Differential effects of activin A on basal and gonadotrophin-induced secretion of inhibin A and progesterone by granulosa cells from preovulatory (F1–F3) chicken follicles

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Previous work has shown that activin A is expressed selectively within the theca rather than the granulosa layer of preovulatory chicken follicles. In the present study, this finding was verified and the potential paracrine actions of activin A on basal and gonadotrophin-induced secretion of inhibin A, inhibin B and progesterone by granulosa cells from the three largest preovulatory follicles (F1–F3) were investigated. Treatment with activin A (0, 0.25, 2.5 and 25 ng ml⁻¹) alone increased inhibin A secretion markedly in a follicle- and time-dependent manner, with the greatest response (up to 15-fold increase; $P < 0.0001$) in F1 follicles after 3 days of treatment. In contrast, activin A alone had no effect on progesterone output at any time. Cells from F3 follicles were more responsive to FSH than were F1 cells in terms of both inhibin A ($P < 0.02$) and progesterone ($P < 0.01$) secretion. Furthermore, activin A greatly enhanced FSH-induced secretion of both inhibin A (up to tenfold; $P < 0.0001$) and progesterone (up to sixfold;

$P < 0.0001$). In terms of LH-induced inhibin A and progesterone secretion, cells from F1, F2 and F3 follicles showed similar responses. Co-treatment with activin A enhanced LH-induced secretion of inhibin A markedly (up to ninefold; $P < 0.0001$) but had only a marginal effect on LH-induced progesterone secretion (up to twofold; $P < 0.001$). The presence of activin receptor subtypes IA, IB, IIA and IIB in cultured granulosa cells from F1, F2 and F3 follicles was demonstrated using immunocytochemistry. These findings support the hypothesis that activin A secreted by the theca layers of avian preovulatory follicles exerts a local paracrine action on granulosa cells to modulate 'basal' inhibin A secretion and to upregulate gonadotrophin-induced secretion of both inhibin A and progesterone. However, the extent to which this local role of activin A contributes to the generation of the preovulatory LH–progesterone surge remains to be established.

Introduction

During preovulatory follicle development in the laying hen, dimeric inhibin A and progesterone are produced selectively by granulosa cells, and maximum production is by the largest follicle (F1) of the preovulatory hierarchy (Etches and Duke, 1984; Etches, 1990; Lovell *et al.*, 1998, 2000, 2001). In contrast, the theca layers of preovulatory hen follicles contain substantially more (> 20-fold) activin A than the corresponding granulosa layers (Lovell *et al.*, 1998, 2001), raising the possibility that theca-derived activin A functions as a local paracrine regulator of granulosa cell function, contributing to the ordered final differentiation of follicles constituting the preovulatory hierarchy in birds.

In mammalian ovarian follicles, in contrast to avian follicles, activins and inhibins are both produced almost exclusively by granulosa cells rather than theca cells (for reviews, see Woodruff and Mather, 1995; Knight and

Glister, 2001). Studies *in vitro* in rodents have shown that activin A can upregulate FSH receptor expression, promote cell proliferation and enhance FSH-dependent processes (for example, inhibin production and steroidogenesis) by granulosa cells (Miro *et al.*, 1991; Xiao *et al.*, 1992; Nakamura *et al.*, 1993; Li *et al.*, 1995). In addition, activin A suppresses basal or LH-induced androgen production by isolated rat (Hsueh *et al.*, 1987), human (Hillier *et al.*, 1991) and bovine (Wrathall and Knight, 1995) theca cells. Such evidence firmly supports the concept that granulosa-derived activin A functions as an intrafollicular modulator of both granulosa cell (autocrine and paracrine action) and theca cell (paracrine action) function in mammals. To the authors' knowledge, there is only one report documenting an effect of activin on the avian ovary, by Rombauts *et al.* (1996), who used mixed ovarian cell cultures derived from chicken embryos and observed an inhibitory effect of activin A on the conversion of pregnenolone to androgens.

The principal aim of the present study was to test the hypothesis that activin A synthesized and secreted by the theca cell layer of preovulatory chicken follicles exerts an intrafollicular paracrine effect on neighbouring granulosa

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cells to modulate their function. After verifying that activin A is produced selectively by theca rather than granulosa cells of preovulatory follicles, the approach taken in the present study was to compare the effects of exogenous activin A on basal and gonadotrophin-induced secretion of inhibin A, inhibin B and progesterone by cultured granulosa cells isolated from the three largest follicles (F1, F2, F3) of the well-characterized preovulatory hierarchy. In addition, immunocytochemistry was used to determine which activin receptor subtypes were expressed by cultured chicken granulosa cells from F1, F2 and F3 follicles.

