G protein expression and second messenger formation in human granulosa cells

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The expression of heterotrimeric ($\alpha\beta\gamma$ subunits) GTP-binding regulatory proteins (G proteins) and the activation of G protein-linked receptors in human granulosa cells were investigated. The cells were obtained from stimulated follicles in women undergoing *in vitro* fertilization and were cultured in serum-supplemented medium. Immunoblotting with specific antibodies showed that granulosa cell membranes express α_s , α_{i3} , $\alpha_{i1,2}$, $\alpha_{q,11}$ and β subunits. Three antibodies against α_o failed to detect this protein. The cells responded to hCG and to prostaglandin E_2 with a dose-dependent increase in cAMP formation, confirming the functional activation of G_{α_s} . The α_2 adrenoceptor agonist, clonidine, inhibited hCG-stimulated cAMP formation and this effect was blocked with pertussis toxin, thus involving a G_i -type protein, most likely $G_{\alpha_{i2}}$. Oxytocin provoked an increase in formation of inositol phosphates and intracellular calcium concentration, which was partly pertussis toxin resistant, providing evidence of $G_{\alpha_{q,11}}$ activation. However, a significant component of the response to oxytocin could be blocked by pertussis toxin, indicating G_i -mediated phospholipase C activation (by either α_i or $\beta\gamma$ subunits). These data demonstrate the presence of G proteins in granulosa cells and suggest a complex regulation of hormonal signalling. The concentration of cAMP in these cells depended on the balance of G_{α_s} : G_{α_i} activation, whereas activation of the inositol phospholipid pathway and rises in intracellular calcium involved both $G_{q,11}$ and G_i pathways.

Introduction

Granulosa cells are essential for follicular development and corpus luteum formation. During follicular development, granulosa and theca cells interact to produce steroids, predominantly oestrogens, and, after ovulation, luteinized granulosa cells develop a large capacity to secrete progesterone (Steele and Leung, 1992; Yong *et al.*, 1992a). The functions of granulosa cells are controlled by gonadotrophins and other hormones whose mechanism of action usually involves binding to receptors on the cell surface and the transmission of information to an effector system within the cells. GTP-binding proteins (G proteins) mediate the transmission of the message from activated hormone receptors to effector systems such as adenylyl cyclase or phospholipase C (Birnbaumer *et al.*, 1990; Olate and Allende, 1991; Hepler and Gilman, 1992). G proteins consist of heterotrimers: $\alpha\beta\gamma$ subunits. The α subunits vary considerably between G proteins and confer specificity for coupling to receptors and effectors, whereas $\beta\gamma$ subunits are relatively common (Hepler and Gilman, 1992). There are at least two types of G protein that interact with adenylyl cyclase: G_{α_s} , which is stimulatory (increasing cyclic AMP formation); and G_{α_i} , which is inhibitory (Gilman, 1990; Levitzki and Bar-Sinai, 1991). Proteins of the $G_{q,11}$ family interact with phospholipase

C (PLC), resulting in the formation of two second messengers: diacylglycerol, which activates protein kinase C, and inositol 1,4,5-trisphosphate ($InsP_3$), which mobilizes intracellular calcium (Smrcka *et al.*, 1991; Taylor *et al.*, 1991). Moreover, $\beta\gamma$ subunits can interact directly with some types of PLC and adenylyl cyclase (Levitzki and Bar-Sinai, 1991; Hepler and Gilman, 1992).

The purpose of this paper was to study G protein expression in human granulosa cells, and to investigate the functional involvement in receptor activation of G proteins by measuring second messenger formation in response to agonists that operate through G protein-linked receptors (Kimura *et al.*, 1992; Lu and Menon, 1994). Human chorionic gonadotrophin was used to stimulate adenylyl cyclase (McFarland *et al.*, 1989; Cooke *et al.*, 1989), and oxytocin was used to activate PLC (Schrey *et al.*, 1987; Phaneuf *et al.*, 1993). In addition, the presence of adenylyl cyclase inhibitory pathways was investigated using clonidine, which activates α_2 -adrenergic receptors thought to be coupled to G_i (Osborne, 1991; Regunathan *et al.*, 1991).

