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Demonstration of a suppressive effect of inhibin α-subunit on the developmental competence of *in vitro* matured bovine oocytes

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Changes in intrafollicular concentrations of different forms of inhibin (free α-subunits and αβ dimers) occur during follicle development and may influence the oocyte maturation process. The aim of this study was to investigate the effects of inhibin A and free α-subunit (pro-αC) isolated from bovine follicular fluid on maturation of bovine cumulus–oocyte complexes, as reflected by their competence for embryo development after *in vitro* fertilization. Bovine cumulus–oocyte complexes were isolated from ovaries obtained from an abattoir and were cultured for 22–24 h at 38.5°C in TCM-199 medium supplemented with 10% oestrous cow serum, pregnant mares' serum gonadotrophin (2.5 iu ml^{-1}) and either inhibin A (0, 0.2 and 1.0 μg ml^{-1}) or pro-αC (0, 2 and 10 μg ml^{-1}). Neither inhibin A nor free α-subunit affected the cleavage rate of cumulus–oocyte complexes after fertilization (approximately 60%). Inhibin A reduced the proportion of cleaved oocytes reaching the eight-cell stage by 19% ($P < 0.05$), but did not affect the yield of blastocysts. However, pro-αC decreased the proportion of cleaved oocytes that reached the eight-cell (25%; $P < 0.05$) and blastocyst (28%; $P < 0.05$) stages. In addition, a negative correlation ($r = -0.55$, $P < 0.001$) was found between concentrations of total immunoreactive (ir) α-inhibin (measured by radioimmunoassay) produced by untreated control cumulus–oocyte complexes and their post-cleavage development to the blastocyst stage. In a second experiment, mouse monoclonal antibodies (20 μg ml^{-1}) against two different regions of the inhibin α-subunit precursor (pro-region and αC fragment) were tested for their ability to neutralize endogenous inhibin α-subunit-related molecules produced by cumulus cells; control cumulus–oocyte complexes were treated with normal mouse IgG (20 μg ml^{-1}). Although the cleavage rate was not affected, the yield of blastocysts was significantly higher in the presence of mouse monoclonal antibodies to both pro-α (77% increase; $P < 0.05$) and αC (48% increase; $P < 0.05$). None of the treatments tested affected endogenous production of activin-A or follistatin by cumulus–oocyte complexes. Overall, these results indicate that the inhibin α-subunit (pro-αC) has an inhibitory role in oocyte maturation which is independent of the modulatory effects of activin and follistatin.

Introduction

It is now well recognized that gonadotrophins are only part of a complex series of factors that regulate the acquisition of developmental competence during oocyte maturation (Gordon, 1994; Bevers *et al.*, 1997). Within the follicle, inhibins and related peptides (free α-subunit, activins and follistatin) are produced mainly by granulosa cells and are present in large amounts in follicular fluid (Ying, 1988; Knight, 1996). In addition to systemic and local autocrine and paracrine roles in regulating follicular growth, differentiation and atresia (Hillier, 1991; Findlay, 1993; Braw-Tal, 1994; Li *et al.*, 1995; Woodruff and Mather, 1995; Knight, 1996), there is increasing evidence that these peptides also regulate oocyte maturation.

In vitro studies with bovine oocytes revealed that addition of activin A to oocyte maturation medium does not effect nuclear maturation, but increases the development of cumulus-enclosed and denuded oocytes after cleavage (Stock *et al.*, 1997; Silva and Knight, 1998). Moreover, follistatin, an activin binding protein, neutralized this effect of activin A and reduced the developmental competence of *in vitro* matured oocytes (Silva and Knight, 1996, 1998). However, the potential effects of inhibin on oocyte maturation remain controversial. In rat oocytes, inhibin A inhibits nuclear maturation (O *et al.*, 1989), whereas in primates, combined treatment with both activin A and

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inhibin A accelerates nuclear maturation and increases fertilization rate (Alak *et al.*, 1996). In contrast, Stock *et al.* (1997) reported that inhibin A had no effect on cleavage rate but enhanced post-cleavage development of bovine oocytes matured in the absence of gonadotrophins.

