Reciprocal regulation of activin A and inhibin B by interleukin-1 (IL-1) and follicle-stimulating hormone (FSH) in rat Sertoli cells *in vitro*

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Abstract

In several biological systems, the inhibin β_A homodimer activin A is stimulated by, and in turn, inhibits the action of interleukin (IL)-1 (both IL-1α and IL-1β) and IL-6. The possibility that a similar regulatory relationship operates within the testis was investigated. Sertoli cells from immature (20-day-old) rats were cultured with human IL-1α or IL-1β, human IL-6 and/or ovine FSH or dibutyryl cAMP. Activin A and the inhibin dimers, inhibin A and inhibin B, were measured by specific ELISA. Immunoreactive inhibin (ir-inhibin) was measured by RIA. Activin/inhibin subunit mRNA expression was measured by quantitative real-time PCR. Both IL-1 isoforms, but not IL-6, stimulated activin A secretion through increased synthesis of β_A-subunit mRNA. IL-1 also stimulated activin A secretion by testicular peritubular cells. In contrast to the effect on activin A, IL-1 suppressed inhibin β_B-subunit and, to a lesser extent, α-subunit mRNA expression, thereby reducing basal and FSH-stimulated inhibin B secretion by the Sertoli cells. Conversely, FSH inhibited basal activin A secretion and antagonised the stimulatory effects of IL-1. Dibutyryl cAMP partially inhibited the action of IL-1 on activin A secretion, but had no significant effect on basal activin A secretion. Secretion of inhibin A was low in all treatment groups. These data demonstrate that IL-1 and FSH/cAMP exert a reciprocal regulation of activin A and inhibin B synthesis and release by the Sertoli cell, and suggest a role for activin A as a potential feedback regulator of IL-1 and IL-6 activity in the testis during normal spermatogenesis and in inflammation.

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Introduction

Testicular function is regulated by the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH), androgens and a complex network of paracrine interactions. Considerable evidence indicates that testicular paracrine regulation involves the inflammatory cytokines, interleukin-1 (IL-1) and IL-6 (Jégou *et al.* 1995), and activin, a member of the transforming growth factor-β (TGFβ) family of cytokines with a broad range of immunoregulatory actions (Phillips *et al.* 2001, de Kretser *et al.* 2004).

Typically, IL-1 and IL-6 are produced by activated monocytes and macrophages during inflammatory responses (Dinarello 1996). IL-1 exhibits two isoforms, IL-1α and IL-1β, which are only 30% identical at the peptide level, but bind to a common receptor to exert an almost identical range of biological actions, including the stimulation of IL-6 production (Dinarello 1996). In the normal testis, IL-1α is produced by the Sertoli cells

(Gérard *et al.* 1991) and is regulated by the spermatogenic cycle (Söder *et al.* 1991). Expression of IL-1α in cultured Sertoli cells is stimulated by phagocytosis of residual bodies, and by lipopolysaccharide (LPS), a bacterial inflammatory stimulus (Gérard *et al.* 1992). As in monocytes, IL-1α stimulates IL-6 production by the Sertoli cell (Syed *et al.* 1995). IL-1β is not significantly expressed in the normal testis, but LPS upregulates expression of IL-1β and IL-1α in Leydig cells (Cudicini *et al.* 1997) and in testicular interstitial macrophages (Hales *et al.* 1992, Jonsson *et al.* 2001).

Sertoli cells are regulated by FSH acting through a cAMP/protein kinase A-mediated pathway, and by androgens (Means *et al.* 1974). *In vitro* studies have confirmed that both IL-1 and IL-6 also have complex effects on Sertoli cell and spermatogenic cell function. IL-1 inhibits FSH-induced aromatase activity (Khan & Nieschlag 1991), and stimulates production of lactate and transferrin in Sertoli cells (Hoeben *et al.* 1997, Nehar *et al.* 1998, Huleihel *et al.* 2002), the proliferation of developing

rat Sertoli cells in culture (Petersen *et al.* 2002), and spermatogonial and preleptotene spermatocyte DNA synthesis in cultured tubules from adult rats (Parvinen *et al.* 1991). Although IL–6 also stimulates transferrin production by Sertoli cells (Boockfor & Schwarz 1991), this cytokine inhibits meiotic DNA synthesis in preleptotene spermatocytes (Hakovirta *et al.* 1995).

Activins are dimers of two homologous protein subunits, β_A or β_B; homodimers of the subunits are called activin A ($\beta_A\beta_A$) and activin B ($\beta_B\beta_B$) (Groome *et al.* 2001). Heterodimers of a distinct α–subunit with one of the two β–subunits produce either inhibin A ($\alpha\beta_A$) or inhibin B ($\alpha\beta_B$). Activin A is produced by Sertoli cells, peritubular cells and Leydig cells from immature testes (Lee *et al.* 1989, de Winter *et al.* 1993, 1994), and Sertoli cells appear to produce some activin B (de Winter *et al.* 1993). The Sertoli cells are also the principal source of inhibin B, which is the predominant inhibin isoform in the male (Illingworth *et al.* 1996). Inhibin B production by Sertoli cells is controlled by FSH, primarily through stimulation of the α–subunit (Toebosch *et al.* 1988, Klaij *et al.* 1992), but there are very few data concerning specific regulation of the β–subunits or the formation of activin in the testis. While inhibin acts as circulating inhibitor of pituitary FSH secretion, the activins possess predominantly paracrine functions, and exert both stimulatory and inhibitory effects on spermatogonial cell proliferation *in vitro*, and inhibitory effects on Sertoli cell proliferation (Mather *et al.* 1990, Hakovirta *et al.* 1993, Boitani *et al.* 1995, Meehan *et al.* 2000).