Materials and Methods

Collection and culture of cells

Granulosa cells were obtained with patients' consent and approval of the Ethics Committee from women undergoing

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Table 1. G-protein antisera used in this study

Antibody	Source	Subunit	Peptide sequence	Binding site
AS/7	NEN	T α , α_{11} , α_{12}	KENLKDCGLF	C-terminus
EC/2	NEN	α_{13} , α_o	KNNLKECGLY	C-terminus
GC/2	NEN	α_o	GCTLSAEERAALERSK	N-terminus
IMI	Milligan	α_o	NLKEDGISAADVK	Residues 22–35
OC1	Milligan	α_o	ANNLRGCGLY	C-terminus
QL	NEN	α_q , α_{11}	QLNLKEYNLV	C-terminus
RM/1	NEN	α_s , α_{olf}	RMHLRQYELL	C-terminus
MS/1	NEN	β -common	MSELDQLRQE	N-terminus

All antisera were raised in rabbits. Sources: NEN-Du Pont, Boston, MA; G. Milligan, Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow.

routine oocyte retrievals for *in vitro* fertilization. Follicular growth was stimulated with daily injections of gonadotrophins in patients previously desensitized with a long course of a GnRH analogue (Forman *et al.*, 1991). Several follicles were aspirated from each patient 35 h after administration of 10 000 iu of hCG (Profasi, Serono Laboratories UK Ltd, Hertfordshire). The mean follicular diameter was 17 ± 2.4 mm (mean $\pm$ SD, $n = 150$). After removing any oocytes present, follicular fluids and flushings were pooled and centrifuged (340 g) at room temperature for 10 min through Ficoll-Paque (Pharmacia, St Albans) to remove red blood cells. Granulosa cell layers were dispersed with collagenase (Sigma Ltd Co, Poole) diluted to 300 U ml⁻¹ and Trasylol (Bayer UK Ltd, Newbury) diluted to 100 U ml⁻¹ for 20 min at 37°C in 1 mmol CaCl₂ l⁻¹. Cell viability after enzymatic dispersion was $73 \pm 4\%$, as assessed by Trypan blue exclusion, and the average yield of viable cells was $0.59 \pm 0.44 \times 10^6$ per patient (mean $\pm$ SD, $n = 48$).

Cells were routinely seeded at a density of 40 000 cells cm⁻² in 24- or 6-well fibronectin-coated wells and were cultured in Hams F12:DMEM (1:1 by volume; GIBCO, Life Technologies, Uxbridge) medium containing 10% (v/v) fetal calf serum, 10% (v/v) horse serum (Advanced Protein Products, Brierley Hill), 1 μ mol vitamin E l⁻¹, 20 nmol selenium l⁻¹ and penicillin/streptomycin 100 units ml⁻¹ (McAllister *et al.*, 1990). Under these conditions, there was cell growth in culture (doubling time approximately 3 days), as demonstrated by reduction of tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) to formazan and by [³H]thymidine uptake.

Steroidogenesis

Oestradiol and progesterone outputs into cell culture supernatants were measured with radioimmunoassay kits (DPC, Los Angeles, CA) following the manufacturers' recommendations. The sensitivity of the progesterone assay was 0.1 pmol ml⁻¹ and the mid-point of the standard curve (50% displacement) was obtained with 6.7 pmol ml⁻¹. The coefficient of variation was 4.7% (intra-assay) and 9.2% (interassay). The sensitivity of the oestradiol assay was 0.03 pmol ml⁻¹ and the mid-point of the standard curve was 1.1 pmol ml⁻¹. The intra- and inter-assay coefficients of variation for this assay were 4.7% and 7.9%, respectively.

