0013-7227/90/1276-3206$02.00/0
Endocrinology

Vol. 127, No. 6
Printed in U.S.A.

Activin Stimulates Spermatogonial Proliferation in Germ-Sertoli Cell Cocultures from Immature Rat Testis

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ABSTRACT. Activin and inhibin are peptide hormones produced in the gonads which may act as autocrine and/or paracrine regulators of testicular function. Sertoli cells produce inhibin, and it has recently been shown that Leydig cells can produce activin *in vitro*. To further explore the local actions of activin and inhibin in the testis, Sertoli and germ cells were isolated from immature rats and cocultured *in vitro*. In these cultures we demonstrate that activin A and activin B, but not inhibin A, stimulated spermatogonial proliferation *in vitro*. Activin increased [^{3}H]thymidine incorporation 2- to 4-fold in cocultures after 48–72 h of treatment. Using autoradiography, the label was localized in the clusters of spermatogonia adhering to the Sertoli cell monolayer. Additionally, activin stimulated a reaggregation of the cultures into tubule-like structures. Fluorescence-activated cytometry was used to analyze the cell population based on size, DNA content, and lipid content. Sertoli cells were identified using Nile Red staining of intracellular lipid droplets; spermatogonia are Nile Red-negative. Activin treatment caused a marked increase in the fraction of Nile Red-negative cells in the cocultures. Activin also caused an increase in the percentage of these cells having 4C DNA. Lastly, specific binding of activin A to 2C, but not 4C, germ cells was demonstrated. These data demonstrate that activin acts as a regulator of spermatogonial proliferation in the male. (*Endocrinology* **127:** 3206–3214, 1990)

INHIBIN A and B are heterodimers made up of an α-subunit combined with a β_A- or β_B-subunit. Inhibin is secreted by testicular Sertoli cells in the male and causes a decrease in FSH secretion from the pituitary (for review see Ref. 1). Activin A and B are homodimers of the β_A- or β_B-subunits, respectively. Activin has been shown to increase FSH secretion from pituitary cultures *in vitro* (2) and to increase circulating FSH levels *in vivo* in adult macaques (3) and rats (4). More recently, it has been demonstrated that testicular Leydig cells produce activin (5, 6). mRNA and protein subunits of the α and β1 chains have been localized in multiple cell types in the testis of rats of various ages (7–9); however, these data cannot differentiate between inhibin and activin production if both α- and β1-subunits are present. These data led us and others to suggest that inhibin and activin act as paracrine and/or autocrine regulators of gonadal function (5, 6, 9, 10) and may play a role in regulating Sertoli-Leydig cell interaction (6, 11–14) or Sertoli-germ cell interaction (9, 15).

Franchimont *et al.* (16) reported a decrease in [^{3}H] thymidine incorporation in the testes of animals treated with follicular fluid. It was suggested that the decrease in spermatogenesis was secondary to a decrease in circulating FSH in the treated animals. More recently, van Dissel-Emeliani *et al.* (15) have shown a decrease in spermatogonial numbers in hamster testis injected locally with both impure and purified inhibin. The latter data supported the hypothesis that inhibin may act locally in the testis, but did not distinguish which cell type(s) may be the primary target of the hormone. We used cocultures of Sertoli and germ cells derived from immature rat testes to determine the *in vitro* effects of purified recombinant activin and inhibin.

While inhibin had no effect on germ cell number or culture morphology, activin increased the proliferation of germ cells in the cocultures and caused a rapid reaggregation of Sertoli and germ cells in the absence of basement membrane or peritubular cells. In addition, we demonstrate that activin binds to isolated germ cells, which suggests that this hormone may act directly at the level of the germ cell.

Materials and Methods

Materials

Insulin, transferrin, testosterone, and α-tocopherol (vitamin E) were obtained from Sigma Chemical Co. (St. Louis, MO).

Received July 31, 1990.
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Aprotinin was obtained from Miles Laboratories (Naperville, IL). Epidermal growth factor, gonadotropins (FSH and PMSG), and growth factors [fibroblast growth factor, nerve growth factor (NGF), interleukin-2, and insulin-like growth factor-I] were obtained from Bethesda Research Laboratories (BRL; Bethesda, MD). Ham's F-12/Dulbecco's Modified Eagle's Medium (F12/DME) was obtained premixed in powdered form from Gibco (Grand Island, NY), prepared in the laboratory, and supplemented with 1.2 g/liter sodium bicarbonate, 10 mM HEPES buffer, and 20 mg/liter gentamycin sulfate. Tissue culture plastics were obtained from Corning (Corning, NY) and Labtek 4-chamber slides from Alameda Scientific (Alameda, CA). Nile Red was obtained from Molecular Probes, Inc. (Eugene, OR), and Hoechst 33342 (Ho 342) from Calbiochem (La Jolla, CA). [^{3}H]Thymidine was obtained from New England Nuclear (Boston, MA).

Human recombinant activin A and B and inhibin A were obtained from Drs. C. Schmelzer and G. Burton of Genentech, Inc., and were expressed and isolated as previously described (3, 17). Concentration and identity were confirmed by amino acid analysis, and the activity of the purified preparations was confirmed by Dr. R. Schwall, Genentech, Inc., using an *in vitro* pituitary bioassay system previously described (18). Recombinant human transforming growth factor-α was obtained from J. Gorrell, Genentech, Inc..