As a consequence of their actions on Sertoli and germ cell activity *in vitro*, it has been suggested that IL–1, IL–6 and activin may play a role in the fine regulation of spermatogenesis. Consequently, interaction between these cytokines is also likely to be important, and there is evidence for such an interaction between IL–1/IL–6 and activin in several tissues. For example, IL–1 stimulates activin β_B mRNA accumulation, as well as the mRNA for its binding protein, follistatin, in the pituitary, thereby regulating FSH secretion (Bilezikjian *et al.* 1998). Activin A is upregulated in monocytes during inflammation (Shao *et al.* 1992) and inhibits proinflammatory and immunoregulatory activities of both IL–1 and IL–6 (Yu *et al.* 1998, Phillips *et al.* 2001). IL–1 potentiates the inhibitory effect of activin on liver cell proliferation, and stimulates activin A production in cultures of bone marrow stromal cells, synoviocytes, chondrocytes and placental cells (Shao *et al.* 1992, Keelan *et al.* 1998, Yu *et al.* 1998, Russell *et al.* 1999, Abe *et al.* 2001).

A similar pattern of interactions might be expected to operate in the testis. In the present study, we investigated regulation of activin and inhibin by IL–1 and IL–6 in the rat Sertoli cell *in vitro*, and their interaction with FSH and cAMP, using a two-site enzyme-linked immunosorbant assay (ELISA), which can discriminate between the activin A, inhibin A and inhibin B dimers (Groome *et al.* 1994,

1996, Knight *et al.* 1996). Interactions between IL–1/IL–6 and activin/inhibin would have important implications both for normal regulation of spermatogenesis and for the progression of spermatogenic disruption during inflammation (O'Bryan *et al.* 2000).

A better understanding of this overlap between the regulation of testicular function and immune processes may help to explain the causes of male infertility commonly associated with illness, infection and inflammation.

Materials and Methods

Isolation and culture of Sertoli cells

Sertoli cells were prepared from immature (20-day-old) male Sprague-Dawley rats (Central Animal House, Monash University, Victoria, Australia) as previously described (Risbridger *et al.* 1989), with minor modifications. Briefly, testes were decapsulated, and the seminiferous tubules were partially drawn apart with forceps, before dissociation in Dulbecco's modified Eagle's medium plus Ham's nutrient mixture F-12 (DMEM:F12; Gibco, Grand Island, NY, USA) through the following series of enzyme digestions in a shaking water-bath (90 cycles/min, 37 °C): 1. collagenase (type II, 300 U/ml; Worthington Biochemical, Lakewood, NJ, USA) and DNase (type I, 5 µg/ml; Sigma), 10 min; 2. collagenase, DNase and hyaluronidase (type II, 0·5 mg/ml; Sigma), 20 min; 3. collagenase, DNase and hyaluronidase, 10 min. The resulting cell preparation was resuspended in DMEM containing 0·1% bovine serum albumin (BSA) (Sigma), penicillin (5000 U/ml) and streptomycin (5000 µg/ml) to a final concentration of approximately $1·0 \times 10^6$ cells/ml, and 1·0 ml aliquots were transferred to Falcon 24-well culture plates (Becton-Dickinson Labware, Bedford, MA, USA). In some experiments the plates were precoated with 1·0 µg/cm^2 mouse laminin (Becton-Dickinson) prior to the addition of the cell suspension. Laminin has been shown to influence Sertoli cell attachment, morphology and hormonal responses *in vitro* (Dym *et al.* 1991). The plated cells were incubated for 24 h at either 37 °C or 32 °C in 5% CO_2/air (pH 7·2–7·3), after which time the medium containing unattached cells was removed. In some experiments, the attached cell layer was subjected to a brief hypotonic shock with water to lyse residual germ cells, the medium was replaced and the attached cells were allowed to recover for an additional 24 h. After the washing step to remove unattached cells, fresh medium containing appropriate test substances was added to quadruplicate wells: human recombinant (hr)IL–1α (160 000U/µg), hrIL–1β (200 000U/µg) (R and D Systems, Minneapolis, MN, USA), hrIL–6 (PeproTech, Rocky Hill, NJ, USA), dibutyryl cAMP (dbcAMP; Sigma), testosterone (Sigma) and ovine FSH (NIH oFSH-20, 4453 IU/mg; NIDDK, Bethesda, MD, USA). Medium was collected and replaced thereafter at 48-h

intervals (days 3, 5 and 7 of culture). In some experiments, after removal of the medium, the attached cells were resuspended in 1 ml 0·01 M PBS, 0·1% Triton X-100 (v/v) and 0·1% BSA, and homogenised in the culture wells. The cell lysates were centrifuged (10 000 g, 10 min) to remove particulates, and the resulting supernatants collected. Media and cell lysates were stored at −20 °C prior to ELISA. For studies on mRNA expression, total RNA was extracted from the Sertoli cells with Trizol reagent (Life Technologies, Rockville, MD, USA). All experiments were repeated at least twice.

Alkaline phosphatase histochemistry

Alkaline phosphatase histochemistry was performed on cultured cells at the end of the culture period by an established protocol (Chapin *et al.* 1987). In four sequential experiments, $8·8 \pm 5·0\%$ (mean ± S.D.) of the cultured cells were alkaline phosphatase-positive peritubular cells at day 3 of culture.

Percoll gradient separation and assessment

In some experiments, Sertoli cells and peritubular cells were separated on discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradients by a modification of the method described previously (Simpson *et al.* 1992). After enzymatic dispersal, the cell suspension was diluted in DMEM:F12 and applied to a 50 ml discontinuous Percoll step gradient (1·025 to 1·075 g/ml) at 1158 g for 20 min at 4 °C. Sertoli cells were collected from a band between 1·045 and 1·055 g/ml, and peritubular cells were isolated between 1·06 and 1·07 g/ml (purity of both fractions was >98%, based on alkaline phosphatase staining). Each fraction was washed three times in culture medium, diluted to approximately $1·010^6$ cells/ml, and plated as described above.

Measurement of DNA content

The attached Sertoli cells were washed with PBS, before 10% trichloroacetic acid was added to each well for 10 min. The plates were then centrifuged at 100 g (10 min, 4 °C), and the supernatant was discarded. The PBS wash, acid treatment and centrifugation were repeated once more, before 0·5 ml ice-cold ethanol was added to each well for 10 min. Plates were centrifuged at 100 g (10 min, 4 °C), and the supernatant was discarded. A 200 µl aliquot of 0·3 M NaOH was added to each well, and the plate was agitated for 10 min. Each well was neutralised by addition of 200 µl 0·3 M HCl before samples were read in a spectrophotometer at 260–280 nm.

