Regulation of steroid production in ovarian stromal tissue from 5- to 8-week-old pullets and laying hens

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Studies were conducted to evaluate the regulation of steroid production in dispersed cells from ovarian stromal tissue from 5- to 8-week-old-pullets (IM cells) and laying hens (MAT cells). Short-term incubation of IM and MAT cells with ovine (o) LH resulted in a dose-dependent increase in progesterone, androstenedione and oestradiol production; progesterone production was greater in MAT cells than in IM cells ($P < 0.05$) in response to 2–200 ng oLH ml⁻¹, whereas androstenedione and oestradiol production was greater in MAT cells following treatment with 20 and 200 ng oLH ml⁻¹ ($P < 0.05$). In both cell populations the cyclic adenosine monophosphate (cAMP) analogue, 8-bromo-cAMP (1 and 10 mmol l⁻¹) stimulated progesterone and androstenedione production, whereas oLH (200 ng ml⁻¹) and forskolin (1–10 µmol l⁻¹) promoted cAMP accumulation ($P < 0.05$ compared with basal values). However, treatment with the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), did not alter basal or oLH-stimulated cAMP accumulation or progesterone production in either IM or MAT cells ($P > 0.10$). PMA did, however, inhibit agonist-induced androstenedione production ($P < 0.05$); co-treatment with the calcium ionophore A23187 potentiated this inhibitory effect. Finally, treatment with transforming growth factor- α (TGF- α ; 1.8–18 pmol l⁻¹) did not affect basal or oLH-stimulated progesterone or androstenedione production by IM cells, MAT cells, theca cells from 6–8 mm follicles or theca cells from the second largest (F2) follicle ($P > 0.10$). We conclude that LH-stimulated steroid production is greater in MAT cells than in IM cells; production of steroids at both stages occurs, at least in part, via the adenyl cyclase/cyclic AMP second messenger pathway. However, we propose that activation of protein kinase C can inhibit agonist-induced cytochrome P450 17 α -hydroxylase, but not cytochrome P450 cholesterol side-chain cleavage, activity in stromal cells. Finally, steroidogenesis in stromal tissue from 5- to 8-week-old pullet and laying hen ovaries is regulated by hormonal and cellular mechanisms most comparable to those that modulate steroidogenesis in theca cells from 6- to 8-mm and F2 follicles.

Introduction

Much work has been accomplished to characterize hormones and second messenger systems that regulate steroidogenesis in hen preovulatory follicles. The gonadotrophin LH stimulates steroid production in granulosa cells from the largest (F1) preovulatory follicle (Zakar and Hertelendy, 1980; reviewed by Johnson, 1990) and theca cells from the second largest preovulatory (F2) follicle (Tilly and Johnson, 1989a) via the cyclic AMP/protein kinase A second messenger system, and these actions depend on calcium mobilization (Asem and Hertelendy, 1986a, b, 1987; Asem *et al.*, 1987;

Levorse *et al.*, 1991). Moreover, the diacylglycerol/protein kinase C second messenger system modulates steroidogenesis in both granulosa and theca cells from preovulatory follicles (Tilly and Johnson, 1988, 1989a; Asem and Tsang, 1989); however, the endocrine/paracrine factors that affect protein kinase C activation within preovulatory follicles have yet to be unequivocally identified. Finally, transforming growth factor- α (TGF- α) and epidermal growth factor (EGF) can regulate steroidogenesis in granulosa cells from preovulatory follicles (Tilly and Johnson, 1990a), but the effects of these growth factors on theca cell steroid production from follicles at any stage of development have yet to be established.

Although preovulatory follicles contribute substantially to the total ovarian steroidogenic output, they represent the least numerous of the types of follicle present within the ovary. It has been demonstrated that ovarian follicles of less than 1 mm from laying hens produce steroids in response to

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incubation with LH (Robinson and Etches, 1986) as do whole ovaries from 1- to 28-day-old chicks (Marrone, 1986). When the mass of ovarian stromal tissue and the large number of primordial follicles within the ovary of both prepubertal and mature birds is considered, it is conceivable that these tissues provide a significant contribution to the total ovarian steroidogenic output. Nevertheless, little is known about the mechanisms that regulate steroidogenesis in ovarian stromal tissue. Thus, the following studies were conducted to characterize the second messenger systems that regulate steroid production in dispersed cells isolated from stromal tissue from both immature (5- to 8-week-old) and sexually mature birds. In addition, when published data were not available we compared cellular mechanisms that mediate steroid production in IM and MAT cells with those active in 6–8 mm follicle theca cells and F2 theca cells. Results of these studies indicate that, although stromal tissue from pullets generally has lower capacity to produce progesterone, androstenedione and oestradiol than does stromal tissue from mature ovaries, steroid production in both tissues is regulated by cellular mechanisms similar to those in theca (but not granulosa) cells of more mature (for example 6–8 mm and F2) follicles.

