Control of oestradiol secretion and of cytochrome P450 aromatase messenger ribonucleic acid accumulation by FSH involves different intracellular pathways in oestrogenic bovine granulosa cells *in vitro*

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Abstract

The objective of this study was to determine the major intracellular signalling pathways used by FSH and insulin to stimulate cytochrome P450 aromatase (Cyp19) mRNA and oestradiol accumulation in oestrogenic bovine granulosa cells *in vitro*. Bovine granulosa cells from small follicles (2–4 mm diameter) were cultured for 6 days under non-luteinizing conditions in the presence of insulin at 100 ng/ml, or insulin (10 ng/ml) and FSH (1 ng/ml). On day 4 of culture, specific inhibitors of phosphatidylinositol 3-kinase (PI3K; LY-294002), protein kinase C (PKC; GF-109203X), protein kinase A (PKA; H-89) or mitogen-activated protein (MAP) kinase activation (PD-98059) were added. The addition of PI3K and PKC inhibitors, but not of PKA inhibitor, significantly decreased insulin-stimulated Cyp19 mRNA levels and oestradiol accumulation ($P < 0.001$). The PKA inhibitor significantly decreased FSH-stimulated Cyp19 mRNA abundance and oestradiol secretion, whereas PI3K and PKC inhibitors decreased oestradiol secretion without affecting Cyp19 mRNA accumulation. Inhibition of MAP kinase pathway significantly increased Cyp19 mRNA abundance in insulin- and FSH-stimulated cells. P450scc mRNA levels and progesterone secretion were not affected by any inhibitor in either experiment. Although FSH stimulates Cyp19 expression predominantly through PKA, oestradiol secretion is altered by PI3K and PKC pathways independently of Cyp19 mRNA levels. In addition, we suggest that Cyp19 is under tonic inhibition mediated through a MAP kinase pathway.

Reproduction (2006) **132** 909–917

Introduction

Ovarian follicular development involves a series of regulated steps of growth and differentiation of follicular cells. One of the better known markers of healthy antral follicles is the steroidogenic capacity of granulosa cells (Richards *et al.* 1995). Oestradiol is a principal steroid produced by granulosa cells before their terminal differentiation is induced by the luteinizing hormone (LH) surge (Fitzpatrick *et al.* 1997). In ruminants and humans, granulosa cells convert theca-derived androgens to oestrogens with the enzyme cytochrome P450 aromatase (Cyp19; Simpson & Davis 2001), and are able to convert androstenedione to testosterone and/or oestrone to oestradiol with 17 β -hydroxysteroid dehydrogenase (17 β HSD; Luu-The 2001).

The expression of Cyp19 mRNA is stimulated by follicle-stimulating hormone (FSH) in rats, humans and ruminants (Steinkampf *et al.* 1987, Fitzpatrick & Richards 1991, Silva & Price 2000), acting mainly through the cAMP/protein kinase A (PKA) intracellular second messenger pathway (Conti 2002). In recent years, it has become clear that FSH activates other intracellular pathways, including extracellular signal-regulated kinases (ERK; Cottom *et al.* 2003). There are PKA-independent pathways of ERK activation via 'exchange factors activated by cAMP' (Epacs; Chin & Abayasekara 2004), and PKA-dependent pathways (Cottom *et al.* 2003). FSH also activates protein kinase B (PKB) through phosphatidylinositol 3-kinase (PI3K) in rat granulosa cells (Gonzalez-Robayna *et al.* 2000), and this pathway is necessary for granulosa cell

differentiation and Cyp19 expression in rats (Zelevnik *et al.* 2003, Alam *et al.* 2004).

FSH is not the sole stimulator of Cyp19 expression/activity, as several other factors have been shown to interact with FSH to enhance oestradiol secretion in various species. Insulin can enhance FSH-stimulated oestradiol production in bovine granulosa cells (Spicer *et al.* 1993, Gong *et al.* 1994, Gutiérrez *et al.* 1997), and can induce Cyp19 expression and/or oestradiol secretion in bovine granulosa cells in the absence of FSH (Gutiérrez *et al.* 1997, Silva & Price 2000). Insulin binds to its receptor, which induces autophosphorylation of the receptor β -subunit, which in turn phosphorylates insulin receptor substrate-1 (IRS-1; Poretsky *et al.* 1999). Phosphorylated IRS-1 activates the inositol phosphate second messenger cascade by activating PI3K. Studies with primarily fibroblasts and muscle cells have shown that phosphorylation of the insulin receptor can also activate ERK pathways, and does not typically act through the PKA pathway (Poretsky *et al.* 1999). However, insulin action appears not to be dependent on PI3K or ERK in human granulosa-lutein cells (Poretsky *et al.* 2001, Seto-Young *et al.* 2003).

