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 Glistér, 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 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 Glistér, 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 A on the avian ovary, by Rombauts et al. (1996), who used mixed avian 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
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) 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 pre-ovulatory 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 and 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.

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).

Immunocytochemistry

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-2 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), ActRIB (Catalogue number AF222), ActRIIA (Catalogue number AF340) or ActRIB (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 (for 1 min each, RT) and again in PBS (2 × 100% for 1 min each, RT) and then rehydrated through the reverse ethanol series (for 1 min each, RT). Then cytoskeletons were stained with Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR). After blocking 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

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

Values are mean ± SEM (n = 11 birds).
*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 μg 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 throughout 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 × FSH × 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.
Although the effect on LH-induced progesterone secretion tended to be greatest in F1 follicles, ANOVA did not reveal a significant follicle position × LH × 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.
stimulated inhibin secretion (LaPolt et al., 1989) and 2000). had virtually no effect on 'basal' progesterone secretion, it required to address this possibility. Although activin A alone enhanced inhibin A secretion by increasing the responsive- Therefore, in the present study, activin A may have increased inhibin A secretion by granulosa cells, especially at about the time of the preovulatory LH surge, when circulat-

Effects of activin A on chicken granulosa cells in vitro 655

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 preovulat-

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.

The authors thank S. A. Feist for skilled technical assistance, P. Smith (NHPP) for supplying purified ovine LH and FSH-1 and recombinant human activin A, and BBSRC for financial support (grant number 45/S11514).

References


Etches RJ (1990) The ovoltary cycle of the hen Critical Reviews in Poultry Biology 2 293–318

Etches RJ and Duke CE (1984) Progesterone, androstenedione and oestradiol content of theca and granulosa tissue of the four largest ovarian follicles during the ovolatory cycle of the hen (Gallus domesticus) Journal of Endocrinology 103 71–76


Lovell TM, Vannontfort D, Bruggeman V, Decuyper E, Groome NP, Knight PG, and Gladwell RT (2000) Circulating concentrations of inhibin-related proteins during the ovolatory cycle of the domestic fowl (Gallus domesticus) and after induced cessation of egg laying Journal of Reproduction and Fertility 119 123–128

Lovell TM, Knight PG, Groome NP and Gladwell RT (2001) Changes in plasma inhibin A levels during sexual maturation in the female chicken and the effects of active immunization against inhibin α-subunit on reproductive hormone profiles and ovarian function Biology of Reproduction 64 188–196

Lovell TM, Gladwell RT, Groome NP and Knight PG (2002) Modulatory effects of gonadotrophins and insulin-like growth factor on the secretion...
of inhibin A and progesterone by granulosa cells from chicken preovulatory (F1–F3) follicles Reproduction 123 291–300
Nakamura M, Minegishi T, Hasegawa Y et al. (1993) Effect of activin A on follicle-stimulating hormone (FSH) receptor messenger ribonucleic acid levels and FSH receptor expression in cultured rat granulosa cells Endocrinology 133 538–544
Rombauts L, Vanmontfort D, Decuyper E and Verhoeven G (1996) Inhibin and activin have antagonistic paracrine effects on gonadal steroidogenesis during the development of the chicken embryo Biology of Reproduction 54 1229–1237

Received 16 May 2002.
First decision 5 July 2002.
Revised manuscript received 22 July 2002.
Accepted 23 July 2002.