Inhibins are disulfide-linked heterodimer proteins, consisting of an α chain and one of two alternate β chains (β_A and β_B), whereas activins are dimers consisting of two β chains ($\beta_A\beta_A$, $\beta_A\beta_B$ or $\beta_B\beta_B$). Granulosa cells synthesize an excess of α subunit over β subunit and consequently secrete substantial amounts of free α-subunit in addition to dimeric inhibins and activins (reviewed by Burger *et al.*, 1995; Knight, 1996). Several forms of α-subunit have been identified in bovine follicular fluid which are generated by proteolytic processing of a full-length α-subunit precursor (Knight *et al.*, 1989; Robertson *et al.*, 1989; Sugino *et al.*, 1989). Similarly, various forms of dimeric inhibin have been detected in bovine follicular fluid, ranging from the fully processed form (32 kDa) to the largest unprocessed forms (110–120 kDa) (Robertson *et al.*, 1985; Miyamato *et al.*, 1986; Sugino *et al.*, 1992; Good *et al.*, 1995). All dimeric forms of inhibin appear able to suppress pituitary FSH secretion (Sugino *et al.*, 1992; Good *et al.*, 1995). In contrast, free α-subunit forms do not possess inhibin-like bioactivity (Knight *et al.*, 1989; Robertson *et al.*, 1989), although there is evidence that the full-length α-subunit precursor (proαNαC) can inhibit the binding of FSH to its receptor (Schneyer *et al.*, 1991). Several studies have reported that intrafollicular concentrations of different forms of inhibin (free α-subunits and dimeric inhibins) vary during follicular growth and atresia (Guilbault *et al.*, 1993; Ireland *et al.*, 1994; Sunderland *et al.*, 1996; Guthrie *et al.*, 1997). These findings indicate that inhibins and free α-subunits have different paracrine roles in the regulation of follicle and oocyte maturation, possibly in combination with the effects of activin and follistatin (Stock *et al.*, 1997; Silva and Knight, 1998).

The objective of the present study was to investigate the effects of purified bovine inhibin A and free α-subunit (pro-αC) on maturation of bovine cumulus–oocyte complexes, as reflected by their developmental competence after fertilization. In addition, the effects of monoclonal antibodies (mAb) raised against different sequences of the α-subunit precursor (pro region and αC region) on oocyte developmental competence were assessed. Endogenous concentrations of total immunoreactive (ir) α-inhibin, inhibin A, activin A and follistatin produced by groups of cumulus–oocyte complexes exposed to the different maturation treatments were also measured and the results were considered in relation to oocyte developmental competence.

Materials and Methods

Isolation of inhibin A and pro-αC from bovine follicular fluid

Pooled bovine follicular fluid was centrifuged (2000 *g*, 15 min, 4°C) and filtered through glass wool to remove cellular debris. Immunoaffinity extraction was performed by mixing 400 ml bovine follicular fluid with 10 mg anti-α monoclonal antibody (clone R1; Groome *et al.*, 1990) covalently linked to HiTrap beads (Pharmacia Biotech, Milton Keynes) and equilibrated with 10 ml wash buffer (PBS, 1% (w/v) BSA, 0.1% (w/v) NaN_3, pH 7.4). After mixing by inversion for 36 h, the beads were recovered from the bovine follicular fluid by centrifugation (1000 *g*, 5 min, room temperature) and washed with buffer until the absorbance of the flow-through was < 0.04 at 280 nm. Inhibins were then eluted from the beads with 6 mol guanidine–HCl l^{-1} containing 0.1 mol Tris–HCl buffer l^{-1} (pH 7). The eluate (approximately 5 ml containing 3–5 mg protein) was applied to a reverse-phase HPLC column (TSK-ODS-120T, 7.8 mm × 300 mm; Anachem, Luton) using an LKB-Pharmacia HPLC pump (model 2249). The column was eluted for 30 min with a linear gradient of 10–80% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 3 ml min^{-1} at room temperature. Column eluant was monitored at 280 nm and 3 ml fractions were collected and stored at –80°C. Fractions were assayed for total ir-α-inhibin (measured by radioimmunoassay) and dimeric inhibin (measured by immunoradiometric assay (IRMA)) and total protein content estimated by absorbance at 280 nm. Two peaks eluting with the same retention times as 26 kDa pro-αC (Knight *et al.*, 1989) and 32 kDa inhibin A (Knight *et al.*, 1990) were recovered and their identities and purity were confirmed by SDS-PAGE and immunoblotting using mAb against pro-α (clone INPRO; Groome *et al.*, 1995), αC (clone 173/29; Knight *et al.*, 1998) and β_A (clone E4; Groome and Lawrence, 1991) inhibin subunits as described by Tannetta *et al.* (1998). The inhibin extraction and purification was performed twice using different batches of the same bovine follicular fluid pool. Fractions containing the pro-αC (four fractions) and inhibin A (three fractions) were pooled separately, diluted in three volumes of buffer (PBS, 1% (w/v) BSA, pH 7.4), divided into aliquots and freeze dried for storage at –70°C. Aliquots of the purified pro-αC and inhibin A used in the cumulus–oocyte complex maturation experiments were subsequently desalted using a PD10 column (Sephadex G25M, Pharmacia Biotech, Milton Keynes) primed and eluted with distilled water. The aliquots were then freeze dried, dissolved in maturation medium and sterilized by filtration (0.22 μm pore size) before use. Maturation medium consisted of TCM-199 medium supplemented with 10% (v/v) oestrous cow serum, 0.4 mmol L-glutamine l^{-1}, 0.2 mmol pyruvate l^{-1}, pregnant mares' serum gonadotrophin (Folligon, 2.5 iu ml^{-1}; Intervet Ltd, Cambridge), 50 iu penicillin ml^{-1} and 50 μg streptomycin ml^{-1}.