It has not been established which pathways are activated by insulin and FSH in oestrogenic ruminant granulosa cells. The emphasis on 'oestrogenic' granulosa cells is important, as granulosa cells undergo a change in responsiveness to FSH/cAMP during luteinization (Gonzalez-Robayna *et al.* 1999), thus potentially changing the physiological response of cells to various ligands. The aim of this study was to determine the major intracellular signalling pathways used by FSH and insulin to stimulate Cyp19 expression and oestradiol accumulation in an established non-luteinizing bovine granulosa cell culture model (Gutiérrez *et al.* 1997, Sahmi *et al.* 2004).

Materials and Methods

Cell culture

The cell culture system was based on the model described previously (Gutiérrez *et al.* 1997) and used in our laboratory (Silva & Price 2000). All materials were obtained from Invitrogen Life Technologies unless otherwise stated.

Briefly, bovine ovaries were collected at a local abattoir and transported to the laboratory at 35 °C in M199 containing Hepes (25 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and fungizone (1 µg/ml). Follicles were dissected free of surrounding tissue and small follicles (2–4 mm diameter) were bisected in culture medium at 37 °C. Granulosa cells were recovered by passing the follicle walls repeatedly through a 1 ml disposable pipette. Cells were washed thrice in M199 containing Hepes (25 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml), then

resuspended in culture medium. Cell viability was estimated at 40–50% by Trypan blue exclusion.

Cells were seeded into 24-well tissue culture plates (Falcon; Becton Dickinson, NJ, USA) at a density of 10^6 viable cells in 1 ml α -MEM with L-glutamine containing sodium bicarbonate (10 mM), Hepes (20 mM), protease-free BSA (0.1%), sodium selenite (4 ng/ml), transferrin (2.5 µg/ml), androstenedione (10^{-7} M), human recombinant insulin-like growth factor I (IGF-I) (10 ng/ml), insulin (10 ng/ml), non-essential amino acid mix (1.1 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were stimulated with FSH and/or insulin as described later. Cultures were maintained at 37 °C in 5% CO₂, 95% air for 6 days, with 700 µl medium being replaced every 2 days.

Experimental design

Cells were cultured under conditions that promote Cyp19 mRNA induction and oestradiol secretion. Cells were stimulated with FSH (USDA bFSH-17; 1 ng/ml) or a higher dose of insulin (100 ng/ml) in the absence of FSH. This dose of FSH stimulates Cyp19 mRNA abundance and oestradiol secretion without affecting cytochrome P450 cholesterol side-chain cleavage (Cyp11a) or progesterone secretion, and the concentrations of insulin and IGF-I (10 ng/ml) in the medium are insufficient to support Cyp19 expression in the absence of FSH (Silva & Price 2000, 2002). The higher dose of insulin used stimulates Cyp19 mRNA levels and aromatase activity in the absence of FSH (Silva & Price 2000).

To determine the major pathways activated by FSH and insulin, specific second messenger inhibitors were added for 2 days on day 4 of culture. The inhibitors were LY-294002, an inhibitor of PI3K; GF-109203X, a protein kinase C (PKC) inhibitor; H-89, a PKA inhibitor and PD-98059, an inhibitor of ERK1/2. All inhibitors were dissolved in DMSO and added directly to each well in a total volume of 5 µl/well. Controls were cultured with and without DMSO (5 µl/well). Inhibitors were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA) and used at the doses indicated in Results.