Materials and Methods

Animals

Single comb white Leghorn chickens (Avian Services, Frenchtown, NJ; or Moyer's Hatchery, Quakertown, PA) were used at 5–8 weeks of age (pullets) or at 35–50 weeks (laying hens). Pullets were housed in floor pens under a photoperiod of 11 h light:13 h dark (lights on at 08:00 h), while hens were housed individually in laying batteries under a 15 h light:9 h dark photoperiod (lights on at 23:00 h). All birds had free access to feed (Broilermaker or Layer Mash, Agway, Inc., Bordentown, NJ) and water. Individual laying records of hens were maintained daily by monitoring time of oviposition. Time of ovulation was predicted using previous laying records and by digital palpation on the day of an experiment. Birds were killed by cervical dislocation between 07:00 and 08:30 h (pullets), or 17–16 h before the second or third ovulation of a sequence (hens).

Reagents

Ovine (o) LH (NIAMDD-oLH-26) was provided by S. Raiti, National Hormone and Pituitary Program, NIH, Baltimore, MD. Eight-bromo-cyclic adenosine monophosphate (8-bromo-cAMP), 3-isobutyl-1-methylxanthine (IBMX), phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187 were obtained from Sigma Chemical Co. (St Louis, MO). Forskolin was from Calbiochem (San Diego, CA), and human recombinant transforming growth factor- α (TGF- α) and human recombinant epidermal growth factor (EGF) were from Bachem, Inc. (Torrance, CA). The growth factors TGF- α and EGF were prepared in advance from stock solutions (34 nmol l^{-1} and $16.4 \mu\text{mol l}^{-1}$, respectively) in incubation medium (see below), whereas 8-bromo cAMP was dissolved in incubation medium immediately before each experiment.

The phorbol ester PMA was prepared from a stock solution (1 mg ml^{-1}) in 95% ethanol, while stock solutions of forskolin (0.05 mol l^{-1}), IBMX (1 mol l^{-1}) and A23187 (0.05 mol l^{-1}) were prepared in dimethylsulfoxide (DMSO). In all instances the vehicle (final concentration of 0.1% or less) had no effect on the parameters measured (data not shown).

Cell incubations

Medium 199 with Hepes supplemented as previously described (M199/Hepes; Tilly and Johnson, 1987) was used as the incubation medium in all experiments. Ovaries were collected from two to six birds and transported to the laboratory in ice-cold 1% saline. Three different tissues were collected from ovaries of laying hens; the F2 follicle, 6–8 mm diameter follicles, and ovarian stromal tissue. Theca cells from the F2 follicle were dispersed and enriched by Percoll gradient as described by Tilly and Johnson (1989a), whereas theca cells from 6–8 mm follicles were prepared and enriched as described by Kowalski *et al.* (1991).

Dispersed cells were prepared from pullet and hen stromal tissue in a manner similar to the isolation of F2 theca cells, with the following exceptions. After collection of pullet and hen ovaries and removing as many follicles as possible from the adult ovary (i.e. follicles greater than 4 mm), the remaining stromal tissue was placed in the lid of a sterile 100 mm Petri dish and crushed with the bottom of the Petri dish. Tissue was placed in M199/Hepes containing 0.3% collagenase for 90 min at 39°C, and was frequently triturated using a flamed-tip Pasteur pipette. Dispersed cells were centrifuged through a 50% continuous Percoll density gradient at 1800 *g* for 20 min. The top 1 ml of the gradient containing cell debris was discarded and fractions with densities between 1.037 and 1.058 were collected and pooled. These are the same fractions collected as for the F2 and 6–8 mm follicles (Tilly and Johnson, 1989a; Kowalski *et al.*, 1991), and they were chosen to represent oLH-responsive cells in terms of androstenedione production (Levors, 1992). After a wash in two volumes of fresh M199/Hepes, cells were centrifuged at 600 *g* for 15 min at room temperature, and then resuspended in fresh M199/Hepes and counted in a haemocytometer. Cell incubations were carried out in 12 mm $\times$ 75 mm polypropylene incubation tubes (Sarstedt, Princeton, NJ) in a final volume of 0.5 ml. All incubations were conducted at 39°C for 4 h under ambient air conditions.