In vitro *maturation*, in vitro *fertilization and embryo culture*

The methods used for *in vitro* maturation, *in vitro* fertilization (IVF) and embryo culture (EC) were those described by Silva and Knight (1998).

In vitro *maturation.* Bovine ovaries were collected from an abattoir and transported to the laboratory in a sterile saline solution supplemented with 1% (v/v) antibiotic and antimycotic solution (Sigma, Poole). Cumulus–oocyte complexes were obtained by aspiration of 2–10 mm follicles. Cumulus–oocyte complexes were washed twice in wash medium (TCM-199 medium with Hepes buffer

supplemented with 10% heat-inactivated calf serum, 50 iu ml^{-1} penicillin ml^{-1} and 50 μg streptomycin ml^{-1}) and once in maturation medium. Groups of 20 oocytes were randomly allocated into different treatment groups and cultured in 60 μl droplets of maturation medium under oil for 22–24 h, at 38.5°C in a humidified atmosphere of 5% CO_2.

In vitro *fertilization.* After maturation, oocytes were washed in fertilization medium (IVF-TALP; Silva and Knight, 1998) and transferred, in groups of approximately ten oocytes, to 20 μl fertilization drops of IVF-TALP under oil. For capacitation of the spermatozoa, frozen–thawed semen was washed four times by centrifugation (800 *g*, 5 min) in sperm TALP medium (Silva and Knight, 1998) and once in IVF-TALP medium. The sperm pellet was then suspended in IVF-TALP medium at a concentration of 2×10^6 spermatozoa ml^{-1} and then added to the IVF drops (final concentration: 1×10^6 spermatozoa ml^{-1}). Semen from the same ejaculate from one bull was used in all experiments. The oocytes and spermatozoa were incubated at 38.5°C in a humidified atmosphere of 5% CO_2 for 22–24 h.

Embryo culture. The presumptive embryos were washed in embryo culture medium (TCM-199 supplemented with 10% heat-inactivated calf serum, 0.4 mmol L-glutamine l^{-1}, 0.2 mmol pyruvate l^{-1}, 10 mmol lactate l^{-1}, 50 iu penicillin ml^{-1} and 50 μg streptomycin ml^{-1}) and placed in co-culture drops (approximately 20 embryos per drop) with granulosa cell monolayers. Granulosa cells were prepared according to Silva and Knight (1998) and cultured for 8–11 days before the embryo co-culture. In all experiments, the same batch of cryopreserved granulosa cells was used. Three days after fertilization (fertilization = day 0), the embryos were assessed for development and the 4–16-cell embryos were transferred to new co-culture drops. The medium was replenished every 2 days (that is, 50% drop volume replaced with fresh medium). On day 10 of culture, the number of blastocysts and hatched blastocysts was evaluated. Cleavage was examined 3 days after fertilization, and represents the proportion of oocytes completing at least one mitotic division. Frequency of development to the eight-cell or blastocyst stage was calculated from the proportion of cleaved oocytes to adjust for differences in the frequency of fertilization.

Experiments

Depending on the number of good quality cumulus–oocyte complexes recovered from each batch of ovaries, between one (that is, 20 cumulus–oocyte complexes) and three (that is, 60 cumulus–oocyte complexes) culture drops were allocated per treatment on a given day. In each culture, equal numbers of drops were allocated to control and treatment groups. In each experiment, treatments were only present during the 22–24 h oocyte maturation period before fertilization.

Experiment 1: effects of inhibin A and free α-subunit

The concentration-dependent effects of inhibin A and free α-subunit on cumulus–oocyte complex maturation were studied. Stock solutions of purified bovine inhibin A and pro-αC were diluted in maturation medium to achieve final concentrations of 0.2 and 1.0 μg ml^{-1} for dimeric inhibin A and 2 and 10 μg ml^{-1} for pro-αC. These concentrations were selected on the basis that endogenous concentrations of inhibin A and total α-subunit immunoreactivity measured in 24 h cumulus–oocyte complex-conditioned maturation medium were approximately 0.2 and 2.0 μg ml^{-1}, respectively. However, it should be noted that average concentrations of inhibin A (approximately 12 μg ml^{-1}) and total α-subunit (approximately 100 μg ml^{-1}) in bovine follicular fluid (Knight *et al.*, 1996) are considerably higher than these values.