Inhibin radioimmunoassay (RIA)

Immunoreactive inhibin (ir-inhibin) was measured in Sertoli cell culture media by heterologous, double-antibody RIA, using a rabbit antiserum (no. 1989) raised against bovine 31 kDa inhibin, with iodinated bovine 31 kDa inhibin as tracer, and an ovarian extract from PMSG-stimulated rats (ROVEA-88) as standard (Robertson *et al.* 1988). This assay, which is directed against the inhibin α-subunit, detects the native inhibin dimer, but cross-reacts with several other proteins derived from the inhibin α-subunit precursor (Groome *et al.* 2001). The assay limit of detection was 0·2 ng/ml. The intra-assay variation was 4·6–9·9%, while the interassay coefficient of variation was 7·0% ($n=12$ assays).

Activin A ELISA

Activin A was measured by specific ELISA (Knight *et al.* 1996) according to the manufacturer's instructions (Oxford Bio-Innovations, Upper Heyford, Oxfordshire, UK) with some modifications. The standard used was human recombinant activin A, as described previously (Robertson *et al.* 1992). Standards and samples were diluted in unconditioned culture medium containing the same additives as were used in the culture. A 6% sodium dodecyl sulphate solution in PBS was added (3% final concentration) followed by boiling for 3 min. The samples were allowed to cool before the addition of H_2O_2 (2% final concentration) and subsequent 30-min incubation. Duplicates were added to the E4 (anti-β_A subunit) monoclonal antibody-coated plates and incubated overnight at room temperature. The plates were washed, and the second detection antibody (biotinylated-E4) was added for 2 h at room temperature. After washing, alkaline phosphatase linked to streptavidin was added to the wells and incubated at room temperature for 1 h. After further washes, the alkaline phosphatase activity was detected with an amplification kit (ELISA Amplification System; Invitrogen, Carlsbad, CA, USA), whereby the substrate was incubated for 1 h at room temperature.

Inhibin A and B ELISA

Inhibin A and inhibin B were measured by specific ELISA (Groome *et al.* 1994, 1996) according to the manufacturer's instructions (Oxford Bio-Innovations) with some modifications. The standards used were an inhibin A standard provided with the kit, which had been calibrated against recombinant 32 kDa inhibin and WHO 96/784 inhibin B reference standard (NIBSC, Potter's Bar, UK). Standards and samples were diluted in unconditioned culture medium and treated according to the manufacturer's protocol. Duplicates were added to the plates and incubated overnight at room temperature. The plates were washed, and the second detection antibody (R1 coupled to alkaline phosphatase) was added for 3 h at room temperature. After washing, the alkaline phosphatase activity was

Table 1 Oligonucleotide primer sequences, primer-specific LightCycler conditions and expected product sizes for rat inhibin family subunits and β-actin

	Nucleotide sequence	Annealing temperature (°C)	Mg (mM)	Product size (bp)
Primer set				
α-Subunit	5′-GCCTTGGTCTCCTGCA-3′ 5′-ACGCGTAGGGACCTCATGCTCC-3′	62	4	300
β$_A$-Subunit	5′-TGGAGTGTGATGGCAAGGTC-3′ 5′-AGCCACACTCCTCCACAATC-3′	60	3	339
β$_B$-Subunit	5′-TCTTCATCGACTTTCGGCTCAT-3′ 5′-TGTCAGGCGCAGCCACACTCCT-3′	60	3	302
β-Actin	5′-GATATCGCTGCGCTCGTC-3′ 5′-TGGGGTACTTCAGGGTCAGG-3′	60	3	202

detected using an amplification kit (ELISA Amplification System; Invitrogen).

mRNA extraction and real-time PCR analysis

Total RNA from Sertoli cell cultures was purified by the guanidinium thiocyanate–phenol–chloroform method of Chomczynski and Sacchi (1987). To remove genomic DNA contamination, samples were then treated with 2 U of DNase I (Ambion, Austin, TX, USA) at 37 °C for 60 min, and the reaction was stopped by adding DNase inactivating reagent. Samples were then assessed by A260/A280 spectrophotometry. One microgram of RNA for each sample was transcribed, using Superscript II and oligo dT13–15 (Gibco-BRL/Invitrogen). For every sample, a no–RT control also was performed, to verify the absence of contaminating genomic DNA in the PCR. mRNA expression was quantified by the Roche LightCycler (Roche, Mannheim, Germany), as previously described (Drummond *et al.* 2000). For PCR, 2 μl of each cDNA preparation were diluted to a final concentration of 1:50 and added to individual capillary tubes with dNTP, Mg^{2+}, SYBR Green and relevant primers. Magnesium concentrations, annealing temperatures, extension times and primer-specific nucleotide locations and sequences are shown in Table 1. Forty cycles of PCR were programmed to ensure that the log-linear phase was reached. At the completion of the reaction, melting curve analysis was performed to establish the specificity of the DNA products produced. PCR products were removed from the capillary tubes and visualised by gel electrophoresis to confirm the product size and integrity of the PCR reaction. In every instance, samples from an individual culture experiment were analysed for each primer set in a single PCR run. The intra-assay variation was 4% for each primer set. The log-linear portion of the PCR amplification curve was identified with the threshold or crossing point (represented in cycle number) defined as the intersection of the best-fit line through the log-linear region and the noise band. In these studies, a normal rat cDNA preparation was employed as a quality control and used in all reactions to ensure cycling conditions remained constant between experimental runs. The levels of expression of each mRNA and their estimated crossing points in each sample were determined with the LightCycler software, and the activin/inhibin subunit mRNAs were normalised against β-actin. These data were used to calculate a relative activity value, determined as the ratio of IL-1-stimulated mRNA to basal mRNA expression at each culture time point.

Statistical analysis

All data were analysed by one-way or two-way analysis of variance (ANOVA) following appropriate transformation to normalise data and equalise variance, where necessary. Mean values were compared by either the Student–Newman–Keuls multiple range test or Dunnett's test for multiple comparisons with a control (Sigmastat Version 1·0; Jandel Scientific Software, San Rafael, CA, USA). Differences between responses were considered statistically significant at the $P<0·05$ level. ELISA dose–response curves were compared by linear regression after log–log transformation of the data. The curves were considered to be parallel if the 95% confidence limits of the slopes overlapped.