Immunoblotting

Cultured granulosa cells were lifted with trypsin-EDTA and resuspended in 25 mmol Tris l⁻¹ buffer, pH 7.4, 250 mmol sucrose l⁻¹–1 mmol EDTA l⁻¹, and were disrupted with a ground glass homogenizer. A membrane pellet was obtained by centrifuging the homogenate at 13 000 g for 30 min, and the protein content was estimated by the Bradford-BioRad method (Richmond, CA). The pellet was solubilized in 10% (w/v) SDS buffer containing 8 mol urea l⁻¹, 0.5 mol dithiothreitol l⁻¹ (DTT) and 0.05 mol Tris l⁻¹, pH 6.8, and the proteins were resolved on a 12.5% polyacrylamide gel containing 0.0625% (w/v) bis-acrylamide for 6 h at 40 mA. Proteins from gels were transferred to nitrocellulose using BioRad electroblotting apparatus with 0.025 mol Tris l⁻¹–0.195 mol glycine l⁻¹ and 20% (v/v) methanol, pH 8.6, at 90 V for 2 h. After transfer, nitrocellulose papers were incubated in PBS, pH 7.5, containing 3% (w/v) skimmed milk (Marvel, Premier Brands UK Ltd, Adbaston) for 1 h at room temperature. The papers were washed three times with PBS and incubated with primary antibodies at dilutions ranging from 1:250 to 1:1000. The antibodies used are given in Table 1. Primary antisera were removed and the blots were washed thoroughly with PBS and incubated with goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase at 1:1000 dilution for 1 h at room temperature, and washed again with PBS. Antibody complexes were detected by enhanced chemiluminescence (ECL) using X-Omat X-ray film (Europe-Finner *et al.*, 1993).

Cyclic AMP estimations

Cells were challenged in serum-free medium with appropriate agonists or drugs. Incubations were carried out at 37°C in the presence of 5 mmol theophylline l⁻¹ (Sigma), and were terminated with 5% (v/v) perchloric acid. Cyclic AMP was extracted by the freon-octylamine method, and was measured by competitive protein-binding using [2,8-³H]adenosine 3',5'-cyclic phosphate (specific activity 1.85 TBq mmol⁻¹, Amersham International, Bucks) as the labelled ligand (López Bernal *et al.*, 1991).

Inositol phosphate formation

Granulosa cells were labelled for 48 h with 2.5 μ Ci per well (0.23 MBq ml⁻¹) of [³H]myo inositol (TRK 912B30; Amersham

International) in inositol-free RPMI medium containing 2% (v/v) fetal calf serum. The cells were washed and challenged by agonists in the presence of lithium chloride (15 mmol l^{-1}) for 30 min at 37°C . The reactions were stopped with 1 ml acidified chloroform/methanol (chloroform/methanol/hydrochloric acid; 50/100/1). Inositol phosphate formation was measured by ion exchange chromatography on Dowex columns as described by Phaneuf *et al.* (1993).

Calcium estimations

Granulosa cells were grown onto 13 mm diameter fibronectin-coated borosilicate glass coverslips. Subconfluent cells were loaded with $5 \mu\text{mol}$ Fura-2 acetoxymethyl ester l^{-1} and 0.1% (w/v) Pluronic (both from Molecular Probes, Cambridge Biosciences, Cambridge) in Hank's medium for 60 min at 37°C . After washing thoroughly, the coverslips were placed in 3 ml plastic PMMA cuvettes (Disposolab-Kartell, Fisons, Loughborough) inside the chamber of a Shimadzu RF-500 1PC fluorimeter (Howe, Banbury) and allowed to settle for 50 s before being challenged with oxytocin. Excitation wavelengths were 340 nm and 380 nm, and emission was measured at 510 nm. The mean intracellular calcium peak concentration ($[\text{Ca}^{2+}]_i$) was calculated from 340:380 nm ratios (Phaneuf *et al.*, 1993).

Statistical analysis

Experiments were carried out at least twice using cells from different donors and all estimations were performed with a minimum of three replicates. Statistical comparisons were assessed by analysis of variance using the Bonferroni multiple comparisons test or by Student's *t* test.

Results

Steroid output

Progesterone output by granulosa cells increased steadily over several days in culture. Oestradiol output increased only moderately over the same period. This steroid profile is reminiscent of the early luteal phase changes that occur in the peripheral circulation *in vivo*, and indicates that the cells maintained their luteinization *in vitro*. Progesterone output was stimulated by hCG with an EC_{50} value of $0.14 \pm 0.10 \text{ iu ml}^{-1}$ (mean $\pm$ SEM, $n = 3$). The cells could synthesize large amounts of progesterone and oestradiol from their immediate precursors (5-pregnen-3 β -ol-20-one and testosterone, respectively), and retained 3 β -hydroxysteroid dehydrogenase and aromatase activities for at least 15 days in culture (data not shown).