Experiment 2: effects of antibodies raised against pro-α and αC-subunit

Two different mAb were used to test whether antibodies against α-inhibin could effect cumulus–oocyte complex maturation. The pro-α mAb was prepared against the pro region of the full-length inhibin α-subunit precursor (clone INPRO; Groome *et al.*, 1995). The anti-αC mAb was prepared against amino acids 1–29 (amino terminus sequence) of the mature αC fragment of the α-subunit precursor (clone 173/29; Knight *et al.*, 1998). For controls, IgG was isolated from normal mouse serum using a protein G-agarose column (Pharmacia Biotech, Milton Keynes). Both mAb and control IgG preparations were desalted using PD10 columns (Sephadex G25M; Pharmacia Biotech, Milton Keynes) primed and eluted with PBS. Stock solutions were sterilized by filtration (0.22 μm filter; Nalgene, Rotherwas) and stored at 4°C. After appropriate dilution in maturation medium, 10 μl aliquots of mAb or IgG solutions were added to cumulus–oocyte complex maturation medium drops (final volume 60 μl) to give a final concentration of 20 μg ml^{-1}.

Hormone determinations

Endogenous production of activin A, follistatin, inhibin A and total ir-α-inhibin during cumulus–oocyte complex maturation was measured to correlate with oocyte developmental potential and also to determine whether exogenous inhibin A, free α-subunit or mAb affected secretion of endogenous hormones. After cumulus–oocyte complex maturation, 40 μl conditioned medium was collected from drops and frozen for hormone assay.

Activin A. Total activin A (free activin and follistatin-bound activin) concentrations in cumulus–oocyte complex-conditioned medium were determined using a two-site enzyme immunoassay (ELISA) as described by Knight *et al.* (1996). Human recombinant (hr) activin A (Genentech Inc., San Francisco, CA) was used as the standard and gave a dilution curve parallel to those for cumulus–oocyte complex-conditioned medium, bovine granulosa cell-conditioned culture medium and bovine follicular fluid. The sensitivity was approximately 100 pg ml^{-1} and mean intra- and interassay coefficients of variation (CV) calculated from five assays were 6.1 and 11%, respectively.

Follistatin. Total follistatin was determined using the ELISA described by Tannetta *et al.* (1998). Pooled bovine follicular fluid was used as the working standard, since hr-follistatin gave a dilution curve that was steeper than those for bovine follicular fluid and cumulus–oocyte complex-conditioned medium. One millilitre bovine follicular fluid working standard was found to be equivalent to 3.93 μg hr-follistatin (Tannetta *et al.*, 1998). Consequently, follistatin concentrations are expressed in terms of the widely available hr-follistatin preparation provided by the National Institute of Diabetes and Digestive Kidney Diseases (Bethesda, MD). The sensitivity was equivalent to approximately 100 pg ml^{-1}. Mean intra- and interassay CV calculated from five assays were 7.3 and 9.6%, respectively.

Inhibin A. Inhibin A concentrations were determined using the two-site IRMA described by Knight and Muttukrishna (1994). Purified bovine 32 kDa inhibin A was used as the standard and gave a dilution curve that was parallel to those for cumulus–oocyte complex-conditioned medium and bovine follicular fluid. The detection limit was 250 pg ml^{-1} and mean intra- and interassay CV calculated from four assays were 5.3 and 5.4%, respectively.

Total ir-α-inhibin. Concentrations of total ir-α-inhibin were measured using the competitive radioimmunoassay reported by Knight *et al.* (1989) and Beard *et al.* (1990). Purified bovine 32 kDa inhibin A was used as the standard and the sensitivity of the assay was approximately 1 ng ml^{-1}. The standard gave a dilution curve that was parallel to those for cumulus–oocyte complex-conditioned medium, bovine follicular fluid and bovine follicular fluid with two concentrations of pro-α antibody (18 and 24 μg ml^{-1}). Mean intra- and interassay CV calculated from four assays were 8.3 and 14%, respectively.

Statistical analysis

The proportion of oocytes or cleaved oocytes reaching defined developmental stages was transformed using the Freeman and Tukey transformation (Zar, 1996) before statistical analysis, but the results are presented as percentages (± SEM) for clarity. The effects of each hormone treatment (different concentrations of inhibin, pro-αC and antibodies) were compared and analysed by one-way ANOVA. When a significant F ratio was obtained, the *post hoc* protected least significant difference test was used to compare individual means. A P value < 0.05 was considered to be significant. Linear regression analysis was used to examine the relationship between endogenous production of inhibin A and total α-inhibin by each group of 20 cumulus–oocyte complexes, and the proportion of cleaved oocytes that developed to blastocysts.