To determine the effects of inhibitors on steroid secretion, triplicate wells were cultured with the indicated doses of inhibitor, and at the end of culture, the medium was harvested for oestradiol and progesterone assays, and cell protein was harvested by incubating cells with 200 µl of 1 M NaOH for 4 h. The extract was neutralized with 200 µl of 1 M HCl and cell protein quantified with the Bradford protein assay (Bio-Rad). To determine the effects of inhibitor on gene expression, 12 wells were cultured with each inhibitor and, at the end of culture, cells were recovered in Trizol (Invitrogen) for DNA and RNA extraction. All experiments were performed at least thrice. Total DNA was quantified by measuring fluorescence in the presence of

Hoechst 33258 (Labarca & Paigen 1980) and compared with a calf thymus DNA standard (Boehringer-Mannheim, Laval, QC, Canada).

Northern hybridization

The relative abundance of mRNA encoding Cyp19 and Cyp11a was determined by northern hybridization. Electrophoresis of 15 µg RNA was performed through a 1% denaturing formaldehyde-agarose gel followed by overnight capillary transfer onto a nylon membrane (Hybond-N; Amersham). Membranes were UV cross-linked in a commercial UV chamber (Bio-Rad) and incubated for 2 h in prehybridization solution, containing 10% dextran sulphate, five-strength saline-sodium phosphate-EDTA buffer (SSPE), five-strength Denhardt's solution, 0.5% SDS and 200 mg/ml herring sperm DNA.

The bovine Cyp19 cDNA probe was prepared in our laboratory (Soumano *et al.* 1996) and encompasses the entire haeme-binding and I-helix regions. The bovine Cyp11a cDNA was a gift from Dr M R Waterman (Vanderbilt University School of Medicine, Nashville, TN, USA) and encompasses the complete coding sequence (John *et al.* 1984).

Probes were labelled with [α -³²P]dCTP by random primer extension using a kit (Boehringer-Mannheim) to a specific activity of $1.5\text{--}3.0 \times 10^9$ d.p.m./mg and purified by centrifugation through a minicolumn using Wizard PCR Preps DNA purification system (Promega). Hybridization to the membranes was performed overnight at 65 °C. After hybridization, membranes were washed in $2 \times$ SSPE-0.1% SDS twice at room temperature (15 min each) and twice at 65 °C (15 min each). Membranes were stripped between hybridizations and finally hybridized to a labelled human 28S ribosomal cDNA probe (Gonzalez *et al.* 1985) for the standardization of RNA loading. The labelled membranes were exposed to Kodak X-Omat film at -70 °C in the presence of an intensifying screen for 1-14 days. Autoradiograms were scanned with a densitometer after different exposure times to ensure films were not overexposed; the representative films used in the figures are those that provided the better photographic image, not necessarily those that were used for analysis.

Assays

Oestradiol was measured in conditioned medium without extraction (Price *et al.* 1995). Inter- and intra-assay coefficients of variation were 12 and 10% respectively. Progesterone was measured as described (Lafrance & Goff 1985), with inter- and intra-assay coefficients of variation of 10 and 7% respectively. The sensitivity of these assays was equivalent to 0.25 and 1 ng/ml medium for oestradiol and progesterone respectively.

Total cAMP production was measured after incubation of cells with FSH or insulin for 48 h in the presence of IBMX. Assay was performed with a DELFIA kit (Perkin-Elmer, Norton, OH, USA) after the addition of lysis reagent to culture wells. Samples and standards were acetylated according to the kit instructions and were run in a single assay.

PKA and PKC activity assays were performed with commercial kits (Upstate Biotechnology, Lake Placid, NY, USA), in which a kinase-specific peptide substrate was phosphorylated in the presence of P³² and 10 µg granulosa cell protein extract (as source of protein kinases). The protein extract was prepared from bovine granulosa cells cultured as mentioned earlier with FSH and lysed in 25 mM Tris-HCl (pH 4) containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β -mercaptoethanol, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 200 µM PMFS and 0.5 mM IBMX. Particulate matter was removed by centrifugation at 14 000 *g* for 10 min at 4 °C. Reactions were performed in duplicate with three independent pools of cell protein. Activity estimates were corrected for non-specific activity, determined by performing reactions in the absence of peptide substrate.

Statistical analyses

The density of hybridization signals was corrected for loading efficiency using hybridization to 28S rRNA. Steroid concentrations are expressed relative to total DNA or total protein content of the wells. Data were transformed to logarithms if they were not normally distributed (Shapiro-Wilk test). ANOVA was used to test the main effects of pathway inhibitors and culture replicate was included as a random variable in the *F*-test for the effect of experiment. Where the main effect of inhibitor was significant, each inhibitor was compared with the control group using orthogonal contrasts. Correlations between steroid secretion and mRNA abundance were determined with the Pearson's correlation coefficient. Analyses were performed with JMP software (SAS Institute, Cary, NC, USA). The data are presented as least square mean $\pm$ S.E.M.