Expression of G proteins

Granulosa cell membranes expressed α_s , α_{i3} , $\alpha_{11,2}$, $\alpha_{q,11}$ and β subunits (Fig. 1). Three antibodies against α_o failed to detect this protein.

Fig. 1. Expression of G proteins in membranes prepared from human granulosa cells. Granulosa cells were cultured in serum-supplemented medium for 14 days and were then lifted with trypsin-EDTA, washed with PBS and disrupted for membrane preparation. Membrane proteins were separated by gel electrophoresis.

Table 2. Effect of several compounds on cAMP production by human granulosa cells

Compound	pmol cAMP mg^{-1} protein
Control	88 ± 19
Forskolin ($1 \mu\text{mol l}^{-1}$)	797 ± 202
hCG (1 iu ml^{-1})	904 ± 149
FSH (1 iu ml^{-1})	157 ± 27
Prostaglandin E_2 ($10 \mu\text{mol l}^{-1}$)	750 ± 128

Granulosa cells were cultured in serum-supplemented medium for 10–14 days. The cells were then washed with PBS and the medium was changed to serum-free Hank's solution with and without the appropriate compounds. Results are means $\pm$ SEM from two to five separate experiments. All the drugs provoked a significant increase in cAMP concentration after 2 h incubation ($P < 0.001$; $P < 0.02$ for FSH).

Stimulation of cAMP production in granulosa cells

Granulosa cells responded to forskolin, which activates the catalytic unit of adenylyl cyclase directly, with a significant increase in cAMP concentrations, demonstrating an operating cyclase system (Table 2). The functional involvement of G_s in the cells was investigated with agonists, the receptors of which are coupled to stimulation of adenylyl cyclase. When granulosa cells were exposed to hCG, there was rapid accumulation of cAMP, and the concentration of cAMP remained stable for at least 4 h (Fig. 2). The effect of hCG was dose-dependent (Fig. 2) with an EC_{50} value of $0.37 \pm 0.14 \text{ iu ml}^{-1}$ (mean $\pm$ SEM, $n = 3$) in the same range as that for stimulation of progesterone production. FSH had only a moderate stimulatory effect (Table 2). Prostaglandin E_2 had a clear stimulatory effect, indicating the presence of EP receptor subtypes coupled to G_s (Bastien *et al.*, 1994; Regan *et al.*, 1994) in these cells (Table 2).

Inhibition of cAMP production

Clonidine inhibited hCG-stimulated cAMP production, providing evidence for an α_2 -adrenoceptor inhibitory pathway opposed to the G_s pathway. Moreover, when the cells were pretreated with pertussis toxin (which inactivates G_i

Fig. 2. Accumulation of cAMP in human granulosa cells stimulated with hCG. Granulosa cells were cultured in serum-supplemented medium for 8–14 days. The cells were then washed with PBS and the medium was changed to serum-free Hank’s solution with and without hCG. In a preliminary experiment (inset), cells from one donor were stimulated with 1 iu hCG ml⁻¹ in the presence of 5 mmol theophylline l⁻¹ for the times indicated. Subsequent incubations were terminated at 2 h. The dose–response curve shows means ± SEM from three different experiments.

by ADP-ribosylation), the effect of clonidine was abolished, thus confirming the involvement of a G_i-like protein (Fig. 3).

Activation of phospholipase C

The cells responded to the G protein activator fluoroaluminate with increased inositol phosphate formation, demonstrating G protein-mediated PLC activity in these cells (Table 3). Oxytocin and arginine-vasopressin stimulated PLC, but the former provoked the strongest stimulation and was used for subsequent experiments. LHRH (Nova Biochem, Nottingham) or the analogue, buserelin acetate (Suprefact: Hoechst UK Ltd, Hounslow), had no stimulatory effect.

The effect of oxytocin was dose dependent (Fig. 4), with an EC₅₀ value of 3.46 ± 1.66 nmol l⁻¹ (mean ± SEM, n = 3), which is in the same range as the K_d value for oxytocin receptors in human ovary (Fuchs *et al.*, 1990). Moreover, the effect of oxytocin was partly inhibited by pertussis toxin (Fig. 4), suggesting that at least two pathways, one pertussis toxin-sensitive (probably mediated by G_i) and one pertussis-toxin-resistant (probably mediated by G_{q,11}) are involved (Phaneuf *et al.*, 1993).