Animals

Twenty- to 25-day-old male Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA) or Charles River Laboratories, Inc. (Wilmington, MA). Animals were used within 24 h of arrival and killed by CO_2 inhalation.

Sertoli and germ cell coculture

Sertoli cells and germ cells were prepared according to the glycine/collagenase method previously described in detail (19, 20). Briefly, testes were removed and decapsulated, and the tubules were teased apart in a buffered hypertonic 1 M glycine solution. The return of the tubules to iso-osmotic medium resulted in lysis of the interstitial tissue without harm to the tubules. The tubules were then minced and treated with collagenase/dispase to remove the basement membranes and peritubular cells. Tubular pieces (1–4 mm in length) that contained Sertoli cells, spermatogonia, and spermatocytes were plated in serum-free Ham's F12/DME medium (1:1, vol/vol) supplemented with HEPES (15 mM) and insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (5 μg/ml) (20, 21), α-tocopherol (5 ng/ml; an antioxidant) (22), and aprotinin (25 mg/ml; a protease inhibitor) (6) (5F). Cells were plated in 12-well plates (1.8 cm^2/well) in 2 ml medium or on four-chamber plastic slides in 0.7 ml medium. Cells were cultured in an atmosphere of 5% CO_2-95% air at saturating humidity and 37 C.

Sertoli cells attached to the substrate and spread to form a monolayer, while spermatogonia and spermatocytes adhered to the monolayer or floated in the medium. The stage of development of germ cells in such coculture is dependent on the age of the animal at the initiation of culture. In cultures prepared from 21-day-old animals, pachytene spermatocytes are the most advanced stage of spermatogenesis present.

Twenty-four hours after plating, the medium was removed, and unattached cells were discarded. Fresh 5F medium was added to all cultures. In addition, 100 ng/ml of recombinant human inhibin A, activin A, or activin B were added to the experimental conditions. All conditions were assayed in triplicate, and each experiment was repeated two or more times. The effects of activin on culture appearance and spermatogonial division described below were observed in 11 separate experiments.

Germ cell preparation

Highly purified germ cells were prepared from 20- to 25-day-old animals by a modification of the technique described above. Tubules were dispersed for 10 min in the glycine solution, washed in serum-free medium, briefly (10 min) exposed to collagenase-dispase (0.1%) and soybean trypsin inhibitor (0.02%), chopped into small pieces, and resuspended vigorously. The released single cells were filtered through a 20-μm Nytex nylon mesh to remove undigested clumps of Sertoli cells and washed extensively. Cells were cultured overnight in 5F-supplemented F12/DME and Sertoli cell-conditioned medium (2:1, vol/vol). Conditioned medium was prepared by collecting 5F medium from the Sertoli cocultures, prepared as described above, after 48 h of culture and filtering the medium through a 0.1-μm filter to remove cells and debris. This medium could be stored under sterile conditions for up to 2 months at 6 C. After 18–20 h of culture the germ cells were more than 95% viable, as assessed by trypan blue staining. The Sertoli cell contamination, as assessed by Nile Red staining (counting >300 cells), was 2–5%.

Electron microscopy

Primary Sertoli and germ cell cocultures were prepared for electron microscopy after 72 h of treatment. Cultures were washed with F12/DME, covered with F12/DME and 2.5% gluteraldehyde in 0.2 M phosphate buffer (pH 7.2; 1:1, vol/vol), and fixed overnight in 2.5% gluteraldehyde. Cultures were postfixed in 1% buffered OsO_4, dehydrated in alcohol, and embedded in Polybed (Polysciences, Inc., Warrington, PA). Sections were cut on a Reichart OmU3 ultratome (Buffalo, NY), stained in 3% aqueous uranyl acetate at 50 C for 1 h, and examined with a Phillips 300 microscope (Mahway, NJ). Multiple samples from one set of cultures were observed by electron microscope.

Thymidine incorporation

One microcurie of [^{3}H]thymidine was added directly to the cocultures (2.0 ml medium/well) containing 1–2 $\times$ 10^6 cells at 0, 24, or 48 h of exposure to the hormones indicated. Incorporation of labeled thymidine was measured after 20 h of further incubation. Cells were detached from the substrate by vigorous pipetting, and the entire contents of the well (including those cells originally attached and floating) were transferred to a 10-ml filter well containing two glass fiber filters and 5 ml cold 20% trichloroacetic acid. The precipitate was caught on the filters, washed twice with cold 5% trichloroacetic acid and once