Results

The dose-responsiveness of granulosa cells to PKA and PKC inhibitors under the present culture conditions was assessed first. H-89, LY-294002 and GF-109203X inhibited oestradiol secretion from FSH-stimulated cells without affecting progesterone secretion (Fig. 1). From this experiment, we chose a single effective dose of each inhibitor and tested the specificity of pathway inhibition in bovine granulosa cell protein extracts (Table 1). The PKA inhibitor H-89 not only strongly inhibited PKA activity, but also reduced apparent PKC activity. The PKC inhibitor (GF-109203X) significantly inhibited PKC

Figure 1 Dose-responses of non-luteinizing bovine granulosa cells to intracellular pathway inhibitors. Cells were cultured for 6 days in the presence of FSH (1 ng/ml) and treatments were added on day 4. The treatments were 0.1% DMSO (inhibitor vehicle, V), H-89 (PKA inhibitors), LY-294002 (PI3K inhibitor), GF-109203X (PKC inhibitor), or untreated controls (C). Steroid concentrations were corrected for total cell protein (cell number), and are least squares mean $\pm$ s.e.m. obtained from three replicate experiments. Asterisks denote doses of each inhibitor that are significantly different from non-treated controls ($P < 0.05$).

activity without affecting PKA activity, and the PI3K inhibitor (LY-294002) altered neither PKA nor PKC activity.

We then assessed the effect of these inhibitors, as well as an ERK1/2 inhibitor (PD-98059) Cyp19 and Cyp11a expression in FSH-stimulated cells. The addition of the PKA inhibitor decreased Cyp19 mRNA abundance to barely detectable levels and significantly inhibited oestradiol secretion (Fig. 2; $P < 0.05$). The PI3K and PKC inhibitors had no significant effect on Cyp19 mRNA abundance ($P > 0.05$), but significantly decreased oestradiol secretion ($P < 0.01$). The ERK inhibitor significantly increased Cyp19 mRNA levels, but was without effect on oestradiol secretion. Overall, oestradiol

Table 1 Relative PKA and PKC activities[†] (mean $\pm$ s.e.m.) in cytosolic protein extracts of bovine granulosa cells in the presence of inhibitors of intracellular signalling pathways.

Inhibitor	PKA activity	PKC activity
H-89 (10 μ M)	5 $\pm$ 3*	39 $\pm$ 16*
GF-109203X (3 μ M)	85 $\pm$ 6	10 $\pm$ 7*
LY-294002 (20 μ M)	90 $\pm$ 7	100 $\pm$ 11

[†]Values expressed relative to kinase activity in the absence of inhibitor (100 $\pm$ 0). *Means are significantly different from control ($P < 0.05$).

Figure 2 Effect of inhibitors of intracellular signalling pathways on Cyp19 mRNA abundance and oestradiol secretion in FSH-stimulated bovine granulosa cells. Cells were cultured for 6 days with FSH (1 ng/ml) and insulin (10 ng/ml). At day 4 of culture, specific inhibitors were added to the cultures. The treatments were control and the inhibitors of PKA (10 μ M H-89), PI3K (20 μ M LY-294002), PKC (3 μ M GF-109203X) or MAP kinase (50 μ M PD-98059). Values are least squares mean $\pm$ s.e.m., and were obtained from three replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and oestradiol is expressed relative to total cell DNA. The upper panel shows a representative northern blot. Asterisks denote significant ($P < 0.05$) differences from control (orthogonal contrasts).

secretion and Cyp19 mRNA levels were not correlated ($r = -0.05$, $P > 0.10$, $n = 15$).

There were no significant effects of any pathway inhibitor on Cyp11a mRNA levels or progesterone secretion (Fig. 3). Progesterone and Cyp11a mRNA levels were not correlated ($r = -0.24$, $P > 0.10$, $n = 15$).