Experiments

Experiment 1 was conducted to determine whether MAT cells and IM cells respond to oLH by a dose-dependent increase in steroid production. MAT or IM cells (5×10^5 cells per tube) were incubated with increasing doses (0.2–200 ng ml^{-1}) of oLH. At the end of the incubation, cells were frozen at -20°C , and the cell plus medium content of progesterone, androstenedione and oestradiol was subsequently measured.

A second experiment was performed to compare progesterone and androstenedione production in MAT and IM cells following treatment with 8-bromo-cyclic AMP, an agent that stimulates steroid production at a site distal to the LH receptor.

IM and MAT cells were incubated with increasing doses of 8-bromo-cAMP (0.1 – 10 mmol l^{-1}) and subsequently assayed for progesterone and androstenedione.

The third experiment was conducted to evaluate further the role of the adenylyl cyclase/cyclic AMP second messenger system as a mediator of steroid production, and to compare the ability of MAT and IM cells to produce cAMP. Cells were incubated in the presence of oLH (2 – 200 ng ml^{-1}) or forskolin (a stimulator of adenylyl cyclase activity; 0.1 – 10 μ mol l^{-1}). All incubations were conducted in the presence of the phosphodiesterase inhibitor, IBMX (0.1 mmol l^{-1}). At the end of incubation, tubes were rapidly frozen to $-70^{\circ}C$ until cells plus medium were assayed for cAMP.

The fourth experiment was designed to determine whether the diacylglycerol/protein kinase C second messenger pathway modulates cAMP accumulation or steroidogenesis in MAT and IM cells. IM and MAT cells were incubated with 0.1 mmol IBMX l^{-1} and increasing doses of oLH (0.2 – 200 ng ml^{-1}) with and without 167 nmol PMA l^{-1} , and cAMP accumulation was measured. Cells were also incubated with PMA (3.3 – 167 nmol l^{-1}) alone or in the presence of 20 ng oLH ml^{-1} or 1 mmol 8-bromo cAMP l^{-1} (for progesterone and androstenedione production). In addition, IM cells, MAT cells and theca cells from 6 to 8 mm follicles (5×10^5 cells per tube) were incubated with A23187 (0.05 – 1 μ mol l^{-1}) alone, in the presence of oLH (20 ng ml^{-1}) or oLH plus PMA (167 nmol l^{-1}). After incubation for 4 h, cells plus medium were frozen at $-20^{\circ}C$ until assayed for androstenedione.

The final experiment was conducted to determine whether the growth factors TGF- α or EGF modulate steroidogenesis in IM cells, MAT cells, 6–8 mm follicle theca cells or F2 follicle theca cells (2×10^5 cells per tube). Cells were incubated with TGF- α (1.8 , 6 or 18 pmol l^{-1}) or EGF (16 , 41 or 164 nmol l^{-1}), alone or in the presence of 20 ng oLH ml^{-1} . Doses of these growth factors were chosen on the basis of results from previous studies (Tilly and Johnson, 1990a, b). After incubation, cells plus medium were frozen at $-20^{\circ}C$ until assayed for progesterone and androstenedione.

Radioimmunoassays

Progesterone, androstenedione and oestradiol were measured by specific radioimmunoassay as described by Tilly and Johnson (1989a, b) and Tilly *et al.* (1991). The inter- and intra-assay coefficients of variation were $< 15\%$ for all steroid assays conducted. The radioimmunoassay for cyclic AMP was performed on succinylated samples, as described by Johnson and Tilly (1988). All samples within an experiment were measured in a single assay, and the resulting intra-assay coefficient of variation was less than 10% .