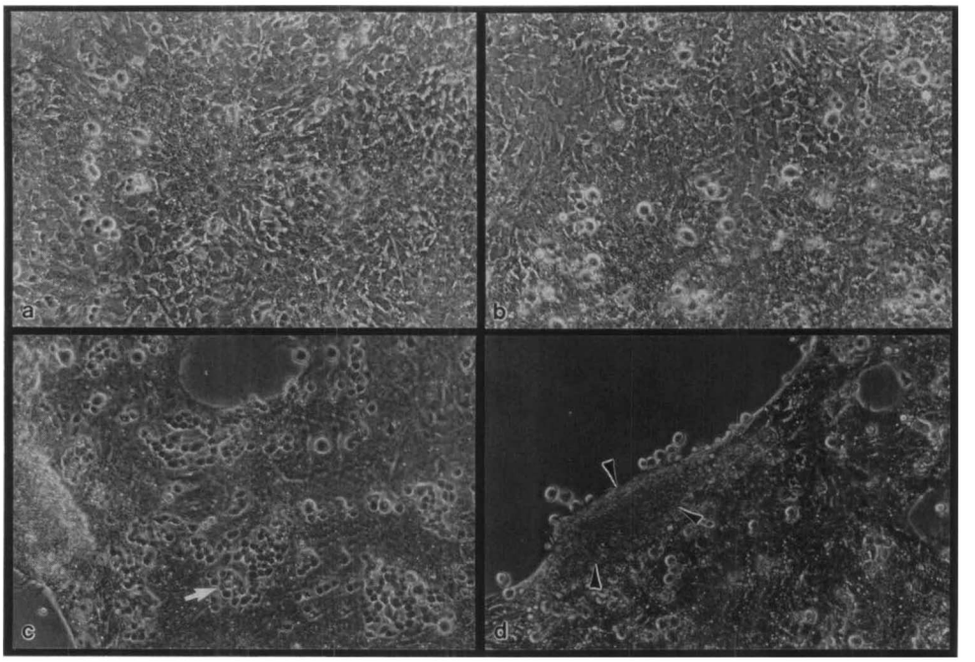

FIG. 1. Phase contrast micrographs of Sertoli and germ cell cocultures. The cultures were prepared as described and are shown 48 h after the addition of hormone. *Arrows* indicate spermatocytes and spermatogonial clusters. The activin-treated cultures are beginning to reaggregate. Cultures shown in a–c are at 200× magnification. a, 5F control; b, 5F plus inhibin A (100 ng/ml); c, 5F plus activin A (100 ng/ml); d, 5F plus activin A (100× magnification).

with cold methanol, and counted wet in scintillation fluid [Ecolite (+), ICN, Cleveland, OH]. Statistical analysis was performed using the unpaired Student's *t* test.

Cells were prepared for autoradiography by adding thymidine to cultures on four-well slides as described above. At the end of the incubation cells were fixed, and the slides were dipped in Ilford K-2 liquid autoradiographic emulsion (Ilford, Ltd., Mobberley, Cheshire, UK) , exposed for 2–4 weeks at 4 C, and developed using Kodak D19 developer (Eastman Kodak, Rochester, NY). Thymidine incorporation was carried out on nine experiments, with autoradiography performed on cultures from three experiments.

Fluorescence-activated cell analysis

The various cell types in the cocultures were quantified by dual laser flow cytometric analysis of Nile Red- and Ho342-stained cultures. Nile Red was excited at a wavelength of 488 nm, and fluorescence was collected with a band pass filter of 525 nm (±25 nm). Ho342 was excited at 351–364 nm, and emission was detected using a 460-nm long pass filter. Nile Red is a hydrophobic fluorescent dye that has maximal emission when in intracellular lipid droplets (23). By simultaneously staining with Ho342, a DNA-specific fluorochrome (24), the percentage of Nile Red gated (positive or negative cells) with 2C or 4C DNA content was determined. A modified Coulter 753 dual beam fluorescence-activated cell sorter (FACS; Coulter Electronics, Hialeah, FL) with a 100-μm orifice was used, as previously described (25). The two lasers were separated by 70 nm (7-μsec delay), with signals correlated to individual cells by a gated amplifier. Ten thousand to 30,000 cells/sample were analyzed using the LIST mode. Four experiments from four separate culture preparations were analyzed.

Hormone binding

Activin A was labeled with fluorescein isothiocyanate (FITC), as previously described (26). Briefly, 10 μg activin A were mixed with 1 μg FITC and allowed to react for 1 h at room temperature. Unconjugated FITC was removed by size-exclusion filtration. One million germ cells were suspended in ice-cold binding medium (F12/DME plus 1 mg/ml BSA) with 20 ng FITC-labeled activin A, with or without a 1000-fold excess of competitor. After incubation at 4 C for 30 min, the cells plus bound ligand were washed twice with PBS-BSA. Cellular fluorescence was measured using a Coulter Elite flow cytometer with 488 nm laser excitation and emission at 525 nm (±25 nm). The 2C and 4C subpopulations were separately analyzed for FITC-activin A binding using combined 90° light scatter and forward-angle light scatter (FALS) gating. The FITC-conjugated activin A was bioactive in the rat pituitary bioassay. Binding was found in 10 experiments on separate germ cell preparations from animals 21–25 days of age.

Results

Coculture

To provide an optimal environment for Sertoli cell function, the medium in all cultures was supplemented with 5F. Neither FSH nor testosterone was added to control medium. In the 5F controls, widely distributed small clusters of 1–2 germ cells were seen in the cultures on day 3 (Fig. 1a). In contrast, the presence of activin A (100 ng/ml) caused a marked change in culture appearance within 24 h. Numerous spermatogonia appeared at 48–72 h of culture in the activin-treated cultures. These cells were in connected clusters of 8–32 cells attached to the Sertoli cell monolayer (Fig. 1c). Activin B also caused an increase in the number of spermatogonia in the cultures (data not shown). No effect was seen on spermatogonial numbers when inhibin was added to the medium (Fig. 1b).