The addition of PI3K and PKC inhibitors to insulin-stimulated cells significantly decreased Cyp19 mRNA abundance and oestradiol secretion (Fig. 4; $P < 0.001$), whereas the addition of the PKA inhibitor had no effect. The addition of the inhibitor of MAP kinase significantly

Figure 3 Effect of inhibitors of intracellular signalling pathways on Cyp11a mRNA abundance and progesterone secretion in FSH-stimulated bovine granulosa cells. Cells were cultured for 6 days with FSH (1 ng/ml) and insulin (10 ng/ml). At day 4 of culture, specific inhibitors were added to the cultures. The treatments were control and the inhibitors of PKA (10 μ M H-89), PI3K (20 μ M LY-294002), PKC (3 μ M GF-109203X) or MAP kinase (50 μ M PD-98059). Values are least squares mean $\pm$ S.E.M., and were obtained from three replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and progesterone is expressed relative to total cell DNA. The upper panel shows a representative northern blot (see Fig. 2 for corresponding 28S rRNA image).

increased Cyp19 mRNA abundance ($P < 0.05$), although it was without effect on oestradiol secretion. There was an overall linear correlation between Cyp19 mRNA and oestradiol secretion ($r = 0.65$, $P < 0.01$, $n = 18$). The addition of DMSO alone had no effect on Cyp19 mRNA or oestradiol secretion.

Again, none of the inhibitors significantly affected Cyp11a mRNA abundance or progesterone secretion (Fig. 5). Cyp11a mRNA abundance and progesterone secretion were not correlated ($r = 0.08$, $P > 0.73$, $n = 18$).

To determine if the pathways activated in insulin-stimulated cells are dependent on the generation of cAMP, we measured cAMP accumulation following stimulation with insulin or with FSH. Compared with untreated control cells (0.7 ± 0.1 nmol cAMP/ml), FSH induced a significant rise in cAMP concentrations

Figure 4 Effect of inhibitors of intracellular signalling pathways on Cyp19 mRNA abundance and oestradiol secretion in insulin-stimulated bovine granulosa cells. Cells were cultured for 6 days with insulin (100 ng/ml) without FSH. At day 4 of culture, specific inhibitors were added to the cultures. The treatments were control, 0.1% DMSO (inhibitor vehicle) and the inhibitors of PKA (10 μ M H-89), PI3K (20 μ M LY-294002), PKC (3 μ M GF-109203X) or MAP kinase (50 μ M PD-98059). Values are least squares mean $\pm$ S.E.M., and were obtained from three replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and oestradiol is expressed relative to total cell DNA. The upper panel shows a representative northern blot. Asterisks denote significant ($P < 0.05$) differences from control (orthogonal contrasts).

(5.0 ± 0.2 nmol/ml; $P < 0.01$), whereas insulin had no effect (0.8 ± 0.6 nmol/ml).

Discussion

Intracellular signalling by gonadotrophic hormones is a complex process, involving many potential pathway interactions. The major pathway activated by FSH is cAMP activation of PKA and PKB, but studies have also demonstrated activation by FSH of ERK and PI3K (Conti 2002). Many discrepancies between studies may

Figure 5 Effect of inhibitors of intracellular signalling pathways on Cyp11a mRNA abundance and progesterone secretion in insulin-stimulated bovine granulosa cells. Cells were cultured for 6 days with insulin (100 ng/ml) without FSH. At day 4 of culture, specific inhibitors were added to the cultures. The treatments were control, 0.1% DMSO (inhibitor vehicle), and the inhibitors of PKA (10 μ M H-89), PI3K (20 μ M LY-294002), PKC (3 μ M GF-109203X) or MAP kinase (50 μ M PD-98059). Values are least squares mean $\pm$ S.E.M., and were obtained from three replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and progesterone is expressed relative to total cell DNA. The upper panel shows a representative northern blot.

be attributed to the differences between species and cell models. For example, culture of pig and bovine granulosa cells, especially in the presence of serum, leads to luteinization and loss of Cyp19 (Henderson & Moon 1979, Pescador *et al.* 1999) that in rats is associated with marked changes in PKA signalling (Gonzalez-Robayna *et al.* 1999). Changes in pathway use have recently been ascribed to changes in gonadotrophin receptor density in immature rat granulosa cells (Donadeu & Ascoli 2005). It is important, therefore, to avoid extrapolating data from one species/cell model to another.