Materials and Methods

Experimental animals

Laying hens (ISA brown) towards the end of their first year of laying were caged individually and maintained under a standard long-day photoschedule of 16 h light:8 h darkness, at an ambient temperature of 21–23°C. Food and water were freely available. Daily oviposition times were recorded and used to predict the timing of oviposition. Hens ($n = 5$ –6 per culture) were killed approximately 4 h after ovulation of a mid-sequence egg and the three largest preovulatory follicles (F1–F3) were removed and placed immediately in sterile Hank's balanced salt solution (HBSS; Gibco-BRL, Uxbridge). Additional birds ($n = 14$) were killed to provide F1, F2 and F3 follicle tissue for measurements *ex vivo* of activin A, inhibin A and inhibin B contents of theca and granulosa layers, and for immunocytochemical staining of cultured granulosa cells.

Isolation of granulosa cells

Granulosa cells were harvested and cultured as described by Lovell *et al.* (2002). Briefly, granulosa and theca layers were separated and the granulosa layers were combined as separate F1, F2 and F3 pools, dissociated using collagenase and cell suspensions were aliquoted into 24-well plates (Falcon 3047) at 3×10^5 viable cells per well. The number of viable cells (> 90%) was estimated using Trypan blue. After 24 h incubation at 39°C, non-attached cells were removed by aspiration and adherent cells were washed three times with 1 ml serum-free incubation medium. All further incubations were done under serum-free conditions. Culture medium (1 ml) with or without test treatments was added to appropriated wells in triplicate. Cells were incubated for three successive 24 h periods, and conditioned media were aspirated and stored at –20°C on each occasion. Culture medium (with or without test treatments) was replenished every 24 h. At the termination of culture, media were removed and the monolayers were washed three times with PBS. The plated cells were sonicated in PBS containing 1% (w/v) BSA (protease free), 1% (v/v) Triton X-100 and 0.1% (w/v) sodium azide (200 µl per well), and the resultant suspension was stored at –20°C.

Treatments

Treatments were added to appropriate wells to give final concentrations of 0 and 10 ng ml^{–1} for both oLH (NIDDK-oLH-25) and oFSH (NIDDK-oFSH-19-SIAPH), and 0, 0.25, 2.5 and 25 ng ml^{–1} for activin A (recombinant human activin A; a gift from P. Smith, NHPP, Torrance, CA).

Immunoassays

Tissue extracts and cell-conditioned media were assayed for inhibin A, inhibin B and activin A using specific two-site ELISAs that use monoclonal antibodies raised against synthetic peptide fragments of the human α -, β_A - and β_B -subunits (Muttukrishna *et al.*, 1994; Groome *et al.*, 1996; Knight *et al.*, 1996). These assays have been validated for domestic fowl as described by Lovell *et al.* (1998, 2001). Recombinant human (rh) inhibin A, inhibin B and activin A were used as assay standards. The detection limits of the inhibin A, inhibin B and activin A assays were 2, 15 and 50 pg per well, respectively. Intra- and inter-plate coefficients of variation were < 10%. Progesterone was determined by direct radioimmunoassay as described by Wrathall and Knight (1995). The total cellular DNA content of each well at the end of culture was determined by fluorimetric assay (Labarca and Paigen, 1980), allowing hormone secretion to be expressed on a per µg DNA basis.

Activin receptor immunocytochemistry

The affinity-purified antibodies used were supplied by R & D Systems Europe Ltd (Abingdon) and were produced in goats immunized against the extracellular domain of purified recombinant human activin receptor (ActR) IA (Catalogue number AF637), ActRIIB (Catalogue number AF222), ActRIIA (Catalogue number AF340) or ActRIIB (Catalogue number AF339). Goat IgG was isolated from normal goat serum (Sigma UK Ltd, Poole) using a protein G-agarose column (Amersham Biosciences, Little Chalfont) and used as a control.