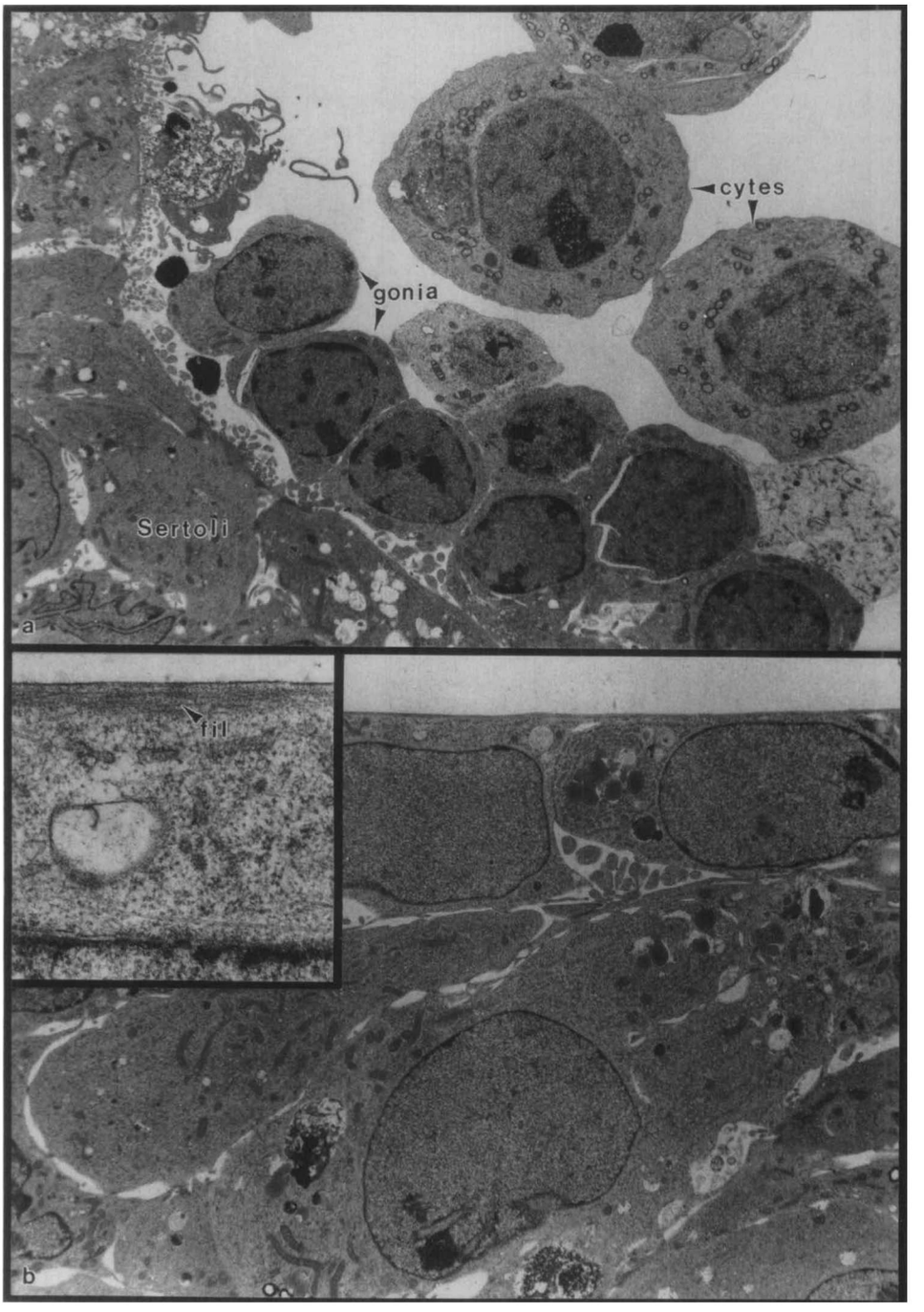

FIG. 2. Transmission electron micrograph of activin-treated cultures such as those shown in Fig. 1, c and d. The section was cut through a reaggregated cell cluster. Sertoli cells with attached spermatogonia (gonia) and spermatocytes (cytes) are shown in a. b, Details of the Sertoli cells. The high magnification *inset* is of the Sertoli cell section. Note the numerous fibrils (fil) at the periphery of the cell.

In addition to the effect of activin on the germ cell number, there was a marked change in the nature of the monolayer. The Sertoli cells began to reassociate into aggregates of cells, with the germ cells adhering to the outside (Fig. 1d). The reaggregation continued until cell clusters and tubule-like structures formed over the entire plate, and the cells detached from the dish at 72–96 h of culture. In both the control and inhibin-treated cultures, the Sertoli cells remained as well spread monolayers throughout this time period.