Data analysis

Steroid production from F2 theca cells (Expt 5) was normalized to ng (androstenedione) or pg (progesterone) per 500 000 cells. Data represent the mean $\pm$ SEM of three to five replicate experiments. Treatment effects were determined by a one- or two-way analysis of variance, as appropriate. Significant

Fig. 1. Ovine (a) LH-induced (a) progesterone, (b) androstenedione and (c) oestradiol production by dispersed cells from ovarian stromal tissue collected from (□) pullets and (▨) laying hens. Data represent the mean $\pm$ SEM of four (pullets) or five (laying hens) replicate experiments. *Denotes the lowest stimulatory dose of oLH ($P < 0.05$ versus basal). †Denotes significant differences in steroid production between pullets and laying hens ($P < 0.05$).

differences ($P < 0.05$) were partitioned by the Newman–Keuls multiple range test.

Results

Experiment 1: effects of oLH on steroid output

Basal concentrations of progesterone, androstenedione and oestradiol were low, but detectable, in IM and MAT cells, and concentrations of each steroid increased following treatment with 2 – 200 ng oLH ml^{-1} (Fig. 1). Progesterone production by MAT cells exceeded production by IM cells at 2 – 200 ng oLH ml^{-1} , whereas androstenedione and oestradiol production in MAT cells exceeded that from IM cells at 20 and 200 ng oLH ml^{-1} ($P < 0.05$).

Experiment 2: effects of 8-bromo cAMP on steroidogenesis

Treatment with 1 and 10 mmol 8-bromo-cAMP l^{-1} significantly increased progesterone and androstenedione production

Fig. 2. 8-Bromo cAMP-induced (a) progesterone and (b) androstenedione production by dispersed ovarian stromal cells from (□) pullets and (▨) laying hens. Data represent the mean $\pm$ SEM from three replicate experiments. *Denotes the lowest stimulatory dose of 8-bromo cAMP ($P < 0.05$ versus basal). †Denotes significant differences in androstenedione production between pullets and laying hens ($P < 0.05$).

Fig. 3. Cyclic AMP accumulation in ovarian stromal cells from (□) pullets and (▨) laying hens following incubation with (a) oLH and (b) forskolin in the presence of 0.1 mmol 3-isobutyl-1-methylxanthine (IBMX) l⁻¹. Data represent the mean $\pm$ SEM from four (oLH) or three (forskolin) replicate experiments. *Denotes the lowest stimulatory dose ($P < 0.05$ versus basal).

in IM and MAT cells (Fig. 2). MAT cells produced higher concentrations of androstenedione in response to 8-bromo-cAMP than did IM cells ($P < 0.05$).

Experiment 3: effects of oLH and forskolin on cAMP concentrations

Incubation of IM and MAT cells with 200 ng oLH ml⁻¹ resulted in an increase in cAMP accumulation ($P < 0.05$; Fig. 3, top panel); there was no significant difference in the amount of cAMP accumulation in IM versus MAT cells. Forskolin also stimulated cAMP accumulation; however, a significant increase in IM cells was detected at 0.1 μmol forskolin l⁻¹, while concentrations of cAMP in MAT cells increased after treatment with 1.0 μmol forskolin l⁻¹ ($P < 0.05$).

Experiment 4: effects of PMA on steroidogenesis

Basal concentrations of cAMP were not different between MAT and IM cells (combined mean, 1.82 ± 0.30 pmol l⁻¹ per 500 000 cells) and oLH enhanced accumulation of cAMP in MAT and IM cells in a dose-dependent fashion (2.09 ± 1.28 , 3.89 ± 0.41 and 8.63 ± 1.57 pmol cAMP l⁻¹ for 2, 20 and 200 ng oLH ml⁻¹, respectively). PMA treatment, however, had no significant effect on basal or oLH-stimulated concentrations of cAMP. Furthermore, PMA failed to alter significantly basal, or oLH- or 8-bromo-cAMP-induced progesterone

production in MAT or IM cells. By contrast, PMA inhibited oLH- and 8-bromo-cAMP-induced androstenedione production in both cell types ($P < 0.05$; Fig. 4).

Although the ionophore A23187 failed to alter basal concentrations of androstenedione, there was a significant inhibitory effect of A23187 on oLH-induced androstenedione production by IM cells, MAT cells and 6–8 mm follicle theca cells (Fig. 5). As previously observed (Fig. 4), PMA inhibited oLH-induced androstenedione, while A23187 had a compounding, inhibitory effect on androstenedione production in the presence of PMA.