Granulosa cells were isolated from F1, F2 and F3 follicles and dispersed, plated and cultured on 13 mm glass coverslips in serum-free conditions for three successive 24 h periods, as described above. At termination of culture, the media were removed and the cells were washed three times in PBS. The cells were fixed in PBS containing 4% paraformaldehyde (Sigma; 30 min at room temperature (RT), pH 7.2), washed in PBS (5 min, RT), dehydrated through an ethanol series (50%, 70%, 90%, 95% and $2 \times 100\%$ for 1 min each, RT) and then rehydrated through the reverse ethanol series (for 1 min each, RT). The cells were washed in PBS (2×10 min, RT), PBS containing 0.2% (v/v) Tween 20 (Sigma; 2×15 min, RT) and again in PBS (2×10 min, RT). After blocking in PBS containing 5% (v/v) normal horse serum (NHS; Sigma; 4 h, RT), primary antibodies (treatment or control) were added to respective wells at 10 µg ml^{–1} in PBS containing 5% (v/v) NHS and 0.1% (w/v) sodium azide and incubated overnight at 4°C. The cells were washed in

Table 1. Contents ex vivo of activin A, inhibin A and inhibin B in theca and granulosa layers of the three largest follicles (F1, F2, F3) of the preovulatory hierarchy in laying hens

	F1		F2		F3	
	Theca	Granulosa	Theca	Granulosa	Theca	Granulosa
Activin A (ng DNA mg ⁻¹)	6.71 ± 0.74	0.40 ± 0.08	8.36 ± 0.71	0.43 ± 0.06	10.32 ± 1.32	0.51 ± 0.10
Inhibin A (ng DNA mg ⁻¹)	0.042 ± 0.004	1.94 ± 0.33 ^a	0.033 ± 0.004	0.12 ± 0.01 ^b	0.030 ± 0.002	0.10 ± 0.01 ^b
Inhibin B (ng DNA mg ⁻¹)	0.036 ± 0.005	0.14 ± 0.03 ^a	0.056 ± 0.01	0.09 ± 0.009 ^a	0.069 ± 0.009	0.34 ± 0.07 ^b

Values are mean ± SEM ($n = 11$ birds).

^{ab}Different superscripts within rows represent granulosa layer contents that differed significantly ($P < 0.05$). There were no significant differences in the theca layer contents of each protein among F1, F2 and F3 follicles.

PBS (3×15 min, RT) and incubated with Alexa 488-labelled donkey anti-sheep IgG secondary antibody (Molecular Probes, Leiden) at $10 \mu\text{g ml}^{-1}$ in PBS containing 5% (v/v) NHS (2 h at RT). Cells were washed subsequently in PBS containing 0.2% Tween 20 (4×15 min, RT) and then in PBS alone (2×15 min, RT). The coverslips were removed, blotted and mounted on glass slides using fluorescent mounting medium (DAKO Ltd, Ely). Images were captured using a scanning laser confocal microscope (Model: TCS NT; Leica Microsystems, Heidelberg).

Statistical analysis

Analysis of variance (ANOVA) was used to compare the hormone contents of tissue extracts, to evaluate treatment effects on hormone secretion and to make comparisons between cells from F1, F2 and F3 follicles. Data for each of the three successive periods of culture were analysed separately. Treatments were tested using triplicate wells and each experiment was repeated in three independent cultures. Values presented are mean ± SEM on the basis of three independent cultures.

Results

Measurements ex vivo in theca and granulosa extracts

Concentrations of activin A, inhibin A and inhibin B measured in extracts of theca and granulosa tissue recovered from F1, F2 and F3 follicles are shown (Table 1). Activin A concentrations were approximately 20-fold higher in theca than in granulosa tissue. In contrast, inhibin A concentrations were higher in granulosa than in theca tissue, particularly in the F1 follicle (46-fold difference). Concentrations of inhibin B were also higher in granulosa than in theca tissue. Moreover, the ratio of inhibin A to inhibin B in granulosa tissue varied according to follicle position: there was a 15-fold excess of inhibin A in F1, approximately equal concentrations of inhibin A and inhibin B in F2, and a threefold excess of inhibin B in F3.

Granulosa cell culture experiments

Concentrations of inhibin A and progesterone in granulosa cell-conditioned media were easy to measure through-

out the study, whereas concentrations of inhibin B and activin A were below assay detection limits.

Effects of activin A on 'basal' inhibin A and progesterone secretion from F1 to F3 granulosa cells

Treatment with activin A (0, 0.25, 2.5 and 25 ng ml^{-1}) alone markedly increased inhibin A secretion in a follicle-dependent manner, with the greatest response (up to a 15-fold increase on day 3; $P < 0.0001$) in F1 follicles and the smallest response (approximately twofold increase on day 3; $P < 0.05$) in F3 follicles (Fig. 1). In contrast, activin A alone had no effect on progesterone output at any time (Fig. 1).