Results

Assay validation for medium samples

In order to measure activin A in the study, it was necessary to validate the assays for tissue culture medium. Initial dose–response studies in standards and samples diluted in culture medium showed a non-linear response in samples and a decrease in sensitivity in the assay compared with assays using a buffer-based standard. Investigations revealed that culture media, in particular carbonate-based media such as DMEM, increased in pH to varying degrees after storage/freezing. The change in the pH had the effect of decreasing the amount of activin A binding to the
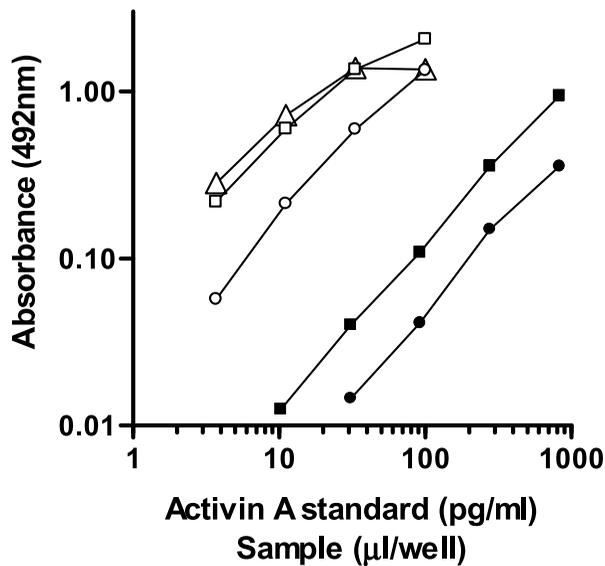
Figure 1 Log-log transformed dose–response curves of activin A standard (filled symbols) and Sertoli cell culture media samples (open symbols) in the activin A ELISA. The dilution of the samples added to the wells is also indicated. Standards and samples were diluted in culture medium (DMEM/0·1% BSA) with different pretreatments: addition of 6% SDS/H$_2$O (●○), addition of 6% SDS/0·05 M PBS (■□) and addition of 6% SDS/H$_2$O after dilution in fresh, buffered medium (△). All values are mean of duplicates.

antibodies and therefore resulted in underestimation of the amount of activin A in the samples and decrease in the sensitivity of the assay (Fig. 1). In the published method (Knight *et al.* 1996), there was no addition of buffer in the activin A ELISA sample treatment, unlike the methods for the inhibin A and B ELISAs. However, when 0·05 M PBS was added to the sample treatment in the form of 6% SDS/0·05 M PBS, instead of the usual 6% SDS solution in water, a uniform pH of all culture medium samples and diluent resulted, regardless of storage. Serial dilution of samples treated in this manner gave dose-dependent responses that were parallel to the standard (Fig. 1). There was also an increase in the signal, resulting in an approximately threefold increase in the sensitivity of the assay. The assay limit of detection was 10 pg/ml. Recoveries of culture medium 'spiked' with activin A were 104·5 ± 6·7 (mean ± S.D.; *n*=3 experiments). Intra- and inter-plate percentage coefficients of variation were 7·4% and 6·3% respectively (*n*=12 assays).

Serial dilutions of samples from Sertoli cell culture medium gave dose-dependent responses which were parallel to the respective standard, also diluted in culture medium, in both the inhibin A and inhibin B ELISAs (data not shown). The detection limits for inhibin A and inhibin B were 2 and 10 pg/ml respectively. Recoveries of culture medium 'spiked' with inhibin A were 101·9 ± 7·8% (mean ± S.D.; *n*=3 experiments); recoveries

of spiked inhibin B were 105·6 ± 4·1% (*n*=5 experiments). All samples were assayed in a single plate for inhibin A and a single plate for inhibin B, with intraplate percentage coefficients of variation of 4·2% and 2·2% respectively.

Effects of IL-1 and IL-6 on activin A production by Sertoli cell cultures

As measured on day 3 of culture (48 h after the initial plating-down period), basal activin A levels in the culture medium were 45 ± 19 pg/ml (mean ± S.D.; *n*=9 cultures). It should be noted that even under the established culture conditions, basal activin A showed considerable variation from culture to culture (range:<10–68 pg/ml). Both IL-1α and IL-1β stimulated the release of activin A from immature Sertoli cells by approximately 10-fold in a dose-dependent manner over an effective range of 5–20 U IL-1/ml (Fig. 2A). The specificity of this response was indicated by the observation that total ir-inhibin release, as an indicator of α-subunit protein secretion, was not significantly affected over the same range of doses (Fig. 2B). The corresponding intracellular content of activin A on day 3 was 7·9 ± 3·3 pg/10^6 cells (mean ± S.D.; *n*=3 cultures) under basal conditions, and this was increased only slightly to 11·6 ± 2·1 pg/10^6 cells by addition of IL-1. Culturing the cells at 32 °C instead of 37 °C reduced IL-1-stimulated activin A levels by approximately 25% without affecting basal activin A release (data not shown). However, neither culturing the Sertoli cells on a laminin-coated surface nor depletion of residual adherent germ cells by hypotonic treatment had any significant effect on basal or IL-1-stimulated activin A release (data not shown).

In contrast to IL-1, IL-6 at doses up to 50 ng/ml had no consistent effect on activin A release, alone or in the presence of either IL-1 isoform (data not shown). All subsequent studies used either the IL-1α or the IL-1β isoform, as indicated.