Finally, in an effort to compare the steroidogenic potential of these three tissues directly, it was determined that basal and oLH-induced androstenedione production (in the absence of A23187 and PMA) was highest in theca cells from 6–8 mm follicles (0.43 ± 0.03 ng and 9.90 ± 2.58 ng, respectively; $P < 0.05$ versus IM and MAT cells) (Fig. 5); consistent with results from experiment 1, oLH-stimulated androstenedione production was greater in MAT cells (2.17 ± 0.21 ng) than in IM cells (1.15 ± 0.06 ; $P < 0.05$).

Experiment 5: effects of TGF-α on steroidogenesis

Neither TGF-α (18 pmol l⁻¹) nor EGF (164 nmol l⁻¹) had an effect on basal progesterone or androstenedione production by IM cells (basal production was 10 ± 5 and 80 ± 10 pg for progesterone and androstenedione,

Fig. 4. Effects of PMA on (□) basal, (▨) oLH- and (■) 8-bromo cAMP-induced progesterone (a and b) and androstenedione (c and d) production in pullet (a and c) and laying hen (b and d) cells. Data are means $\pm$ SEM of three replicate experiments. *Denotes the lowest inhibitory dose of PMA ($P < 0.05$ versus agonist in absence of phorbol myristate 13-acetate (PMA)).

respectively), MAT cells (23 ± 22 and 90 ± 20 , respectively), 6–8 mm follicle theca cells (19 ± 14 and 210 ± 70 pg, respectively) or F2 follicle theca cells (190 ± 27 and 1950 ± 580 pg, respectively) ($P > 0.10$). In addition, TGF- α and EGF failed to alter oLH-induced steroid production from IM cells (72 ± 9 and 820 ± 80 pg for progesterone and androstenedione, respectively), MAT cells (205 ± 47 and 3120 ± 430 pg, respectively), 6–8 mm follicle theca cells (119 ± 19 and 8070 ± 2090 pg, respectively) or F2 follicle theca cells (1063 ± 181 and $41\,530 \pm 15\,480$ pg, respectively) ($P > 0.10$). Similarly, lower doses of TGF- α (1.8 and 6 pmol l^{-1}) and EGF (16 and 41 nmol l^{-1}) had no effect on androstenedione production (data not shown).

Discussion

The most significant findings from the present studies are that while ovarian stromal tissue from both pre- and post-pubertal animals produces progestin, androgen and oestrogen, LH-induced steroid production is greater in MAT than in IM cells and that stimulation and modulation of such steroid production occurs via cellular mechanisms similar to those that function within theca cells from 6–8 mm and preovulatory (F2) follicles. While Robinson and Etches (1986) concluded that whole follicles less than 1 mm in diameter collected from the laying hen ovary are steroidogenically active, they did not evaluate steroid production by ovarian stromal tissue, or speculate on the site of production (theca and/or granulosa tissue). Our results indicate that stromal cells from the laying hen (MAT cells) respond to LH. Moreover, the finding that dispersed IM cells produce progesterone, androstenedione

and oestrogen is consistent with previous studies that used whole ovary incubations (Tanabe *et al.*, 1979; Marrone, 1986; Pedernera *et al.*, 1988).

The ability of oLH and forskolin to stimulate cAMP accumulation and steroid production, together with the ability of 8-bromo cAMP to mimic the effect of oLH, in both IM cells and MAT cells, indicate that the adenylyl cyclase/cyclic AMP second messenger pathway has a role in the regulation of steroidogenesis (Marrone, 1986). It is of interest, however, that whereas a dose of 2 ng oLH ml^{-1} stimulates steroid production from IM and MAT cells, a dose of 200 ng oLH ml^{-1} is required to detect a significant increase in cAMP accumulation. This observation is similar to that reported for F2 theca (Tilly and Johnson, 1989a) and for theca from 6–8 mm steroids (Kowalski *et al.*, 1991). Such results may indicate that low doses of gonadotrophin may act via a cAMP-independent second messenger pathway such as the calcium-calmodulin system (Levors *et al.*, 1991). Alternatively, it is possible that low doses of oLH may induce significant increases in cAMP within localized subcellular compartments and that such changes are not detectable with measurements of whole cells.