Effects of activin A on gonadotrophin-induced inhibin A and progesterone secretion from F1 to F3 granulosa cells

On the basis of previous observations using the same culture system (Lovell *et al.*, 2002), a 10 ng ml^{-1} dose of FSH and LH was selected for activin A co-treatment experiments, as this dose elicited submaximal responses in terms of inhibin A and progesterone secretion. As reported by Lovell *et al.* (2002), cells from F3 follicles were more responsive to FSH (10 ng ml^{-1}) than were cells from F1 follicles in terms of both inhibin A ($P < 0.02$) and progesterone ($P < 0.01$) secretion. Furthermore, activin A greatly enhanced FSH-induced secretion of both inhibin A (up to tenfold; $P < 0.0001$) and progesterone (up to sixfold; $P < 0.0001$; Fig. 2). The inhibin A response to co-treatment tended to be greater in F1 follicles (approximately tenfold on day 3) than in F3 follicles (approximately fivefold on day 3), although ANOVA only revealed a significant follicle position $\times$ FSH $\times$ activin A interaction during the first 2 days of treatment. Conversely, the progesterone response to co-treatment tended to be greater in F3 follicles than in F1 follicles, but this difference was not significant on any day of culture.

In terms of LH-induced inhibin A and progesterone secretion, cells from F1, F2 and F3 follicles showed similar responsiveness (Fig. 3). Co-treatment with activin A markedly enhanced LH-induced secretion of inhibin A (up to ninefold; $P < 0.0001$) but had only a marginal effect on LH-induced progesterone secretion (up to twofold; $P < 0.001$).

Fig. 1. Dose-dependent effect of activin A on the secretion of (a,c,e) inhibin A and (b,d,f) progesterone by hen granulosa cells from F1 (○), F2 (●) and F3 (□) follicles. Data for three consecutive 24 h incubations are plotted: (a,b) 24–48 h; (c,d) 48–72 h; and (e,f) 72–96 h. Values are mean ± SEM ($n = 3$ independent cultures). Results of ANOVA are indicated.

Fig. 2. Effect of activin A, alone and in combination with FSH, on the secretion of (a,c,e) inhibin A and (b,d,f) progesterone by cultured hen granulosa cells derived from F1 (■), F2 (▨) and F3 (□) preovulatory follicles. Data for three consecutive 24 h incubations are plotted: (a,b) 24–48 h; (c,d) 48–72 h; and (e,f) 72–96 h. Values are mean ± SEM ($n = 3$ independent cultures). Results of ANOVA are indicated on each panel. Foll: follicle; ActA: activin A.

Fig. 3. Effect of activin A, alone and in combination with LH, on the secretion of (a,c,e) inhibin A and (b,d,f) progesterone by cultured hen granulosa cells derived from F1 (■), F2 (▨) and F3 (□) preovulatory follicles. Data for three consecutive 24 h incubations are plotted: (a,b) 24–48 h; (c,d) 48–72 h; and (e,f) 72–96 h. Values are mean $\pm$ SEM ($n = 3$ independent cultures). Results of ANOVA are indicated on each panel. Foll: follicle; ActA: activin A.

Although the effect on LH-induced progesterone secretion tended to be greatest in F1 follicles, ANOVA did not reveal a significant follicle position $\times$ LH $\times$ activin A interaction on any day of culture.

Effects of activin A on cell proliferation

Measurement of total cellular DNA content at the end of the culture period indicated that LH and FSH alone had no effect on proliferation of granulosa cells from F1, F2 or F3 follicles. In contrast, activin A, either alone or in combination with LH or FSH, promoted a small but highly significant increase in the number of cells (20–30%; $P < 0.0001$), which was not affected by follicle position in the hierarchy (data not shown).

Immunostaining of activin receptors in cultured granulosa cells

Representative confocal images of F1 granulosa cells immunostained for the activin receptor subtypes (IA, IB, IIA and IIB) are shown (Fig. 4). Immunofluorescent staining for all four activin receptor subtypes was clearly greater than background, although the intensity of fluorescence was greater for activin receptors IA and IIB than for IB and IIA. There was considerable heterogeneity of staining within a given granulosa cell population. Given this finding, and the fact that each antibody was used only to immunostain a

single preparation of F1, F2 and F3 cells from a single hen, it was not considered appropriate to attempt a quantitative comparison of the relative fluorescence intensity of F1, F2 and F3 cells. However, subjective assessment indicated that the overall signal intensity for each receptor subtype was similar in F1, F2 and F3 granulosa cultures.