Regulation of activin A, inhibin B and mRNA for β$_A$, β$_B$ and α-subunits time-course studies

Under basal culture conditions, both activin A and inhibin B were progressively secreted into the culture medium over the initial 48 h of incubation, with detectable levels (>10 pg/ml) present by 8 h (Fig. 3A and B). IL-1α stimulated activin A and suppressed inhibin B secretion across this same time period. Stimulation of activin A by IL-1 was consistent with the effects seen at the mRNA level, with IL-1α inducing maximal expression of β$_A$-subunit mRNA around 8 h of culture (Fig. 3C). The dynamic effects of IL-1 on the inhibin B subunits were quite different, however, with progressive inhibition of the β$_B$-subunit by IL-1α across the entire 48-h period (Fig. 3D).
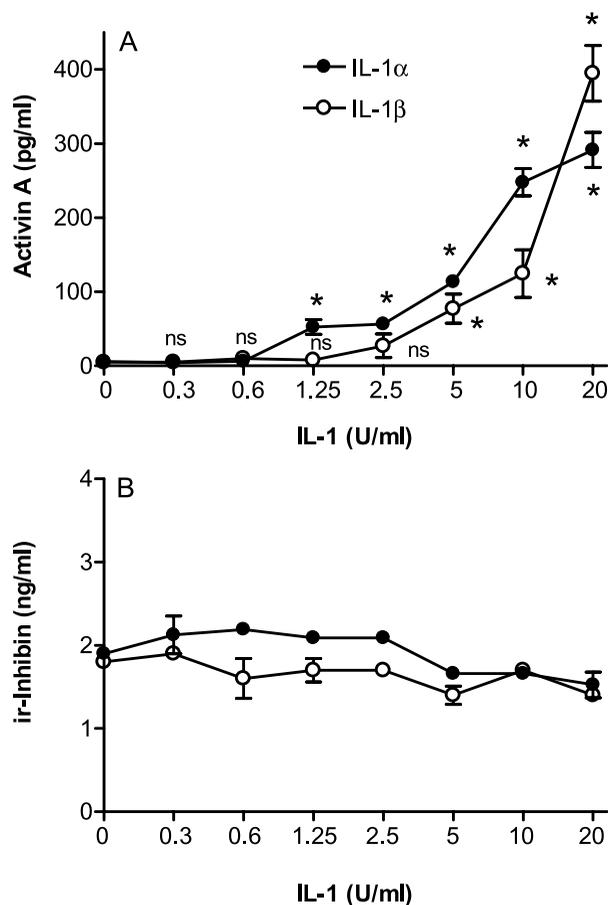
Figure 2 IL-1α (●–●) and IL-1β (○–○) log dose–response curves for secretion of activin A (A) and ir-inhibin (B) by immature rat Sertoli cells, collected on day 3 of culture (incubation time 48 h). All values are mean ± S.E.M.; *n* = 4 wells. Asterisk indicates significantly different from control (in absence of IL-1) at *P* < 0·05. Ir-inhibin values are not significantly different from control at any IL-1 dose.

In contrast to the other subunits, inhibition of the α-subunit was transient, and significant inhibition was observed only at the 24-h time point (Fig. 3E). This latter observation was consistent with the failure to detect significant inhibition of α-subunit protein in 48-h cultures by ir-inhibin assay (Fig. 2B).

Although the Sertoli cells showed a reduction in response to IL-1 at the longer culture periods (days 3 and 5), cells maintained basal activin A release, and retained responsiveness to IL-1 for up to 7 days in culture (Fig. 4A). Cell numbers, as measured by DNA content, appeared to decline slightly between days 3 and 7 of culture, but IL-1 had no significant effect on this parameter (Fig. 4B). Intracellular activin A levels were unaffected by the presence of IL-1 over the entire 7 days of culture (data not shown).

Effects of testosterone, FSH and dbcAMP on activin A production by Sertoli cell cultures

Testosterone (50 or 100 ng/ml) had no effect on basal or IL-1-stimulated activin A release by Sertoli cells (data not shown). In contrast, activin A was significantly inhibited by ovine FSH (500 ng/ml) (Fig. 5A). Moreover, FSH consistently antagonised the action of IL-1 on the release of activin A. IL-1β significantly inhibited the release of dimeric inhibin B regardless of the presence or absence of FSH in the culture (Fig. 5B). Likewise, IL-1β inhibited dimeric inhibin A release by these cultures, although the levels of inhibin A were extremely low compared with either activin A or inhibin B (Fig. 5C). In direct contrast to its effects on inhibin B, FSH on its own inhibited the release of inhibin A, and when it was combined with IL-1β, a further inhibition was observed.

Similar to the action of FSH, dbcAMP antagonised the effects of IL-1β on activin A release, although even very high doses of dbcAMP were unable to suppress activin A to basal levels in these cultures (Fig. 6). Moreover, in contrast to FSH, dbcAMP did not significantly affect basal activin A secretion.

Comparison of Sertoli cell and peritubular cell responses to IL-1

After the separation of these two cell types by Percoll density gradients, it was found that peritubular cells also respond to IL-1β by increasing release of activin A (Fig. 7). While the peritubular cells secreted less activin A under basal conditions than Sertoli cells, they produced between 2–3 times more on a per cell basis after IL-1 stimulation. However, since peritubular cells comprised less than 10% of the total cells in the Sertoli cell cultures, their *in vitro* contribution would be relatively minor overall.