Fig. 3. Effect of clonidine on hCG-stimulated cAMP concentration in human granulosa cells. Granulosa cells were cultured in serum-supplemented medium for 14 days. The cells were then washed with PBS and the medium was changed to serum-free Hank’s solution with and without the appropriate compounds. The inhibitory effect of clonidine ($P < 0.01$) was abolished by incubating the cells with 1 µg pertussis toxin ml⁻¹. Results are means ± SD of triplicate estimations in a representative experiment. Similar results were obtained in three separate experiments with cells from different donors. (□) control; (▨) clonidine (100 µmol l⁻¹); (■) clonidine + pertussis toxin (1 µg ml⁻¹).

Table 3. Effect of several peptides on inositol phosphate (IPs) formation by human granulosa cells

Peptide	Total IPs formation (% of control)
Control	100
Fluoroaluminate	317 ± 52*
Arginine/vasopressin	246 ± 31*
Oxytocin	498 ± 57*
Buserelin	82 ± 9
LHRH	93 ± 12

Granulosa cells were cultured in serum-supplemented medium for 7 days. The cells were then washed with PBS and the medium was changed to serum-free Hank’s solution with and without the appropriate agonists. Fluoroaluminate was a mixture of 20 mmol sodium fluoride l⁻¹ and 10 µmol aluminium fluoride l⁻¹. Peptides were used at 1 µmol l⁻¹. Results are means ± SEM of quadruplicate estimations. *Significantly different from control ($P < 0.01$).

Effect on intracellular calcium

Oxytocin increased [Ca²⁺]_i in human granulosa cells in a dose-dependent manner (Fig. 5). The source of Ca²⁺ was not investigated in these experiments, but it is likely to be a combination of Ca²⁺ mobilization from InsP₃-sensitive stores and Ca²⁺ entry from the extracellular medium (Phaneuf *et al.*, 1993). Moreover, pretreatment of the cells with pertussis toxin resulted in partial attenuation of the response (Fig. 5), again suggesting the involvement of two pathways: one resistant to pertussis toxin (possibly mediated by G_{q,11}) and another sensitive to pertussis toxin (possibly involving G_i).

Fig. 4. Effect of oxytocin on inositol phosphate formation in human granulosa cells. For these experiments, granulosa cells were cultured in serum-supplemented medium for 7–14 days. Results are means $\pm$ SD of quadruplicate estimations in cells from one donor. The inset is a different experiment showing that cells incubated with 1 μ g pertussis toxin ml⁻¹ for 24 h (■) had a lower (* P < 0.01) response to oxytocin. Similar results were obtained in two other experiments.

Discussion

Cultured human granulosa cells retain steroidogenic capacity and hormone responsiveness for at least two weeks and provide a useful model to study second messenger pathways. The cells express a variety of G proteins. RM/1 is a well characterized antibody that has been used successfully to demonstrate G_{α_s} in a variety of animal and human tissues (Morris *et al.*, 1990; Europe-Finner *et al.*, 1993). There are four splice variants of α_s , but human granulosa cells express a single band, probably corresponding to the 'long form' of α_s (Europe-Finner *et al.*, 1993). Strong functional evidence for an active G_{α_s} pathway is given by the finding that two unrelated agonists, hCG and PGE₂, the receptors of which belong to the G protein-linked family (Hipkin *et al.*, 1993; Bastien *et al.*, 1994; Lu and Menon, 1994; Regan *et al.*, 1994), provoke a large and rapid increase in cAMP production by granulosa cells.

Of particular interest is the expression of G_{α_i} subtypes, since this inhibitory pathway is often overlooked when studying cAMP-mediated events in granulosa cells. The antibodies AS/7 and EC/2 were raised against α_{i1}/α_{i2} and α_{i3}/α_o , respectively, and the immunoblot data suggest that at least two different members of the G_i family (most likely $\alpha_{i1,2}$ and α_{i3}) are present in these cells. The absence of α_o was confirmed with three different antibodies. Functional evidence for the existence of a G_{α_i} pathway was obtained with clonidine, which activates α_2 adrenoceptors linked to $G_{\alpha_{i2}}$ (Osborne, 1991) and possibly to other α_i -subtypes (Raymond *et al.*, 1994). Human granulosa cells possess α adrenoceptors that inhibit hCG-induced progesterone secretion (Fohr *et al.*, 1993). Furthermore, the inhibitory effect of clonidine was blocked completely by pertussis toxin, confirming that it is a G_{α_i} -mediated event.