Although oLH stimulates steroid production in both IM and MAT cells, it does so to a somewhat different degree; MAT cells produce more progesterone, androstenedione and oestradiol in response to oLH than do IM cells. It is unlikely that such differences in steroid production result from more functional LH receptors on MAT cells, as the accumulation of cAMP following oLH treatment in this study was not significantly different in IM versus MAT cells. Furthermore, the increase in cAMP accumulation following forskolin treatment is comparable, and responsiveness perhaps greater, in IM versus MAT cells, indicating competence of the post-receptor adenylyl

Fig. 5. The calcium ionophore A23187 failed to affect (□) basal androstenedione production by ovarian stromal cells from (a) pullets, (b) laying hens and (c) 6–8 mm follicle theca cells collected from laying hens. By contrast, A23187 inhibited oLH (20 ng ml⁻¹)-stimulated androstenedione production (▨; *denotes lowest inhibitory dose). Phorbol myristate 13-acetate (PMA) (167 nmol l⁻¹) inhibited oLH-stimulated androstenedione production in the absence of A23187 ($P < 0.05$; denoted by †), while A23187 had an additive, inhibitory effect in the presence of PMA (■; **denotes lowest inhibitory dose versus absence of A23187; $P < 0.05$). Data represent the mean $\pm$ SEM of three (laying hens) or four (pullets and 6–8 mm follicle theca) replicate experiments.

cyclase complex in IM cells. Although at this time we cannot explain why progesterone production in response to 8-bromo cAMP was not different in MAT versus IM cells, it is evident that this agonist stimulates androstenedione to a greater extent in MAT cells than in IM cells. Thus, generally higher amounts of steroid production by MAT cells indicate greater cytochrome P450 side-chain cleavage (P450scc) activity, 3 β -hydroxysteroid dehydrogenase activity, or cytochrome P450 17 α -hydroxylase (P450 17 α -OH) activity, or alternatively, reflect a greater availability of cholesterol substrate for metabolism to progesterone. It is possible that the greater potential for steroid production by MAT cells reflects a longer period of exposure to circulating gonadotrophins, and in particular, FSH. FSH can increase P450scc and P450 17 α -OH mRNA abundance and induce enzyme activity in hen granulosa cells (Li and Johnson, 1993a, b).

Previously published studies of granulosa and theca cells prepared from preovulatory follicles have implicated the protein kinase C second messenger pathway in the regulation of ovarian steroidogenesis (Asem and Tsang, 1989; Tilly and Johnson, 1988, 1989a, b). For instance, in F1 granulosa cells, PMA and the diacylglycerol analogue 1-oleoyl-2-acetyl-glycerol (OAG) attenuate agonist-induced progesterone and androstenedione production. This attenuation is mediated via the prevention of cAMP accumulation and by the inhibition of P450scc activity (Tilly and Johnson, 1988). By comparison, PMA stimulates progesterone and androstenedione production in theca cells from 6–8 mm and F2 follicles, and this stimulation occurs in the absence of a detectable increase in cAMP accumulation (Tilly and Johnson, 1989a; Kowalski *et al.*, 1991). In contrast, PMA inhibits LH-induced androstenedione (but not progesterone) production in theca cells from 6–8 mm and F2 follicles, and this modulation occurs distal to the formation of cAMP, via the inhibition of P450 17 α -OH enzyme activity. In the present studies, there was no detectable increase in basal concentrations of progesterone or androstenedione from IM or MAT cells in response to PMA treatment. This lack of a detectable increase may be due to the inherently low basal concentrations of steroid production in IM and MAT cells. However, PMA was found to inhibit oLH- and 8-bromo-cAMP-stimulated androstenedione (and not progesterone) production from IM and MAT cells. These results indicate that, as previously determined for theca cells from 6–8 mm and F2 follicle theca cells, protein kinase C activation in stromal tissue inhibits P450 17 α -OH activity, but not P450scc activity.