Results

Confirmation of identities of pro-αC and inhibin A containing fractions isolated from bovine follicular fluid

SDS-PAGE and immunoblotting (data not shown) confirmed that the major protein present in the first purified fraction of bovine follicular fluid was pro-αC. Under non-reducing conditions the major protein band (27 kDa) was detected by both αC and pro-αC mAb. Under reducing conditions, the major protein ran as a 22 kDa band that was only detected by the αC mAb (that is, the αC fragment). Weak bands corresponding to extended α-subunit forms (44–46 kDa and 48–50 kDa) and inhibin A (32 kDa) were also detected under non-reducing conditions. The presence of inhibin A dimer in this free α-subunit preparation was confirmed by IRMA, which indicated approximately 5% contamination of total α forms with inhibin A. SDS-PAGE and immunoblotting of the second purified fraction revealed, under non-reducing conditions, a major band of 32 kDa detected by the β_A mAb, corresponding to inhibin A. Surprisingly, under the conditions used, the αC mAb did not recognize this non-reduced form, although after reduction, a major band of 22 kDa was detected, corresponding to the free αC subunit of inhibin A. Lightly stained bands of approximately 48 kDa, and 66 kDa and > 100 kDa, corresponding to inhibin A precursors, and 27 kDa (pro-αC) were also observed under non-reducing conditions. Therefore, the identities of the two purified bovine follicular fluid fractions were confirmed as highly enriched preparations of pro-αC (27 kDa) and inhibin A (32 kDa), respectively.

Experiment 1: effects of purified bovine inhibin A and pro-αC on oocyte developmental competence

Oocyte cleavage rate (54–60%) was not affected significantly by the addition of inhibin A or pro-αC to the maturation medium (Table 1). Inhibin A did not affect the total number of ≥ eight-cell embryos or blastocysts obtained. However, the higher concentration of inhibin A (1 μg ml^{-1}) resulted in a 19% ($P < 0.05$) decrease in the proportion of cleaved oocytes that reached at least the eight-cell stage by day 5. This reduction was not observed in the development to blastocysts after cleavage. Indeed, the proportion of eight-cell embryos that reached the blastocyst stage increased by 52% ($P < 0.05$) when 1 μg inhibin A ml^{-1} was added to oocyte maturation medium (Table 1). In contrast, the highest concentration of pro-αC (10 μg ml^{-1}) decreased by approximately 30% ($P < 0.05$) both the total yield of blastocysts and the proportion of cleaved oocytes that developed to blastocysts (Table 1). Both concentrations of pro-αC also reduced the proportion of cleaved oocytes that reached at least the eight-cell stage. Hatching rates (hatched blastocysts per blastocyst) were not affected by the different treatments, and ranged between 45 and 55%.

Addition of inhibin A or pro-αC to cumulus–oocyte complex maturation medium did not have a significant effect on endogenous concentrations of either activin A or follistatin produced by cumulus–oocyte complexes during the 22–24 h culture period (results not shown).

A negative correlation ($r = -0.55$, $P < 0.001$) was found between endogenous total ir-α-inhibin (measured by radioimmunoassay) produced by groups of control (untreated) cumulus–oocyte complexes during the 22–24 h

Table 1. Effect of the presence of inhibin A and pro-αC during *in vitro* maturation of bovine cumulus–oocyte complexes on embryonic development

Culture treatment	Number of oocytes cultured*	Percentage of oocytes that developed to			Percentage of cleaved oocytes that developed to		Percentage of hatched blastocysts
		two-cell	eight-cell	blastocyst	eight-cell	blastocyst	
Control	395 (20)	56 ± 3	37 ± 3[a]	10 ± 1[ab]	68 ± 4[a]	18 ± 2[a]	45
Inhibin (0.2 μg ml^{-1})	392 (20)	55 ± 3	33 ± 4[ab]	11 ± 2[ab]	59 ± 4[ab]	19 ± 2[a]	54
Inhibin (1.0 μg ml^{-1})	391 (20)	59 ± 3	33 ± 3[ab]	13 ± 2[a]	55 ± 3[b]	22 ± 3[a]	45
Pro-αC (2.0 μg ml^{-1})	398 (20)	60 ± 3	33 ± 2[ab]	10 ± 2[ab]	56 ± 3[b]	16 ± 2[ab]	54
Pro-αC (10.0 μg ml^{-1})	392 (20)	54 ± 3	28 ± 3[b]	7 ± 2[b]	51 ± 4[b]	13 ± 3[b]	55

Values are means ± SEM.
*Number of replicate culture drops shown in parentheses.
[ab]Different superscripts within columns indicate significant differences among treatment groups ($P < 0.05$).