Discussion

The availability of specific, two-site ELISAs that can discriminate between the inhibin A, inhibin B and activin A dimers has relatively recently enabled the measurement of the production of these proteins by testicular cells (Depuydt *et al.* 1999, Clifton *et al.* 2002). Earlier studies employed radioimmunoassays that did not readily discriminate between the inhibin/activin dimers and monomeric α-subunit or β-subunit proteins, relied on extrapolation from mRNA data, or used semiquantitative approaches such as Western blot analysis to assess production of dimeric inhibin and activin by testicular cells (Toebosch *et al.* 1988, Risbridger *et al.* 1989, Kaipia *et al.* 1992, Klaij *et al.* 1992, Simpson *et al.* 1992, de Winter *et al.* 1993, 1994). Using these dimeric immunoassays, we have established that both Sertoli cells and peritubular cells from immature rat testes produce physiologically
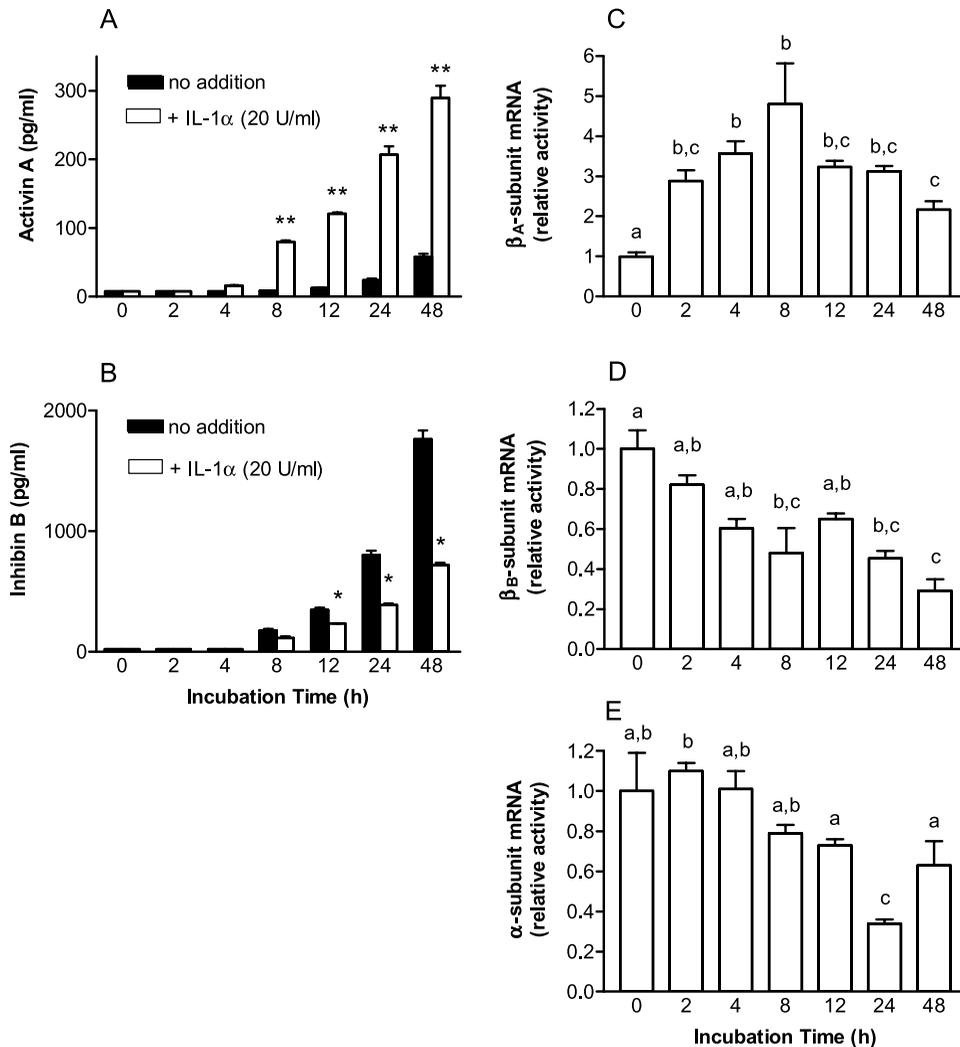
Figure 3 Time course (0–48 h) of secretion of (A) activin A and (B) inhibin B, and expression of mRNA for β_A-subunit (C), β_B-subunit (D) and α-subunit (E) in cultured immature rat Sertoli cells, in the presence of 20 U/ml IL-1α (open histograms) or in the absence of any addition (solid histograms). All values are mean ± S.E.M.; $n=4$ wells. Statistical comparisons (panels A and B) are between basal and IL-1-stimulated secretion levels: *$P<0.05$; **$P<0.01$ (all other comparisons were not significant at $P<0.05$). Values with same superscript letter (panels C–E) are not significantly different at $P<0.05$.

significant amounts of dimeric activin A *in vitro*, and that IL-1, but not IL-6, is a specific stimulator of activin A synthesis by these cells. Studies in a number of other tissues have reported a stimulatory effect of IL-1 on activin production, which appears to involve IL-1-responsive promoter elements in the β_A-subunit regulatory region (Tanimoto *et al.* 1996, Ardekani *et al.* 1998). In opposition to the action of IL-1, FSH inhibits basal activin A production by the Sertoli cell, and both FSH and cAMP inhibit IL-1-stimulated activin A secretion. These observations, together with the fact that IL-1 also inhibits both basal and FSH-stimulated inhibin B production, indicate for the first time the existence of a mechanism of reciprocal

formation of activin A and inhibin B in the Sertoli cell regulated by IL-1 and FSH.

