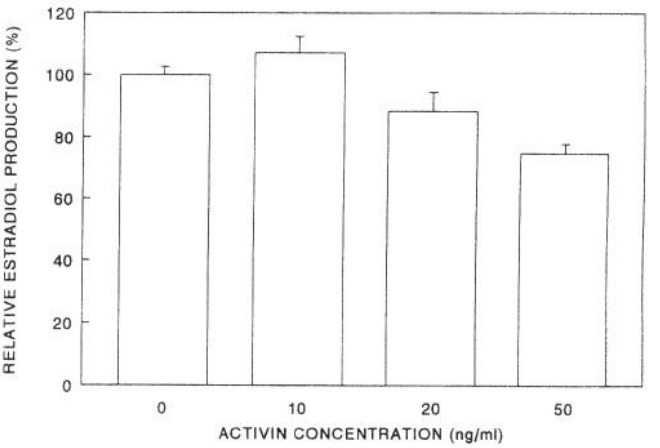

FIG. 4. Effects of different doses of recombinant bovine activin-A on oFSH-stimulated (20 ng/ml) aromatase activity after 72 h of culture. Values are expressed as percentages of oFSH-stimulated estradiol production per μg Sertoli cell DNA. The results represent the mean ± SEM of one experiment (n = 4 incubations).

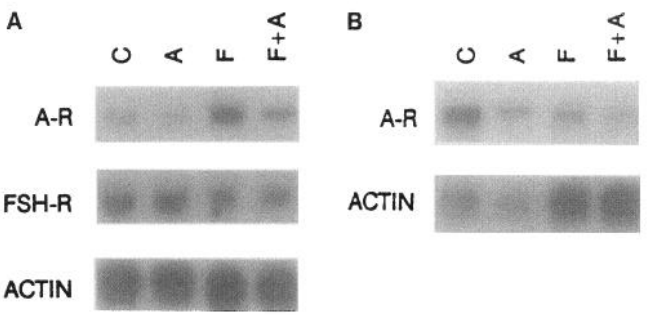

FIG. 5. A, Effects of recombinant bovine activin-A (50 ng/ml) and oFSH (50 ng/ml) on androgen receptor (A-R) and FSH receptor (FSH-R) mRNA expression in rat Sertoli cells isolated from 21-day-old rats after 24 h of culture. B, Effects of recombinant bovine activin-A (50 ng/ml) and oFSH (50 ng/ml) on androgen receptor (A-R) mRNA expression in rat Sertoli cells isolated from 17-day-old rats after 4 h of culture.

a recent study we used this assay to show that Leydig cells and Leydig cell tumors do not produce activin (11).

Culture of *Xenopus* animal pole tissue in diluted conditioned medium from immature rat Sertoli cells and mouse TM4 cells revealed the secretion of a heat-stable mesoderm-inducing factor, which was retained by a filter with a mol wt cut-off at 10 kDa. This excludes the mesoderm-inducing effects of a number of the above-mentioned factors. Basic fibroblast growth factor, which is secreted by Sertoli cells (32), is heat labile (33). TGFβ was detected in SCCM, using the mink lung epithelial cell bioassay, which is sensitive for TGFβ1, TGFβ2, TGFβ3, and TGFβ5 (26, 34). However, the amount of TGFβ present in nonconcentrated SCCM and conditioned medium of TM4 cells was below the concentration necessary for mesoderm induction [TGFβ2, 3–12 ng/ml (25); TGFβ3, 0.8–1.4 ng/ml (26)]. Furthermore, Sertoli cells from immature rat testes only secrete TGFβ1 (35, 36), which has no mesoderm-inducing activity (26). *int*-1 encodes a secretory glycoprotein associated with the cell surface, probably functioning locally in cell to cell signalling (for review, see Ref. 37). In the mouse, *int*-1 mRNA is only expressed in

TABLE 2. Effects of recombinant bovine activin-A and FSH on specific [^{3}H]R1881 binding to rat Sertoli cells after 24 h of culture

Addition	[^{3}H]R1881 binding (% of control)
Control	100.0 ± 1.1
Activin (50 ng/ml)	87.6 ± 2.0[a]
FSH (50 ng/ml)	111.3 ± 1.4[b]
FSH (50 ng/ml) + activin (50 ng/ml)	102.7 ± 3.0[c]

Sertoli cells were isolated from 17-day-old rats and incubated with the indicated additions for 24 h. Subsequently, R1881 binding was estimated, as described in *Materials and Methods*. Values are calculated as percentages of R1881 binding in control cells; the mean absolute number of receptors was 2156/cell. The results represent the mean ± SEM of two experiments, with triplicate incubations.

[a] Significantly different from binding to control cells ($P = 0.002$, by Tukey's test).