Table 2. Effect of the presence of monoclonal antibodies (mAb) against αC and pro-α during *in vitro* maturation of bovine cumulus–oocyte complexes on embryonic development

Culture treatment	Number of oocytes cultured*	Percentage of oocytes that developed to			Percentage of cleaved oocytes that developed to		Percentage of hatched blastocysts
		two-cell	eight-cell	blastocyst	eight-cell	blastocyst	
Mouse IgG (20 μg ml^{-1})	237 (12)	62 ± 4	39 ± 4	14 ± 2[a]	62 ± 4	22 ± 2[a]	66
αC mAb (20 μg ml^{-1})	237 (12)	63 ± 3	38 ± 5	20 ± 3[ab]	59 ± 6	31 ± 3[b]	56
Pro-α mAb (20 μg ml^{-1})	235 (12)	64 ± 3	39 ± 4	24 ± 2[b]	62 ± 6	38 ± 4[b]	72

Values are means ± SEM.
*Number of replicate culture drops shown in parentheses.
[ab]Different superscripts within columns indicate significant differences among treatment groups ($P < 0.05$).

maturation period and the proportion of cleaved oocytes that subsequently developed to blastocysts (Fig. 1). In contrast, no significant correlation was found between endogenous inhibin A (measured by IRMA) produced by cumulus–oocyte complexes and their subsequent developmental capacity (Fig. 1).

Experiment 2: effects of antibodies against pro-α and αC regions of the inhibin α-subunit precursor on oocyte developmental competence

The addition of mAb against either the pro-α or αC region to cumulus–oocyte complex maturation medium did not affect the rate of oocyte cleavage (62–66%) or embryo development to the eight-cell stage (Table 2). However, the presence of mAb against αC increased the proportion of cleaved oocytes that developed to blastocysts by 48% ($P < 0.05$). A more substantial increase (70–77%, $P < 0.001$) of both the total number of blastocysts and the proportion of cleaved oocytes that developed to blastocysts was achieved by the presence of mAb against pro-α (Table 2).

The addition of αC mAb to cumulus–oocyte complex maturation medium did not affect endogenous concentrations of activin A or follistatin (results not shown). For technical reasons, endogenous inhibin A and ir-α-inhibin could not be measured in the presence of this antibody, although confirmation was obtained that these assays were not affected by the presence of the pro-α monoclonal antibody. Addition of pro-α monoclonal antibody promoted a 55% increase ($P < 0.05$) in the amount of total ir-α-inhibin measured in cumulus–oocyte complex-conditioned medium, but did not affect endogenous production of inhibin A, activin A or follistatin (Fig. 2).

Discussion

The present study demonstrates for the first time a biological effect of free inhibin α-subunit (pro-αC form) on oocyte maturation. Addition of exogenous pro-αC during cumulus–oocyte complex maturation significantly decreased the developmental potential of oocytes, as expressed by the proportion of either total oocytes or cleaved oocytes that reached the eight-cell and blastocyst stages. This effect is the reverse of the stimulatory action of activin A on oocyte maturation reported previously (Silva and Knight, 1996, 1998; Stock *et al.*, 1997). Since follistatin also reduces oocyte maturation by blocking the effects of activin (Silva and Knight, 1998), the possibility was considered that the inhibitory action of pro-αC on cumulus–oocyte complexes was a consequence of a change in the relative amounts of endogenous activin A or follistatin. However, the concentrations of activin A and follistatin and the ratio of