The growth factors TGF- α and EGF inhibit LH-induced progesterone production in hen F1 granulosa cells (Pulley and Marrone, 1986; Tilly and Johnson, 1990a). TGF- α and EGF were shown to block FSH-induced increases in P450scc and P450 17 α -OH mRNA and enzyme activity in granulosa cells from 6–8 mm follicles (Li and Johnson, 1993a, b). It was proposed that some of the effects of these growth factors on granulosa cells may be mediated, at least in part, via activation of protein kinase C (Tilly and Johnson, 1990a). Although TGF- α and EGF have been found not to affect plasminogen activator activity in theca cells from preovulatory follicles (Tilly and Johnson, 1990b), the effects of these growth factors on theca cell steroidogenesis have, until now, not been evaluated. Results from the study reported here indicate that neither TGF- α nor EGF alter basal or LH-stimulated steroid production in theca cells or stromal tissue following a short-term (4 h) incubation. On the basis of these data, we propose that neither TGF- α nor EGF activates the protein kinase C second messenger pathway in these cells, and that these growth factors do not play a role in modulating either steroid production or plasminogen activator activity in theca cells; this lack of effect by TGF- α and EGF in follicles cannot be attributed to the absence of EGF receptors, as both EGF receptor mRNA (Johnson, unpublished data) and immunoreactive EGF receptor protein (O. M. Onagbesan and M. Peddie, personal communication) have been identified within theca tissue.

Although IM and MAT cells have a lower capacity to produce androstenedione in response to oLH than do 6–8 mm follicle and F2 follicle theca cells, the mechanisms that regulate steroid production in stromal (IM and MAT) cells are similar to

those reported for theca cells (Tilly and Johnson, 1989a; Kowalski *et al.*, 1991; this study). Progesterin and androgen synthesis in avian theca occurs within the theca interna, whereas oestrogens are produced from a discrete population of cells located within the theca externa (Porter *et al.*, 1989; Nitta *et al.*, 1991). By contrast, granulosa cells fail to express aromatase activity at any stage during development (Armstrong, 1984). In the study reported here, the observation that the cell population collected from the Percoll gradient produces oestradiol indicates that there are theca externa-like cells. Nevertheless, our criterion for selection of cells from the Percoll gradient was based on ability to produce androstenedione, and not oestradiol; in fact, the greatest number of oestradiol producing cells was eluted at a density of 1.065 (Levorso, 1992). Furthermore, it is possible that ovarian stromal tissue is the predominant, if not sole, source of oestrogen in the immature pullet. Finally, studies have shown that dispersed granulosa cells from follicles less than 8 mm in diameter do not produce progesterone or androstenedione, *in vitro*, owing to the absence of P450_{scc} and P450 17 α -OH activities (Tilly *et al.*, 1991; Li and Johnson, 1993a, b). Taken collectively, these observations indicate that the steroidogenically active IM and MAT cells prepared from stromal tissue are of theca cell origin.

In agreement with results obtained using F2 theca cells (Levorso *et al.*, 1991), A23187 had no effect on basal steroid production in IM cells, MAT cells or theca cells from 6–8 mm follicles. Moreover, A23187 inhibited oLH-stimulated androstenedione production in each cell type examined, and this inhibitory effect was enhanced in the presence of PMA (Levorso *et al.*, 1991; this study). The inhibitory effects of A23187 in theca and stromal tissue are in contrast to the stimulatory effects on progesterone and androstenedione production from F1 granulosa tissue (Tilly and Johnson, 1988). As discussed with reference to F2 theca cells, there are several possible explanations for the inhibitory effects of A23187 on androstenedione production in theca and stromal tissue. For example, high concentrations of intracellular calcium activate protein kinase C (Ho *et al.*, 1988), and the results reported here indicate that protein kinase C activation (using PMA) inhibits LH- and 8-bromo-cAMP-stimulated androstenedione production. Alternatively, Johnson *et al.* (1990) provided evidence that A23187 stimulates the release of arachidonic acid in F2 theca cells, and that arachidonic acid inhibits androstenedione production in F2 theca cells. Finally, it is possible that the ionophore causes a supraphysiological rise in intracellular calcium concentrations, which subsequently inhibits cellular function (White *et al.*, 1989).

In summary, results from the present experiments demonstrate that dispersed cells from ovarian stromal tissue of both sexually immature and mature birds are steroidogenically active and responsive to the gonadotrophin, LH. On a per cell basis, theca cells from 6–8 mm and F2 follicles produce more steroid; nevertheless, considering the mass of stromal tissue present at both stages of ontogeny, it is possible that such tissue contributes significantly to the total steroidogenic output of the ovary. Finally, the qualitative and quantitative pattern of steroidogenesis, and the cellular mechanisms regulating steroid production in IM and MAT cells are similar to those in theca cells from growing and preovulatory follicles. We conclude that the steroidogenically active cellular component

of ovarian stromal tissue is the progenitor of follicle theca cells.

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