Aggregates formed in the activin-treated cultures, and control and inhibin-treated monolayers were fixed 72 h after the addition of hormone and prepared for transmission electron microscopic observation. The aggregates contained healthy Sertoli cells with characteristic nuclei, lipid droplets, and microfibrils (Fig. 2, a and b, *inset*). Clusters of spermatogonia adhered directly to the Sertoli cells, and primary spermatocytes were more loosely connected (Fig. 2a). Junctional complexes could be seen between Sertoli cells and between Sertoli and

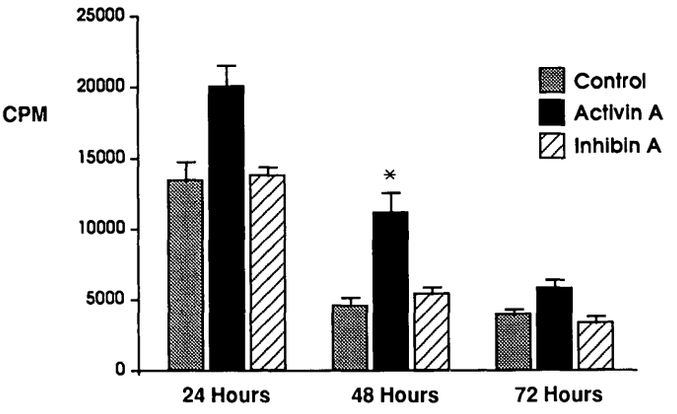

FIG. 3. Time course of stimulation of germ cell proliferation. Thymidine incorporation is shown on days 2, 3, and 4 of cultures 24, 48, and 72 h after hormone addition. In all cases the thymidine pulse was for 20 h. Activin A and inhibin A were added at 100 ng/ml. The figure shows the mean and SEM of triplicate samples. *, Significantly different from control at $P < 0.02$. Animals were 21 days old at the initiation of culture.

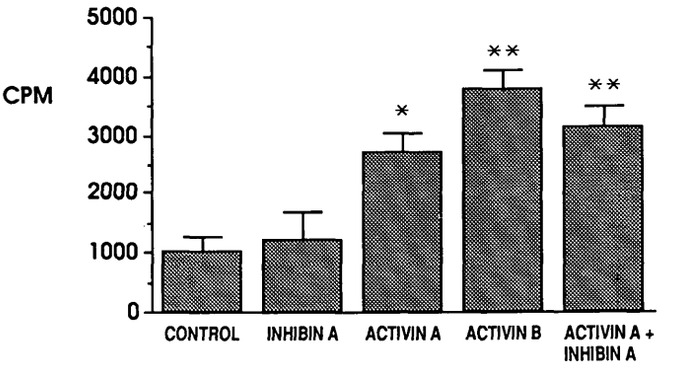

FIG. 4. Comparison of the effects of various hormones on [^{3}H]thymidine incorporation. Sertoli and germ cell cocultures were prepared as described and treated with activin A, activin B, inhibin A, or both activin A and inhibin A as indicated, each at 100 ng/ml. The incorporation shown was on day 4 of culture, 72 h after the addition of hormone. The figure shows the mean ± SEM of triplicate samples. Samples significantly different from the control are shown: *, $P < 0.02$; **, $P < 0.01$. Activin A, activin B, and activin plus inhibin are not significantly different from each other. Animals were 20 days old at the initiation of culture.

germ cells. Cytoplasmic bridges were sometimes observed between spermatogonia. Sections cut from control and inhibin-treated cultures contained Sertoli cells similar to those shown in Fig. 2b. No peritubular or germ cells were seen in the sections examined.

Thymidine incorporation

To determine whether the apparent increase in spermatogonia was due to proliferation of these cells *in vitro*, the cultures were pulse labeled with [^{3}H]thymidine. The 5F control cultures had a high level of incorporation of [^{3}H]thymidine into DNA during the initial 24-h period after the medium change, which decreased on the second day and remained at this level (Fig. 3). Incorporation was higher in the activin-treated cultures than in the control and inhibin-treated cultures at all time periods. The period of maximum stimulation of spermatogonial proliferation by activin A occurred during the period 48–72 h after a single addition of hormone (Figs. 3 and 4). Maximal stimulation of thymidine incorporation into DNA varied from 2–4 times control levels in nine separate experiments.

Activin A increased germ cell number, culture reaggregation, and thymidine incorporation at concentrations of 10–200 ng/ml. The effect was time and dose dependent, with all effects appearing earlier at higher doses (data not shown). In addition, the removal of germ cells from the monolayer reduced thymidine incorporation in activin-treated cultures to control levels (data not shown).

Activin B stimulated both thymidine incorporation and the appearance of germ cell clusters (Fig. 4). Simultaneous addition of 100 ng/ml inhibin A and activin A resulted in [^{3}H]thymidine incorporation and morphological appearance indistinguishable from those of cultures treated with activin A alone (Fig. 4). Other factors tested that showed no reproducible stimulatory effect on thymidine incorporation in three separate experiments include transforming growth factor-α (100 ng/ml), fibroblast growth factor (50 ng/ml), NGF (100 ng/ml), insulin-like growth factor-I (50 ng/ml), FSH (37 IU/ml), testosterone (5×10^{-8} M), and interleukin-2 (4.5 nmU). Forskolin (10 μM) was slightly stimulatory in repeated experiments (data not shown).