[b] Significantly different from binding to control cells ($P = 0.004$, by Tukey's test).

[c] Significantly different from binding to FSH-stimulated cells ($P = 0.034$, by Tukey's test).

postmeiotic male germ cells and midgestational embryos (38). It is, therefore, not likely that the product of *int*-1 is present in conditioned medium of Sertoli cells obtained from immature rat testes. Activin, in contrast, is heat and acid stable (28), is retained by filters with a cut-off at 10 kDa, and can be secreted by Sertoli cells, which express inhibin βB-subunit mRNA (39). Immunoprecipitation of [^{35}S]methionine-labeled Sertoli cell-secreted proteins and Western blotting of SCCM showed the secretion of a 24- to 25-kDa protein, which is recognized by antisera against the inhibin β-subunits. This protein can be reduced to a 14-kDa protein, which is also recognized by the inhibin-βB antiserum. Finally, Grootenhuis *et al.* (12) described a 25-kDa protein in SCCM that can stimulate FSH release from dispersed pituitary cells. This mol wt correlates well with the mol wt of 25.9 kDa described for recombinant human activin-B (40) and 25 kDa described for native porcine activin-B (41). Taking these results together, we conclude that activin is secreted by Sertoli cells from 21-day-old rats *in vitro*.

Sertoli cells isolated from immature rat testes express activin receptor type II mRNA (13). Here we demonstrate that recombinant bovine activin-A can inhibit the FSH-stimulated conversion of testosterone to estradiol. The relative effect after long term (72-h) culture is smaller than after 24 h of culture, possibly because of the endogenous production of activin, the breakdown of the added activin, and the secretion of activin-binding proteins. This can also be the explanation for the relatively high dose of activin needed for a significant response. A similar inhibition of aromatase activity was shown in immature porcine Sertoli cells for TGFβ1 (42). These researchers suggested enhancement of cAMP-phosphodiesterase activity by TGFβ1. In contrast, activin and TGFβ stimulate aromatase activity in granulosa cells (43, 44).

FSH administration to Sertoli cells obtained from immature rats results in a transient down-regulation of androgen receptor mRNA expression at 5 h, followed by an up-regulation of androgen receptor mRNA expression and androgen binding at 24–72 h (21a). Here, we show an inhibitory effect of recombinant bovine activin-A on basal, short term down-regulated and long term up-regulated androgen receptor mRNA expression. In the long term experiments, a similar, but less pronounced, suppressive effect was found on the amount of binding of the synthetic androgen R1881. The smaller effect on androgen binding might be explained by a shorter half-life of the mRNA compared to the half-life of the protein, as described by Blok *et al.* (21a). In contrast, no significant effect was found on FSH receptor mRNA expression.

As discussed above, the effects of activin on the investigated parameters of Sertoli cell function were relatively small, possibly because of endogenous production of activin by the Sertoli cells. However, *in vivo* the ratio of the expression of inhibin α- and βB-subunits varies during the cycle of the seminiferous epithelium (45). This might result in changing activin/inhibin ratios, which, in turn, could affect androgen receptor mRNA expression. Variations in androgen receptor mRNA expression during the cycle of the seminiferous epithelium of the rat testis have actually been described (46). This suggests that Sertoli cells may regulate their sensitivity to androgen, one of the main regulators of spermatogenesis, by changing the ratio between inhibin α- and β-subunits.

The ratio of the expression of inhibin α- and βB-subunit mRNAs in total testes of rats of various ages is constant from day 21 onward (47). This suggests that activin can also be produced by Sertoli cells in the adult testis, provided that the translation efficiencies of these mRNAs do not change. Indeed, Shintani *et al.* (9) detected relatively high levels of immunoreactive activin in rat testicular extract, which are unlikely to be produced by Leydig cells (11).

Apart from the autocrine effects of Sertoli cell-secreted activin, it may also have a paracrine effect on spermatogenesis: Mather *et al.* (8) showed activin-A binding to spermatogonia and stimulation of spermatogonial proliferation by activin-A and -B, de Winter *et al.* (48) detected a high expression of activin receptor type II mRNA in pachytene spermatocytes and round spermatids around meiosis, and Woodruff *et al.* (49) reported binding of fluorescein isothiocyanate-labeled activin-A to spermatogonia, late pachytene-diplotene spermatocytes, and spermatids.

In conclusion, production of activin by immature rat Sertoli cells was demonstrated. Activin can act as a specific autocrine regulator of Sertoli cell function, inhibiting FSH-stimulated aromatase activity, androgen receptor mRNA expression, and androgen binding, without significantly affecting FSH receptor mRNA expression. To our knowledge, activin is the first Sertoli cell-secreted product shown to regulate androgen receptor expression in Sertoli cells.

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