For measurement of the proteins in culture medium containing bicarbonate buffer, the assays were optimised and validated. A similar problem had been reported previously for the inhibin B ELISA (Clifton *et al.* 2002). Failure to account for pH variations in samples can lead to significant over- or underestimation of these molecules. According to the data from the modified ELISA, the amount of activin A produced after IL-1 stimulation is physiologically significant (i.e. in the high pg range). Consistent with other studies (Illingworth *et al.* 1996, Depuydt *et al.* 1999), secretion of inhibin A by Sertoli cells
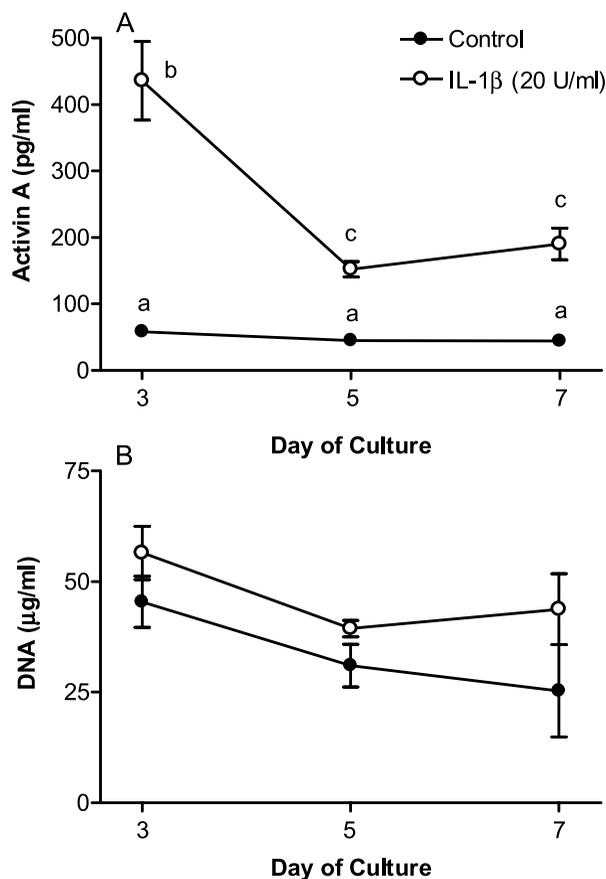
Figure 4 Time course (3–7 days) of activin A secretion (A) and DNA content (B) in cultures of immature rat Sertoli cells in the absence (●) or presence (○) of IL-1β (20 U/ml). All values are mean ± S.E.M.; $n=4$ wells. Values with same superscript letter are not significantly different at $P<0.05$. DNA content values are not significantly different from each other at any time point.
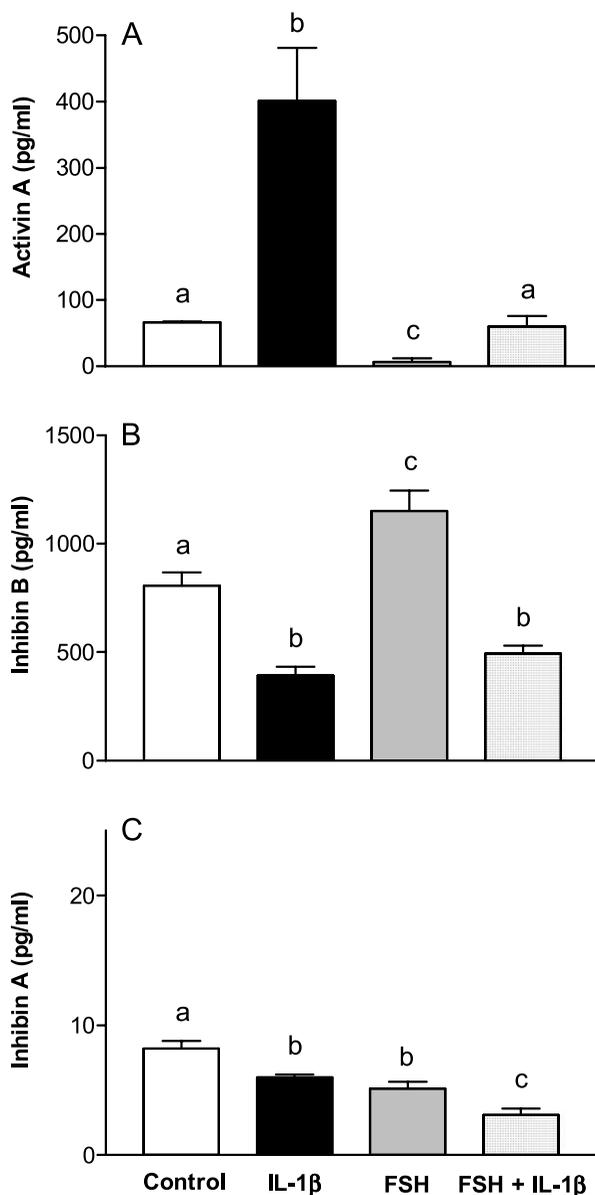

Figure 5 Secretion of activin A (A), inhibin B (B), and inhibin A (C) by immature rat Sertoli cells in the absence of cytokines (control), and in the presence of IL-1β (20 U/ml), of FSH (500 ng/ml) or a combination of IL-1β and FSH, collected at day 3 of culture (incubation time: 48 h). All values are mean ± S.E.M.; $n=4$ wells. Values with same superscript letter are not significantly different at $P<0.05$.

was exceedingly low in comparison with either inhibin B or activin A, although small but significant changes in levels were detectable. Although it also was confirmed that peritubular cells produce activin A (de Winter *et al*. 1993, 1994) at levels comparable to those produced by Sertoli cells on a per cell basis, peritubular cells were only a minor cellular component of the cultures studied. *In vivo*, however, peritubular cells probably make a highly significant contribution to local activin A levels, as the number of these cells is approximately 25% of the total number of Sertoli cells (Ariyaratne & Mendis–Handagama 2000). It is interesting that these cells were also stimulated by IL-1, and their role in the interaction between IL-1 and activin A warrants further exploration. Several culture variables also known to influence Sertoli cell responsiveness and/or inhibin/activin subunit expression were assessed: attachment to laminin (Dym *et al*. 1991), the presence of residual germ cells (Pineau *et al*. 1990, Clifton *et al*. 2002) and

incubation temperature (Gonzales *et al*. 1989). Only incubation temperature had any effect on activin A production *in vitro*.

The differential regulation of inhibin and activin within the testis through independent regulation of the subunits, which then associate intracellularly to form the relevant dimers, remains poorly understood. Early data suggested
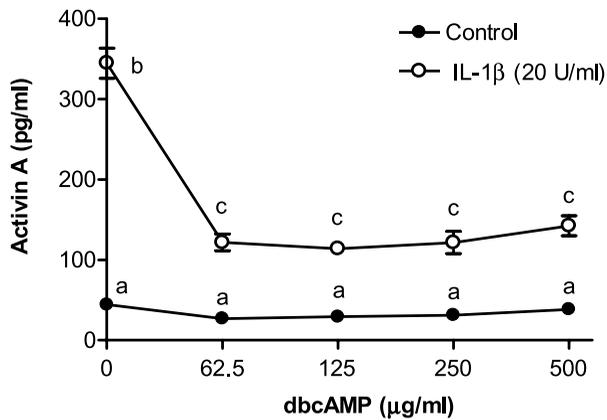
Figure 6 Dibutryl cAMP log dose–response curve for secretion of activin A by immature rat Sertoli cells, in the absence (●-●) or presence (○-○) of IL-1β (20 U/ml), collected on day 3 of culture (incubation time: 48 h). All values are mean ± S.E.M.; *n*=4 wells. Values with same superscript letter are not significantly different at *P*<0·05.