Discussion

This study confirms a previous report that activin A concentrations in hens are 20-fold higher in preovulatory theca tissue than in the corresponding granulosa tissue, whereas inhibin A concentrations are much higher in the granulosa tissue, particularly in the F1 follicle (Lovell *et al.*, 1998). This finding is in contrast with the situation in mammals, in which follicular expression of both activins and inhibins is largely confined to granulosa cells, and prompted the present investigation of the possibility that theca-derived activin A exerts a paracrine action on granulosa cells in avian follicles. Under the culture conditions used, the secretion and residual cell content of activin A in granulosa cells were below the assay detection limit, which was perhaps not surprising given the very low activin A content of freshly isolated granulosa cell layers. Therefore, an autocrine–paracrine role of granulosa cell-derived activin A seems unlikely, despite the firm evidence supporting such an action in mammalian follicles (for reviews, see Findlay,

Fig. 4. Confocal images of chicken F1 follicle granulosa cells immunostained with primary antibodies raised in goats against human activin receptor (a) IA (b) IB, (c) IIA and (d) IIB. (e) Control goat IgG. Primary antibodies were localized using Alexa 488-labelled donkey anti-sheep IgG antibody. All images were captured under the same laser power and amplification to facilitate comparisons. Scale bars represent 10 μm .

1993; Knight and Glister, 2001). Inhibin B was detectable in *ex vivo* homogenates of F1–F3 granulosa layers (and was more abundant in F3 than in F1 follicles) but it was not possible to measure secretion *in vitro* by hen granulosa cells cultured under the present conditions.

Treatment of cultured granulosa cells from F1, F2 and F3 follicles with activin A enhanced 'basal' inhibin A secretion despite the fact that, in absolute terms, inhibin A secretion declined during the culture period. However, the maximum response in F1 cells increased substantially over the 3 days of treatment, whereas the response in F3 cells had almost disappeared by day 3. This observation supports the contention that theca-derived activin A acts selectively in F1 follicles to enhance inhibin A output, and is consistent with the finding of Lovell *et al.* (1998), confirmed in the present study, that the F1 granulosa layer contains substantially more inhibin A than do either the F2 or F3 granulosa layers.

The inter-follicle differences observed in activin A-induced enhancement of inhibin A secretion may reflect differential expression of receptors for activin, FSH and LH, or differences in post-receptor signal transduction pathways. However, no gross differences in expression of activin receptors among F1, F2 and F3 granulosa cells were detected by immunocytochemistry. The enhancement of LH-stimulated inhibin A secretion indicated that theca-derived activin A, like insulin-like growth factor I (IGF-I) (Lovell *et al.*, 2002), may act in a paracrine manner to increase inhibin A secretion by granulosa cells, especially at about the time of the preovulatory LH surge, when circulating inhibin A concentrations are highest (Lovell *et al.*, 2000).

In mammals, activin A stimulates basal and FSH-stimulated inhibin secretion (LaPolt *et al.*, 1989) and steady-state concentrations of inhibin α (LaPolt *et al.*, 1989; Xiao *et al.*, 1990; Su and Hsueh, 1994) and inhibin-activin β -subunit mRNA-protein (LaPolt *et al.*, 1989). Endogenous IGF-I production is essential for activin- and FSH-dependent inhibin α production in rat granulosa cells (Kubo *et al.*, 1998; Li *et al.*, 1998). In the present study, gonadotrophins and activin stimulated inhibin A secretion, whereas an IGF-I analogue promotes inhibin A secretion by hen granulosa cells (Lovell *et al.*, 2002). Moreover, Davis *et al.* (1999) found that both LH and FSH increase expression of inhibin α mRNA and protein in hen granulosa cells, consistent with the present findings for inhibin A dimer. As expression of IGF-I mRNA was detected in hen theca tissue but not in granulosa tissue (Armstrong and Hogg, 1996), further work is warranted to explore potential interactions between theca-derived IGF-I and activin A in modulating inhibin A and progesterone secretion by granulosa cells in hens.