Bovine granulosa cells cultured in serum-free medium secrete oestradiol and are responsive to FSH at physiological doses (Gutiérrez *et al.* 1997, Silva & Price 2000). They express Cyp19 mRNA predominantly

from the ovarian-specific Cyp19 promoter (Hamel *et al.* 2005) and do not luteinize (Sahmi *et al.* 2004). The present data show that under these culture conditions, FSH stimulates Cyp19 mRNA and oestradiol secretion predominantly through cAMP signalling, most likely to PKA. These data are entirely in agreement with the major pathway employed in other granulosa cells models (Fitzpatrick & Richards 1991). This conclusion is based on the inhibition of Cyp19 mRNA abundance and oestradiol secretion in FSH-stimulated cells by the PKA inhibitor H-89, and the increased cAMP accumulation caused by FSH in these cells. The mechanism of action is widely believed to involve phosphorylation of cAMP-binding protein (CREB), which interacts directly with the cAMP-response element (CRE) within the ovary-specific aromatase promoter (Carlone & Richards 1997).

However, the present data suggest that other reported FSH-signalling pathways are not important for steroidogenesis in this cell model. In rat granulosa cells, FSH activated ERK (Cameron *et al.* 1996, Das *et al.* 1996, Cottom *et al.* 2003) and PI3K second messengers (Gonzalez-Robayna *et al.* 2000), and activation of PKB via PI3K is suggested to be obligatory for Cyp19 expression in rats (Zelevnik *et al.* 2003). Inhibition of PI3K with LY-294002 reduced or blocked FSH-stimulated phosphorylation of PKB and several FSH-stimulated genes in rats (Gonzalez-Robayna *et al.* 2000, Alam *et al.* 2004, Ongeri *et al.* 2005), whereas the same inhibitor had no effect on Cyp19 mRNA levels in bovine granulosa cells under the present culture conditions. While these data do not preclude a role for PI3K for other FSH-responsive genes, they argue against PI3K activation for Cyp19.

Inhibition of ERK phosphorylation with PD-98059 enhanced FSH-stimulated progesterone secretion and intracellular steroidogenic acute regulatory (StAR) protein levels in rat granulosa cells (Seger *et al.* 2001), and a different ERK inhibitor stimulated Cyp19 mRNA levels in rat granulosa cells (Moore *et al.* 2001). Therefore, FSH-stimulated ERK activity appears to attenuate steroidogenesis. The present data suggest that this inhibitory pathway is also active in oestrogenic bovine granulosa cells, at least for FSH-stimulated Cyp19 expression. FSH also activates a kinase similar to ERK, p38 MAP kinase (Maizels *et al.* 1998, Gonzalez-Robayna *et al.* 2000) that is not inhibited by PD-98059. Whether FSH activates p38 through PKA is unclear, as p38 phosphorylation was inhibited by H-89 in one study (Maizels *et al.* 1998) but not altered in others (Gonzalez-Robayna *et al.* 2000, Alam *et al.* 2004). It should be noted that H-89 may not be highly specific for PKA in all granulosa cell models, as it inhibited apparent PKC activity in bovine granulosa cells in the present study.

An intriguing observation was the reduction in oestradiol secretion from FSH-stimulated cells after culture with PI3K and PKC inhibitors, despite maintained Cyp19 mRNA levels. These data suggest that PI3K and

PKC pathways favour oestradiol secretion at a site distal to Cyp19 gene expression. This is in contrast with studies showing that phorbol esters (activators of PKC) reduced oestradiol secretion from FSH/cAMP-stimulated pig, rat and cow granulosa cells (Hylka *et al.* 1989, Fitzpatrick *et al.* 1997, Legault *et al.* 1999). Since FSH stimulates PI3K and downstream kinases including PKB in rats (Gonzalez-Robayna *et al.* 2000), our data are compatible with potential regulation of aromatase activity (but not gene expression) in bovine cells by FSH through PI3K, possibly involving PKB. Alternatively, other enzymes involved in oestrogen biosynthesis, such as NADPH-cytochrome P450 reductase and 17 β HSD, may be regulated through PKA-independent pathways. Phorbol ester increased 17 β HSD type 1 expression in JET-3 choriocarcinoma cells (Piao *et al.* 1997), although 17 β HSD is regulated by FSH in bovine granulosa cells (Sahmi *et al.* 2004).