Labeled cells were identified by autoradiography. This technique was not quantitative, because many of the nonadherent and loosely adherent cells were lost in the processing. In the activin-treated cultures the label was incorporated into the clusters of germ cells attached to the monolayer (Fig. 5). Each cluster contained both labeled and unlabeled cells, consistent with proliferation occurring throughout the 0- to 72-h period of treatment, although [^{3}H]thymidine was present only during the last 24 h of this time.

Analysis of germ cell DNA content

Sertoli cells, which contain large lipid droplets (see Fig. 2b), stained brightly with Nile Red, a lipophilic dye (21) (Fig. 6), while spermatogonia, which contain little or no lipid, did not stain. The pachytene spermatocytes contained small lipid droplets after 2–3 days in coculture, but due to their large volume, had a high fluorescence intensity per cell. Cultures were simultaneously stained with Nile Red and Ho342. The Nile Red-negative subpopulation was identified and electronically gated for analysis of DNA content (Fig. 7A). Activin treatment

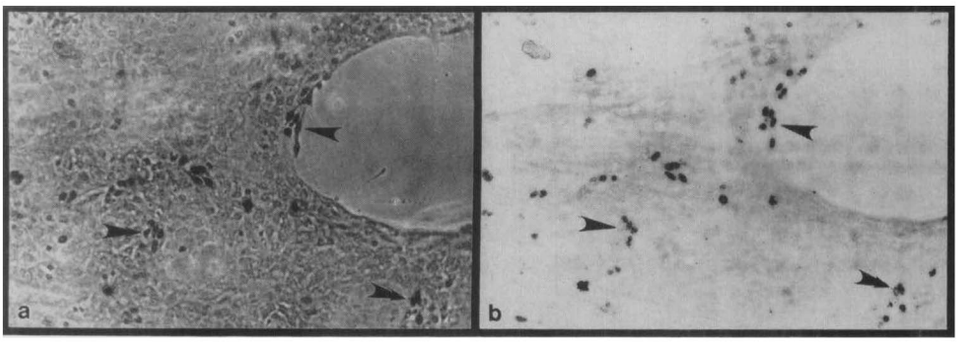

FIG. 5. Autoradiography of an activin-treated culture. Cultures were thymidine labeled between 48–72 h after the addition of hormone. a, A phase contrast photograph; b, a *trans*-illuminated photograph of the same area to enhance the appearance of the silver grains. The clusters of spermatogonia seen in phase can be seen to be heavily, but not uniformly, labeled.

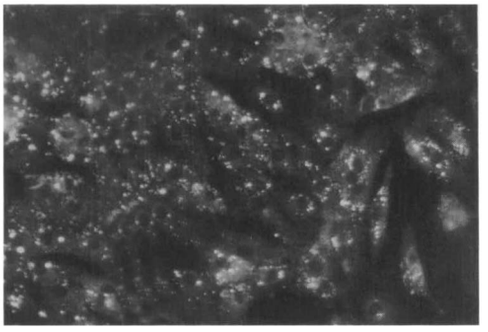

FIG. 6. Nile Red staining of the monolayer. Brightly fluorescent lipid droplets can be seen in the Sertoli cells.

resulted in a large increase in the fraction of germ cells with a 4C DNA content 48 h after treatment (Fig. 7C). Inhibin-treated cultures showed no change from control (Fig. 7B). A higher percentage of the total cells were Nile Red negative after activin treatment, and a higher percentage of these negative cells had a 4C DNA content (Table 1). Combining all of the subpopulations of germ cells, the ratio of germ cells to Sertoli cells was 0.9 in the control and inhibin-treated cultures and 1.8 in the activin-treated cultures

FITC-activin A binding

To determine whether it was possible that activin was acting on the germ cells directly, we assessed the binding of FITC-labeled activin A in purified germ cell cultures. Binding of FITC-activin is reflected as a shift of the frequency histogram toward a higher mean fluorescence per cell. The extent of the shift is proportional to the number of labeled molecules bound per cell (24). Cells could be gated by FACS into a population of small cells, which have a 2C DNA content, and a population of large cells having predominantly a 4C DNA content. These two distinct germ cell populations had different activin-A binding characteristics, as shown in Fig. 8. A subpopulation (~54%) of the 2C cells bound FITC-activin A (Fig. 8B), and this binding was 82% competed by a 1000-fold excess of unlabeled activin A (Fig. 8C). There was no competition with a 1000-fold excess of inhibin A (data not shown). In contrast, the 4C population of germ cells did not show a shift in the peak fluorescence in the presence of labeled activin A, indicating no activin binding to these cells (Fig. 8, D–F).