Activin enhances expression of FSH receptors in rat granulosa cells (Xiao *et al.*, 1992; Nakamura *et al.*, 1993). Therefore, in the present study, activin A may have enhanced inhibin A secretion by increasing the responsiveness of granulosa cells to FSH treatment; further work is required to address this possibility. Although activin A alone had virtually no effect on 'basal' progesterone secretion, it

moderately enhanced LH-stimulated progesterone secretion, and this effect tended to be greatest in F1 granulosa cells (although the difference was not significant). This observation provides only limited support for the contention that theca-derived activin A is involved in promoting the LH–progesterone positive feedback mechanism that leads to the preovulatory LH surge and ovulation. Similarly, it remains to be established whether the much more pronounced activin A-induced enhancement of inhibin A secretion is a key component of the preovulatory surge-generating mechanism. However, as indirect evidence for this, active immunization of female chickens against inhibin α -subunit resulted in increased activin A in F1 and F2 theca layers (Lovell *et al.*, 2001). Moreover, inhibin-immunized hens showed a transient increase in plasma progesterone for 6 weeks after the onset of laying. Thus, further work is needed to test the hypothesis that theca-derived activin A acts in a paracrine manner to increase progesterone secretion by granulosa cells via an inhibin A-dependent mechanism. Activin A also regulates granulosa cell steroidogenesis in mammalian cells (Hutchinson *et al.*, 1987; Xiao *et al.*, 1990; Li *et al.*, 1992; Ford and Howard, 1997; Alak *et al.*, 1998). However, unlike in avian species, oestradiol rather than progesterone is involved in the generation of the preovulatory LH surge; therefore, caution is required when making comparisons between mammalian and avian models.

The potential role of the activin-binding protein, follistatin, in follicular development should be considered as follistatin-bound activin is devoid of biological activity (Kogawa *et al.*, 1991). A new two-site ELISA was used to show that follistatin, like activin A, is most abundant in the theca layer of hen follicles (Lovell *et al.*, 2000). Although detectable in follicle granulosa layers, follistatin concentrations are significantly lower in preovulatory (F1–F4) than in pre-hierarchical (5–9 mm) follicles, possibly increasing the likelihood that theca-derived activin A would reach and interact with activin A receptors on granulosa cells, as proposed in the present study. In accordance with this observation, Davis and Johnson (1998) reported that expression of follistatin mRNA was undetectable in the preovulatory follicle granulosa cells of hens. No attempt was made in the present study to measure follistatin secretion in granulosa cells *in vitro*.

An association between type I and II activin receptors is required to mediate the biological response to activin with type II receptors determining ligand binding specificity (for a review, see Peng and Mukai, 2000). Although an activin type II receptor (ActRII) has been cloned in chickens (Ohuchi *et al.*, 1992), its regional distribution during follicular development has not been investigated. In the present study, positive immunofluorescence staining with antibodies raised against human activin receptors indicated that all four subtypes (IA, IB, IIA and IIB) are expressed by chicken granulosa cells from F1, F2 and F3 follicles, supporting the functional evidence that theca-derived activin A can influence granulosa cell function. However,

no obvious differences were observed in receptor expression between cultured granulosa cells from F1, F2 and F3 follicles using this approach. However, as cultured cells were examined only under basal conditions, the possibility cannot be excluded that differences in activin receptor expression arise after stimulation with gonadotrophins. A more comprehensive evaluation of activin receptor expression (at both mRNA and protein levels) in granulosa cells throughout follicle development in hens is warranted. Although specific binding sites for inhibin A have been demonstrated in gonadal tumours in inhibin α knockout mice (Draper *et al.*, 1998), cognate inhibin receptors have not yet been cloned (for a review, see Robertson *et al.*, 2000). However, inhibins also compete with activin for binding to ActRII receptor, albeit with a lower affinity (Attisano *et al.*, 1992; Xu *et al.*, 1995). Therefore, a potential role of inhibin A in the direct regulation of the action of activin A also needs to be taken into account when looking at tissue responsiveness.

In conclusion, these observations support the hypothesis that theca-derived activin A contributes to the regulation of preovulatory follicle development in hens by exerting a local paracrine action on granulosa cells, leading to enhanced output of inhibin A and progesterone. The finding of differential activin A (present study) and IGF (Lovell *et al.*, 2002) responsiveness amongst granulosa cells from F1, F2 and F3 follicles implies developmental stage-dependent roles of these theca-derived peptides that may contribute to ordered follicular development.

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