Fig. 5. Effect of oxytocin on $[Ca^{2+}]_i$ concentration in human granulosa cells. Cells were cultured onto fibronectin-coated glass coverslips in serum-supplemented medium for 7–14 days and were loaded with Fura-2. Results are means $\pm$ SD of triplicate estimations in cells from one donor. The inset is a different experiment showing that cells incubated with 1 μ g pertussis toxin ml⁻¹ for 24 h (■) had a lower (* P < 0.01) response to oxytocin. Similar results were obtained in two other experiments.

The $G_{\alpha_{q,11}}$ proteins are ubiquitous (Mitchell *et al.*, 1991) and are expressed in granulosa cells. The oxytocin receptor is thought to be coupled to PLC activation via $\alpha_{q,11}$ and, when the cells were challenged with oxytocin, there was rapid accumulation of inositol phosphates and an increase in intracellular calcium, demonstrating an active PLC–InsP₃ pathway. However, the mechanism of action of oxytocin is complex, as both PLC activation and $[Ca^{2+}]_i$ rises have a pertussis toxin sensitive component that cannot be accounted for by G_q . This component may represent α_i stimulation of PLC. Moreover, some PLC isozymes (e.g., PLC β_3) can be activated directly by $\beta\gamma$ subunits (Exton, 1993; Sternweis and Smrcka, 1993), and it is possible that the pertussis sensitive component of oxytocin-induced PLC activation is due to liberation of $\beta\gamma$ subunits from G_i -like proteins. Human granulosa cells express PLC β_1 (a target for G_q), as well as PLC β_2 and PLC β_3 (C. H. Hamilton, S. Phaneuf and A. López Bernal unpublished observations); hence it is possible that there are multiple signalling pathways for oxytocin. Oxytocin is produced by granulosa/luteal cells in ruminants (Jones and Flint, 1988; Camier *et al.*, 1991) and humans (Guillou *et al.*, 1992), and the oxytocin receptor is expressed in the ovary (Fuchs *et al.*, 1990; Zhang *et al.*, 1991; Kimura *et al.*, 1993). It is possible that oxytocin has a paracrine/autocrine role in follicular steroidogenesis, modulating the effect of gonadotrophins (Pitzel *et al.*, 1990; Voss and Fortune, 1991; Clamagirand *et al.*, 1991). The data presented here demonstrate that mature granulosa cells are a target tissue for oxytocin, but its role and mechanism of action deserve further investigation.

In conclusion, the data demonstrate the presence of G_{α_s} in human granulosa cells and reinforce the notion that this G protein mediates the stimulatory effect of hCG. Prostaglandin E_2 is a luteotrophic agent in some species and may participate in the events leading to ovulation (Zelinski-Wooten and Stouffer, 1990). PGE_2 can interact with a variety of EP receptor subtypes, and granulosa cells clearly respond via G_{α_s} . However, the presence of other signalling pathways for PGE_2 in this tissue merits further investigation. There are at least two subtypes of G_{α_i} and the functional involvement of these proteins is suggested by direct inhibition of hCG-stimulated cAMP production (most likely an α_{i2} -mediated effect) and by their contribution to oxytocin-mediated PLC activation (by either α_i or $\beta\gamma$ subunits). Granulosa cells play a vital role in steroidogenesis and oocyte maturation. The role of cAMP in gonadotrophin-mediated control of granulosa cell function is well established (Yong *et al.*, 1992b). Assuming a constant level of catabolism by phosphodiesterase, cAMP concentrations in granulosa cells are determined by the balance of stimulatory, G_{α_s} -mediated, and inhibitory, G_{α_i} -mediated, pathways. Response to gonadotrophins, for example, may depend not only on the amount of LH or FSH reaching the cells, but also on the concurrent amount of G_{α_i} stimulation generated by other receptors. These findings apply to 'mature' granulosa cells obtained from stimulated IVF cycles at the preovulatory stage and may not necessarily represent the characteristics of these cells at earlier stages of development. Nevertheless, they illustrate the complexity of this endocrine tissue and the various potential sites for hormonal interaction.

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