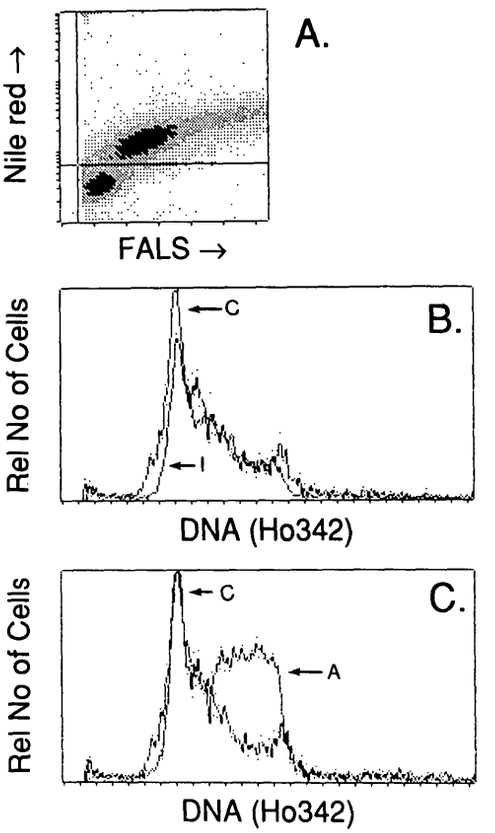

FIG. 7. FACS analysis of the DNA content of mixed cultures treated with 5F and inhibin A or activin A. Cells were stained using Nile Red and Ho342 and analyzed by dual laser flow cytometry. A illustrates a bivalent FALS (forward light scatter) *vs.* Nile Red histogram used to gate the low FALS (*i.e.* small size) Nile Red-negative spermatogonia (cells *below the horizontal line*) for analysis of DNA content (*i.e.* Ho342 fluorescence). Cultures pretreated for 48 h with either inhibin (I; panel B) or activin (A; panel C) were gated using the Nile Red/FALS plots, and total cellular DNA content was analyzed in the Nile Red-negative low FALS field. Each DNA histogram is overlaid with the appropriate 5F control (C) histogram for comparison. The *abcissa* represents cell number, and the *ordinate* represents the fluorescence intensity of the Ho 342/cell.

TABLE 1. Distribution of cells into Nile Red-positive and -negative and 2C and 4C populations

Treatment	Cell no.				Ratio total germ/Sertoli
	Nile red positive		Nile red negative		
	2C	4C	2C	4C	
5F control	100	45	27	16	0.9
+ Activin A	100	37	56	83	1.8
+ Inhibin A	100	49	31	13	0.9

The Nile Red-positive and -negative populations were gated as shown in Fig. 7. Each field was then analyzed for DNA content by Ho342. The number of cells in each group was normalized to 100 cells in the NR+, 2C group, which was identified as Sertoli cells. The other three fields contained spermatogonia (NR−, 2C, and 4C) and spermatocytes (NR+, 4C) and were summed to give total germ cells. The effect of activin on total germ/Sertoli ratio is significant ($P < 0.001$), while that of inhibin is not ($P > 0.05$).

Discussion

There was a marked proliferation of germinal elements in cocultures of immature Sertoli and germ cells treated with activin *in vitro*. The increase in clusters of spermatogonia seen in phase contrast microscopy was reflected by an increase in [^{3}H]thymidine incorporation in the total culture after treatment with activin. Moreover, autoradiography of the treated cultures shows that label was predominantly incorporated into the clusters of germ cells attached to the monolayer. These data are consistent with the hypothesis that activin is stimulating division of spermatogonia *in vitro*.

The Nile Red-staining method described is a novel approach to analyzing cultures of mixed testicular cell types without physical separation of the cells. Using this stain and fluorescence-activated cell analysis, we could selectively analyze changes in the distribution of the germ cells by electronically gating these cells in the mixed cultures containing Sertoli cells. The results showed a marked increase in the overall percentage of germ cells after treatment with activin. There was also an increase in the fraction of cells in the proliferative phase (4C DNA content) after activin treatment. These data again support the hypothesis that activin is stimulating spermatogonial division, and perhaps differentiation, in these cultures.

Previous reports of Sertoli, Sertoli-peritubular, or Sertoli-peritubular-germ cell cocultures have shown that reassociation of cells in the cultures was dependent on the presence of peritubular cells (27) or basement membrane components (28). In this report we show that a single hormone, activin, can rapidly cause the reassociation of the Sertoli and germ cells in the cultures into tubular elements. This reassociation begins within 24 h of the addition of the hormone. After 3 days of hormone treatment, electron microscopic observation could detect no peritubular cells and no formation of basement membrane in the cultures. Thus, the reassociation seems to be occurring via a different mechanism than that reported previously, or activin, presumably produced *in vitro*, is involved in the reassociation seen in these other culture systems. These effects seem to be specific for activin A and B.