Aside from FSH, the culture medium also contained IGF-I which is widely believed to activate the same signalling pathway as insulin (Poretsky *et al.* 1999). Therefore, the addition of PKC and PI3K inhibitors could potentially alter Cyp19 expression and oestradiol secretion in FSH-stimulated cells by inhibiting IGF-I rather than FSH signalling. However, withdrawal of IGF-I from culture did not affect Cyp19 expression or oestradiol secretion in a previous study (Silva & Price 2002). Furthermore, withdrawal of both IGF-I and insulin caused a decrease in Cyp19 mRNA levels but did not affect oestradiol secretion (Silva & Price 2002), which is inconsistent with the present results (decrease in oestradiol but no change in Cyp19 mRNA). Therefore, we suggest that the effects of PKC and PI3K on FSH-stimulated oestradiol secretion in the present study reflect inhibition of the FSH signalling pathway and not that of IGF-I.

Insulin alone also stimulates Cyp19 mRNA levels in the granulosa cell culture system used herein. This action is independent of FSH and specific for Cyp19 as increases in P450scc are not observed (Silva & Price 2000). Few studies have evaluated the mechanism of insulin action in ovarian cells. In luteinized porcine cells, insulin augmented LH-stimulated low-density lipoprotein receptor expression through PI3K- and ERK/MAP kinase-dependent mechanisms, as determined using the same pathway inhibitors used here (Sekar & Veldhuis 2001). Insulin and IGF-I have been shown to stimulate PI3K activity in bovine luteal cells (Chakravorty *et al.* 1993). IGF-I has been shown to stimulate PKB phosphorylation in rat granulosa cells and this action was blocked by PI3K inhibitor (LY-294002) but not by PKA (H-89) or ERK (PD-98059) inhibitors (Gonzalez-Robayna *et al.* 2000). Consistent with this, IGF-I stimulated PI3K activity and PKB phosphorylation in porcine granulosa cells (Westfall *et al.* 2000). In this study, we have determined that insulin stimulates Cyp19 principally through PI3K and PKC pathways, and not

PKA or ERK/MAPK pathways, showing that the pathways used are similar to those described above for rat and porcine granulosa cells. It is a distinct possibility therefore that in oestrogenic bovine granulosa cells, insulin may activate PKB via PI3K.

The MAP kinase and PI3K pathways have been implicated in apoptosis (Westfall *et al.* 2000). We did not specifically measure cell death in the present study, but we did observe a decrease in 28S rRNA in insulin-stimulated cells treated with the PI3K inhibitor. This is consistent with loss of cell integrity and apoptosis, as observed in rat cells also treated with PI3K inhibitor (Westfall *et al.* 2000). Interestingly, this effect on 28S rRNA was not observed in FSH-stimulated cells, which suggests that FSH afforded some protection against PI3K-inhibited apoptosis. This is consistent with the observation that LH but not IGF-I maintained viability of LY-294006-treated hen granulosa cells (Johnson *et al.* 2001).

In the present study, we did not determine by which pathway Cyp11a expression is stimulated, as Cyp11a mRNA levels are not upregulated by the doses of FSH and insulin used (Silva & Price 2000). Nevertheless, it is likely that the basal expression levels observed here are constitutive, as none of the pathway inhibitors used affected Cyp11a mRNA levels or progesterone secretion.

In summary, the present report describes the major intracellular pathways used to stimulate Cyp19 mRNA abundance by FSH and insulin in oestrogenic bovine granulosa cells. FSH acts principally through the PKA pathway, as expected. Insulin acts principally through PI3K- and PKC-dependent pathways, but not through PKA- or ERK/MAP kinase-dependent pathways. We also provide evidence for two alternative regulatory pathways, a tonic inhibition of Cyp19 mRNA levels by ERK/MAP kinase, and post-transcriptional regulation of oestradiol secretion through PI3K and PKC pathways.

Acknowledgements

We thank Drs A K Goff and A Bélanger for steroid antibodies, Dr M R Waterman for the bovine Cyp19 cDNA and Dr A F Parlow and the NIDDK National Hormone & Peptide Program for providing bovine FSH. This work was supported by NSERC Canada and J M S was supported by a scholarship from CONACYT, Mexico. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 6 June 2006

First decision 7 August 2006

Accepted 15 August 2006