that regulation of inhibin production by FSH in the Sertoli cell involved stimulation of the α-subunit via a cAMP-mediated pathway, with little or no apparent effect on either β-subunit (Toebosch *et al.* 1988, Klaij *et al.* 1992). More recent studies employing assays for the inhibin B dimer have led to a reassessment of this concept (Depuydt *et al.* 1999), with recognition that β_B mRNA expression in the Sertoli cell may be stimulated by cAMP analogues (Najmabadi *et al.* 1993, Depuydt *et al.* 1999). The data in the present study clearly indicate that FSH inhibits formation of both of the β_A-subunit-containing dimers (activin A and inhibin A) in the Sertoli cell. This is quite different from the situation in the ovary, where FSH and

Figure 7 Comparison of activin A secretion, normalised against DNA content by Sertoli cells (A) or peritubular cells (B) separated by Percoll density gradient, in the absence (control) or presence of IL-1β (20 U/ml), collected on day 3 of culture (incubation time: 48 h). All values are mean ± S.E.M.; *n*=4 wells. Values are significantly different from control at *P*<0·05 or **P*<0·01.

cAMP stimulate inhibin A formation (Tuuri *et al.* 1996, Drummond *et al.* 2000). It is equally interesting that, even though IL-1 stimulates β_A-subunit expression by the Sertoli cell, this does not result in an increase in inhibin A. It appears that most of the available β_A-subunit produced in the Sertoli cell is preferentially directed to form the activin A homodimer. On the other hand, IL-1 inhibited secretion of the inhibin B dimer, primarily by inhibition of synthesis of the β_B-subunit. This was quite different from the stimulatory effect of IL-1 on β_B-subunit expression in the pituitary (Bilezikjian *et al.* 1998), although it should be noted that βA, and therefore activin A, is not normally expressed in the pituitary (Meunier *et al.* 1988, Schneider *et al.* 2000). Overall, it appears that the regulation of the activin/inhibin subunits and formation of the mature dimeric proteins in the Sertoli cell may be specific to this cell type.

In an earlier study, Khan and Nieschlag (1991) demonstrated that IL-1β can block FSH-stimulated, but not basal or dbcAMP-stimulated, aromatase activity in immature rat Sertoli cells, suggesting a site of action prior to the formation of cAMP. In the present study, IL-1 stimulated activin A production even in the presence of a saturating dose of dibutyryl cAMP, suggesting a site of action after cAMP formation. These data suggest that IL-1 can act both before and after cAMP formation in the Sertoli cell. A similar situation has been observed in rat Leydig cells, where IL-1 has been shown to have an inhibitory effect upon both LH/ human chorionic gonadotrophin-induced cAMP production and cAMP-stimulated testosterone production (Moore & Moger 1991). A variable in the present studies was the wide variation in basal activin A production by the cultured Sertoli cells. The reasons for this variability are not clear, but endogenous IL-1α production by the Sertoli cells is one likely explanation. Consequently, it was curious that FSH itself was able to inhibit basal activin A secretion, whereas dbcAMP had no effect, suggesting that the inhibitory effect of FSH on activin A may involve both cAMP-dependent and cAMP-independent pathways.

Regardless of the pathways involved, there is no doubt that FSH and IL-1 act through separate signalling pathways in the Sertoli cell. The fact that the IL-1 signalling pathway in the Sertoli cell does not involve the cAMP pathway through which FSH acts is entirely consistent with results in other tissues where cAMP is generally not involved in IL-1 signalling (Dinarello 1996). Recent reports of the involvement of the phospholipase A/ceramide pathway, a common regulatory pathway for IL-1 in other cells, in regulation of Sertoli cell function suggests that this pathway might be involved (Meroni *et al.* 1999). However, while they may act through separate pathways, the effects of IL-1 and FSH on the Sertoli cell are not uniformly antagonistic. Recent studies by Huleihel and colleagues (Huleihel & Lunenfeld 2002, Huleihel *et al.* 2002) have shown that the effects of IL-1 and FSH on
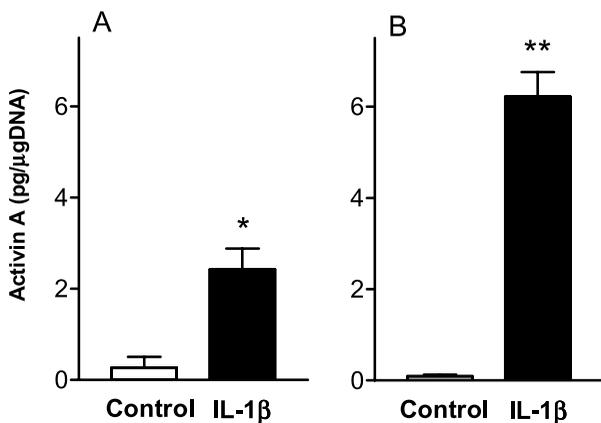
transferrin secretion by immature mouse Sertoli cells are additive.

The data in the present study further implicate activin A as a locally regulated cytokine within the testis, driven by IL-1 and suppressed by FSH. As a result, one potential role for activin A could be as a negative feedback inhibitor of IL-1 and IL-6 production and action within both the seminiferous tubules and interstitial tissue. Although there is no doubt that spermatogenesis can persist in mice deficient in IL-1, activin A, or even FSH production and/or signalling, albeit at a reduced level in some cases (Cohen & Pollard 1998, Chang *et al.* 2001, Sairam & Krishnamurthy 2001), the presence of these hormones/cytokines and the intersection of their signalling pathways in this tissue must have physiological consequences. In this regard, it is interesting to note that there is cyclical regulation of both IL-1α and β$_A$-subunit mRNA across the cycle of seminiferous epithelium (Söder *et al.* 1991, Kaipia *et al.* 1992). These observations suggest that IL-1α and FSH may be involved in the regulation of activin A during the seminiferous cycle, with activin A potentially acting as a cyclical negative feedback regulator of local IL-1 and IL-6 action within the seminiferous epithelium. Recent data have confirmed that inhibin B is also regulated by specific germ-cell stages (Clifton *et al.* 2002), and it is reasonable to suggest that local production of IL-1α may be involved in cyclical regulation of inhibin B as well. Finally, considerable evidence indicates that both inflammatory and immune responses in the testis are inhibited (Hedger 1997). The local production of activin A in response to the upregulation of IL-1 and IL-6 during a local inflammatory episode in the testis may be an important restraint upon the inflammatory response in the testis.

Acknowledgements

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