Inhibin had no affect on either culture morphology or

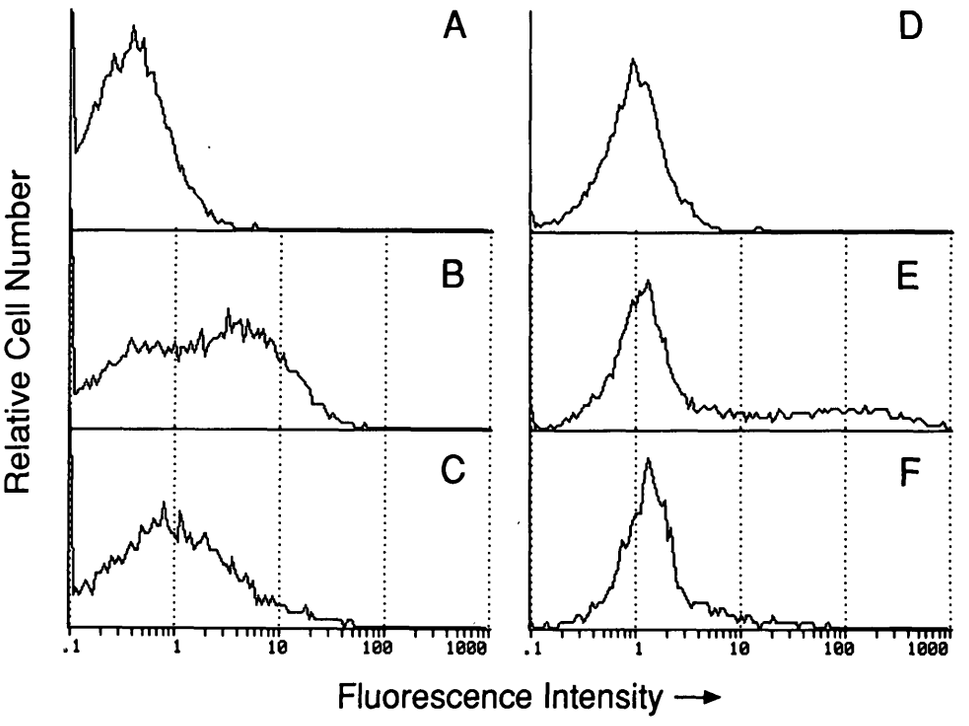

FIG. 8. Activin binding to germ cells. FITC-labeled activin A was incubated to saturation with a purified germ cell population derived from 21-day-old animals, as described in the text. The germ cell culture was divided into two populations on the basis of size and analyzed for FITC-activin A binding and DNA content. The population shown in A–C was composed predominantly of small cells with a 2C DNA content. The population shown in D–F was composed predominantly of large cells with a 4C DNA content (meiotic prophase). A and D are the control (no hormone) autofluorescence; B and E are germ cells binding FITC-activin A. A rightward shift in the intensity of fluorescence indicates an increased binding of the hormone. The histogram shows the distribution of cells binding different amounts of hormone. C and F show competition of FITC-activin A by excess unlabeled activin A.

[^{3}H]thymidine incorporation in the cultures. Inhibin, at equivalent concentrations, did not block or decrease the effect of activin on these cultures. This is in contrast to the opposing effects reported for these hormones on FSH release by pituitary cultures (18), thymocyte proliferation (29), and the differentiation of K549 cells (30). It is of interest that the inhibition of spermatogonial number (15) seen *in vivo* after the injection of purified inhibin in the hamster is not seen in these cultures. Other factors, such as testosterone, known to be involved in regulating spermatogenesis *in vivo*, also did not affect germ cell proliferation in the culture system described here. Thus, inhibin and testosterone may be acting on other cell types in the testis or on stages of spermatogenesis not represented in these cultures, or may require another hormone or growth factor for activity which is not present in this culture system.

Activin binding is exhibited by early stage germ cells (2C DNA content) and not by meiotic prophase (4C DNA content) cells. These are the same cells that proliferate in response to activin *in vitro*. This demonstration of activin A binding to germ cells raises the intriguing possibility that activin is acting directly on the spermatogonia themselves, rather than exerting an indirect affect via Sertoli cells. However, such an indirect affect of activin cannot be ruled out. The cellular source of the activin *in vivo* could be either Sertoli cells or Leydig cells, since the spermatogonia are in the basal compartment during their proliferative phase.

The germ cells might, in this instance, be directing the monolayer reaggregation *in vitro*. Other instances of potential germ to Sertoli interactions are the recently reported production of NGF by germ cells, with NGF receptors appearing on Sertoli cells in a stage-dependent fashion (31), and germ cell regulation of Sertoli transferrin message levels (32). The β_A- and β_B-subunit mRNAs and proteins have been found in Sertoli cells, but not germ cells, while β_A-protein (but not mRNA) has been localized in germ cells (8), suggesting that these cells are a target for activin, rather than a site of production.

van Dissel-Emiliani *et al.* (15) have reported that a partially purified fraction of Sertoli cell-conditioned medium containing inhibin, but not purified inhibin, can stimulate the proliferation of undifferentiated spermatogonia when injected *in vivo* in hamsters. The activity in the Sertoli cell-conditioned medium was not identified, but Sertoli cells may secrete activin which initially copurifies with inhibin in many systems (2).

Activin and/or inhibin seem to be produced and act at a number of sites of stem cell proliferation and differentiation (10, 33, 34). The data presented above suggest that activin plays a role in regulating germ cell proliferation in the male. Activin A and related peptides have been shown to play a role in embryonic organization during development (35–37). The rapid and specific reassociation of Sertoli cells into tubule-like structures *in vitro* when treated with activin suggests that this factor may also play a role in gonadal organization during development.

Acknowledgments

We would like to acknowledge the technical assistance of Jane Battaglia, Jim Borree, John Woronicz, and Stan Hansen and helpful discussions with Dr. Joseph Orly.

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