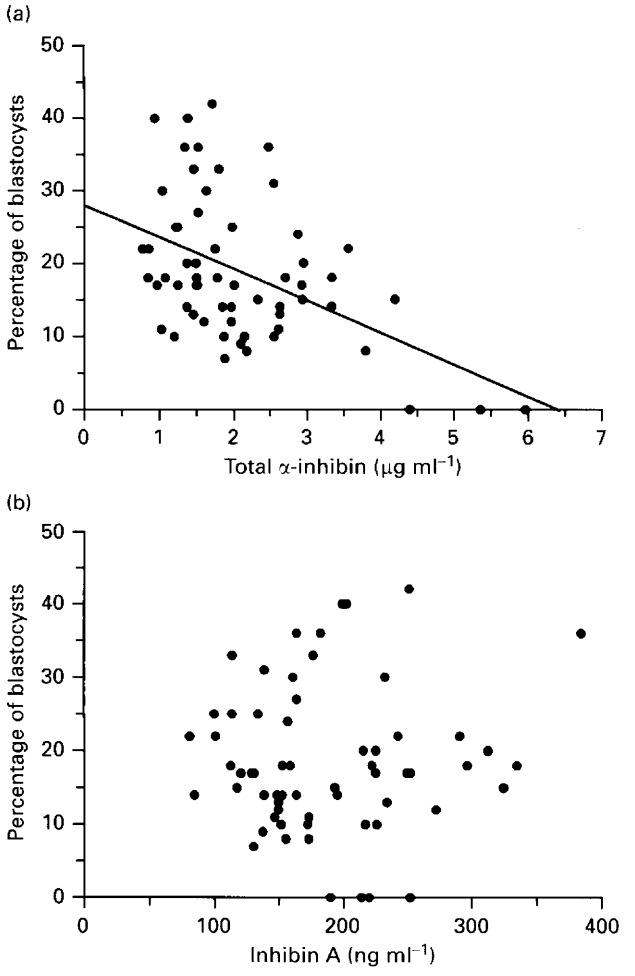

Fig. 1. Correlation between endogenous (a) total ir-α-inhibin and (b) inhibin A produced during maturation of bovine cumulus–oocyte complexes and their subsequent developmental capacity (percentage of cleaved oocytes developing to the blastocyst stage). (a) $r = -0.551$, $P < 0.001$, $n = 63$; (b) $r = 0.082$, $P = 0.53$, $n = 63$.

activin A:follistatin were not changed by the addition of pro-αC. This indicates that pro-αC modulates cumulus–oocyte complex maturation independently of activin and follistatin. Whether pro-αC acts directly on the oocyte or whether its effect is mediated by cumulus cells was not resolved in this study. It is possible that the pro-αC form inhibits binding of FSH to receptors on cumulus cells, as has been suggested for the proαNαC form (Schneyer *et al.*, 1991), thereby reducing the stimulatory action of gonadotrophin on cumulus–oocyte complex maturation. However, it has been reported that ir-α-inhibin is detectable in ooplasm before maturation and that concentrations fall markedly after maturation (Izadyar *et al.*, 1998, Silva *et al.*, 1998). Given that α-inhibin mRNA is not expressed in bovine oocytes (Izadyar *et al.*, 1998), this observation raises the possibility that pro-αC produced by cumulus cells has direct effects on the oocyte to influence nuclear or cytoplasmic maturation.

Neither inhibin A nor pro-αC affected the cleavage rate of oocytes, indicating that there was no effect on nuclear maturation. Although O *et al.* (1989) reported that inhibin inhibited nuclear maturation, recent studies involving bovine oocytes revealed that inhibin A accelerates nuclear maturation without affecting the proportion of MII oocytes after 24 h (Stock *et al.*, 1997). Classically, inhibin has been considered to oppose the effects of activin (Woodruff and Mather 1995; Knight, 1996), either by binding to type II activin receptors and preventing activin-induced type I–type II receptor dimerization (Mathews and Vale, 1991) or by interacting with a distinct inhibin receptor on the same cell (Draper *et al.*, 1998). The present findings and those of Silva and Knight (1988) do not support reciprocal effects of inhibin A and activin A on oocyte maturation in terms of the extent of development to blastocysts after cleavage. Indeed, recent studies indicate that inhibin and activin may act synergistically to enhance oocyte maturation (Alak *et al.*, 1996; Stock *et al.*, 1997). In the present study, addition of inhibin A to cumulus–oocyte complex maturation medium reduced slightly the proportion of cleaved oocytes that reached the eight-cell stage without affecting the total number of blastocysts. However, the proportion of eight-cell embryos that reached the blastocyst stage was increased by the same treatment. As reported by Silva and Knight (1998), the addition of activin A increased the proportion of cleaved oocytes that reached the blastocyst stage. Given that the addition of inhibin A to cumulus–oocyte complex maturation medium did not affect endogenous production of either activin A or its binding protein (follistatin), the observed increase in the proportion of eight-cell embryos forming blastocysts may therefore reflect an activin-independent effect of inhibin A on oocyte maturation. Alternatively, inhibin A and activin A may differentially regulate the progression of nuclear and cytoplasmatic maturation of oocytes. Early development (until the eight-cell stage) relies on the storage of maternal mRNA and proteins within the oocyte, but in order to support further development embryos must activate their own genome (Frei *et al.*, 1989; Thompson, 1997). Therefore, induced differences in nuclear and cytoplasmic maturation may result in different patterns of development after cleavage.

The lack of a relationship between endogenous inhibin A produced by cumulus–oocyte complexes and post-cleavage development to blastocysts also brings into question the potential role of inhibin A in regulating oocyte maturation. Silva and Knight (1998) reported a positive correlation between endogenous activin A produced by cumulus–oocyte complexes and blastocyst yield. In contrast, a negative correlation was found between total endogenous α-inhibin concentration and the proportion of cleaved oocytes that developed to the blastocyst stage. This indicates that free inhibin α-subunits, rather than inhibin A, oppose the positive effect of activin A on oocyte maturation. Since α-subunits do not bind to activin receptors, their action cannot be explained by competition with activin for binding to its type II receptor, as is proposed for inhibin αβ dimers.

Additional support for a negative effect of free inhibin α-subunits on oocyte maturation was provided by immunoneutralization studies using mAb against either the mature αC region or the pro region of the inhibin α-subunit precursor. In the present study, the presence of either

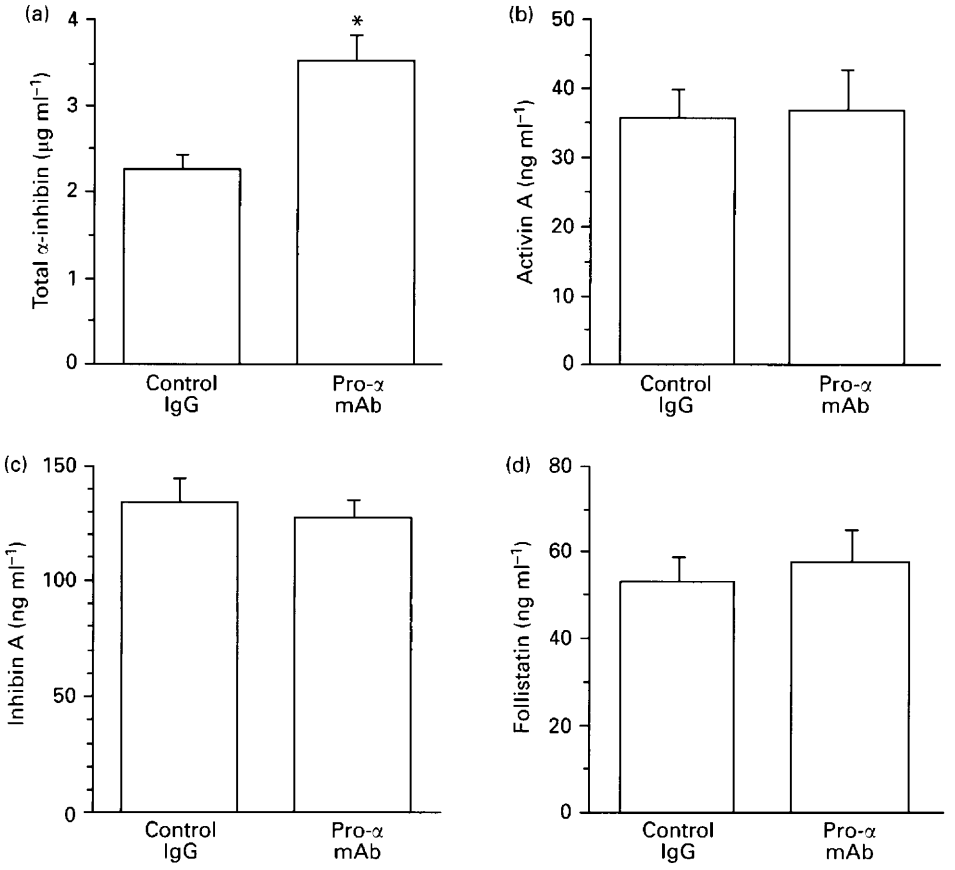

Fig. 2. Effect of the addition of a monoclonal antibody (mAb) against pro-α to maturation medium on endogenous concentrations of (a) total ir-α-inhibin, (b) activin A, (c) inhibin A and (d) follistatin produced by groups of 20 bovine cumulus–oocyte complexes. Values are means ± SEM (n = 12). *P < 0.05 versus control.

antibody during cumulus–oocyte complex maturation resulted in a significant increase in the developmental potential of oocytes, as expressed by the proportion of cleaved oocytes that reached the blastocyst stage. Given that endogenous activin A and follistatin concentrations and the ratio of activin A:follistatin did not change in the presence of either mAb, their stimulatory effect is presumably a direct consequence of the neutralization of one or more forms of endogenous α-inhibin secreted by cumulus cells during cumulus–oocyte complex maturation. The pro-α mAb was more effective than the αC mAb in enhancing oocyte maturation (70% increase in blastocyst yield). Furthermore, the main immunoreactive forms detected by western blotting of cumulus–oocyte complex-conditioned medium using the pro-α mAb were the 27 kDa (pro-αC) and 48–50 kDa (proαNαC) forms (data not shown). It is thus likely that both these forms were neutralized in the second experiment. Therefore, it is proposed that, together with pro-αC, endogenous proαNαC also participates in the modulation of oocyte maturation.

In summary, these *in vitro* observations support the hypothesis that pro-αC, and possibly other forms of free inhibin α-subunit (proαNαC) secreted in large amounts by cumulus cells, inhibit the developmental potential of cumulus–oocyte complexes. This indicates that free inhibin α-subunit, along with activin A and follistatin, plays an intrafollicular paracrine role in regulating bovine oocyte maturation *in vivo*. Further work is required to assess the potential regulatory effects of inhibin A in this regard.

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